IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

GENENTECH, INC. and CITY OF HOPE, Plaintiffs, v. AMGEN INC., Defendant.	C.A. No. 18-924-GMS [redacted] PUBLIC VERSION FILED: July 25, 2018

FIRST AMENDED COMPLAINT

Plaintiffs Genentech, Inc. ("Genentech") and City of Hope (collectively, "Plaintiffs") bring this First Amended Complaint for declaratory and injunctive relief against Defendant Amgen Inc. ("Amgen") to address Amgen's infringement of patents relating to Genentech's groundbreaking breast cancer drug Herceptin®.

NATURE OF THE CASE

1. Breast cancer is a serious disease affecting over 2.8 million women in the United States. Approximately 20-25% of those women suffer from "HER2-positive" breast cancer. This is a particularly aggressive form of the disease characterized by overexpression of human epidermal growth factor receptor 2 (i.e., "HER2") proteins due to excessive HER2 gene amplification.

2. In the early 1990s, a diagnosis of HER2-positive breast cancer was effectively a death sentence: patients had an average life expectancy of only 18 months. The quality of life for those patients was markedly poor—the disease rapidly metastasized (*i.e.*, spread to other parts of the body). The only available treatments were invasive and disfiguring surgery and

chemotherapeutic drugs with harsh side effects, and those treatments added little to the patient's life span.

3. The treatment of HER2-positive breast cancer, and the lives of millions of women suffering from the disease, changed dramatically with Genentech's development of Herceptin®. Herceptin® was the first drug of its kind—an antibody called trastuzumab that specifically targeted the biological mechanism that makes HER2-positive breast cancer such an aggressive form of the disease.

4. Although the scientific community was initially skeptical that such an antibody-based therapy could work, Genentech's specific methods of using Herceptin® proved remarkably effective. Indeed, after Genentech revealed the results of its clinical studies, the scientific community hailed Herceptin® as "the beginning of a whole new wave of biological drugs that modulate the causes of cancer"[1] and a sign that "the whole field of cancer research has turned a corner."[2]

5. Since FDA approval of Herceptin® in 1998, Genentech has worked diligently to develop new methods of using Herceptin®—including improved dosing schedules and broader indications—to expand access to therapy and improve the quality of life for millions of patients worldwide. This research has greatly expanded the number of patients who are able to benefit from Herceptin®. To further expand access to this lifesaving drug, Genentech also provides Herceptin® free of charge to patients who are uninsured or cannot afford treatment and assists with out-of-pocket prescription-related expenses. All told, Genentech has spent over two decades, and billions of dollars, developing Herceptin® into the life-saving drug it is today.

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[1] Gina Kolata and Lawrence M. Fisher, *Drugs to Fight Breast Cancer Near Approval*, NEW YORK TIMES (FRONT PAGE) (Sept. 3, 1998).

[2] Robert Langreth, *Breast-Cancer Drug Is Backed by FDA Panel*, Wall Street J. (Sept. 3, 1998).

6. Genentech's groundbreaking work developing Herceptin® was the result of years of research from a group of talented scientists. The United States Patent and Trademark Office recognized that innovative work by granting Genentech numerous patents claiming Herceptin®, its manufacture, and its use. And as one of the pioneers in the biotechnology field, Genentech collaborated with scientists at research institutions such as the City of Hope to make foundational inventions, such as efficient techniques for making antibodies that can be used as drugs.

7. Seeking to profit from the success of Plaintiffs' innovations, Amgen is seeking FDA approval of a biosimilar version of Herceptin® called ABP 980. ABP 980 is a copycat product for which Amgen is seeking the same label indications and usage as Herceptin®. In fact, Amgen is relying upon Genentech's own studies demonstrating the safety and efficacy of Herceptin® to obtain approval of its biosimilar product.

8. In 2010, Congress provided a pathway for resolving patent disputes relating to biosimilar products through the Biologics Price Competition and Innovation Act ("BPCIA"). Amgen initially purported to follow the process outlined in the BPCIA, which requires biosimilar applicants and innovator companies to exchange certain information concerning the biosimilar product and the patents that may be infringed by the manufacture and sale of the biosimilar product. *See* 42 U.S.C. § 262(*l*).

9. Plaintiffs thus bring this action for infringement pursuant to 35 U.S.C. § 271(e)(2) based upon Amgen's submission of its aBLA for ABP 980. Plaintiffs also seek a declaratory judgment pursuant to 42 U.S.C. § 262(*l*)(9) and 28 U.S.C. § 2201 that the manufacture, use, offer to sell, sale, or importation into the United States of Amgen's biosimilar product would infringe the patents described below. Pursuant to 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), and/or 35 U.S.C. § 283, Plaintiffs also seek a preliminary and/or permanent

injunction barring Amgen's manufacture, use, offer to sell, sale, or importation of its biosimilar product prior to the expiration of those patents. In the event that Amgen imports, manufactures, or launches its biosimilar product, and/or otherwise practices the patented inventions in the United States prior to the expiration of those patents, Plaintiffs also seek monetary damages, including lost profits, and any further relief as this Court may deem just and proper.

PARTIES

10. Plaintiff Genentech is a corporation organized and existing under the laws of the State of Delaware with its corporate headquarters at 1 DNA Way, South San Francisco, California 94080.

11. Genentech was founded in 1976 and for four decades has been at the forefront of innovation in the field of therapeutic biotechnology. Today, Genentech employs a large number of researchers, scientists, and post-doctoral staff members who routinely publish in top peer-reviewed journals and are among the leaders in total citations to their work by researchers. Genentech currently markets numerous approved pharmaceutical and biologic drugs for a range of serious or life-threatening medical conditions, including various forms of cancer, heart attacks, strokes, rheumatoid arthritis, and respiratory diseases.

12. Plaintiff City of Hope is a California not-for-profit organization, with its principal place of business at 1500 East Duarte Road, Duarte, California 91010.

13. Founded in 1913, the City of Hope is a leading research hospital that incorporates cutting-edge research into patient care for cancer, diabetes, and other serious diseases.

14. Upon information and belief, Defendant Amgen is a company organized and existing under the laws of the State of Delaware with its principal place of business located at One Amgen Center Drive, Thousand Oaks, California 91320.

15. Amgen is, among other things, engaged in the development of biologic drugs, including a proposed biosimilar version of Genentech's Herceptin® product, ABP 980 ("Amgen's aBLA product"). Upon information and belief, Amgen's aBLA product will be distributed and sold in the State of Delaware and throughout the United States.

JURISDICTION AND VENUE

16. This action arises under the BPCIA, 42 U.S.C. § 262(*l*) and the Patent Laws of the United States, Title 35, United States Code, and the Declaratory Judgment Act, 28 U.S.C. §§ 2201-2202. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331, 1332, and 1338.

17. Venue is proper with respect to Amgen in this Court pursuant to 28 U.S.C. §§ 1391 and 1400(b) because Amgen is incorporated in Delaware.

18. This Court has personal jurisdiction over Amgen because it is incorporated in Delaware. In addition, among other things, Amgen has filed an Abbreviated Biologics License Application ("aBLA") for ABP 980 with the FDA seeking approval to market it, which reliably indicates that it will market its proposed biosimilar product in Delaware if approved.

THE PARTIES' EXCHANGES UNDER THE BPCIA

19. On July 31, 2017, Amgen announced that it had submitted an aBLA for ABP 980 to the FDA seeking approval for the commercial manufacture, use, offer for sale, or sale of the Amgen aBLA product, a biosimilar version of trastuzumab, which is subject to BLA No. 103792 to Genentech.[3]

20. The FDA accepted Amgen's aBLA for review [redacted].

[3] http://www.amgen.com/media/news-releases/2017/07/amgen-and-allergan-submit-biosimilar-biologics-license-application-for-abp-980-to-us-food-and-drug-administration/

21. On October 16, 2017, Amgen provided Genentech with a copy of Amgen's aBLA, which included a small amount of manufacturing information.

22. On November 3, 2017, Amgen provided Genentech with additional manufacturing information regarding Amgen's aBLA product.

23. Genentech responded on November 20, 2017, to identify deficiencies in Amgen's production of manufacturing information and request specific information concerning the manufacture of Amgen's biosimilar product. Amgen provided additional manufacturing information on December 1, 2017, and December 4, 2017, but did not satisfy its disclosure obligations. Genentech then responded on December 15, 2017, to explain that Amgen's production was deficient in that it failed to provide all of the requested information in contravention of 42 U.S.C. § 262(*l*)(2).

24. Amgen did not disclose all of the information relevant to establishing whether the manufacture of Amgen's aBLA product will infringe each of the patents identified on Genentech's operative list pursuant to 42 U.S.C. § 262(*l*)(3)(A), despite Genentech's request that Amgen provide sufficient "other information that describes the process or processes used to manufacture" as required by 42 U.S.C. § 262(*l*)(A). Amgen's failure to provide sufficient information under those circumstances justifies Genentech's contention that manufacturing Amgen's aBLA product will infringe such patents.

25. Despite Amgen's non-compliance (and without waiving Genentech's objection to such non-compliance), Genentech provided its operative list of 36 patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) on December 15, 2017.

26. Amgen replied on December 20, 2017, to assert its position that it had complied with its disclosure obligations based on Amgen's earlier production of its aBLA and two manufacturing documents.

27. Genentech responded on December 27, 2017, to reiterate that Amgen's production was insufficient to provide Genentech with a complete understanding of Amgen's trastuzumab manufacturing process.

28. Amgen replied on February 1, 2018, with an additional supplemental production.

29. On February 6, 2018, Genentech supplemented its § 262(*l*)(3)(A) list to include a newly issued manufacturing patent: U.S. Patent No. 9,868,760.

30. On February 13, 2018, Amgen purported to provide its detailed statement concerning non-infringement and invalidity pursuant to 42 U.S.C. § 262(*l*)(3)(B) ("Amgen's 3B Statement"). Amgen's 3B Statement was deficient in numerous ways. For example, it—like Amgen's document productions—failed to fully describe Amgen's manufacturing process, such that Genentech was unable to evaluate many of Amgen's non-infringement arguments.

31. On [redacted] and [redacted], Amgen produced additional documents regarding [redacted]. These supplemental productions still failed to fully describe Amgen's manufacturing process.

32. On April 13, 2018, and subject to its objections, Genentech provided its response to Amgen's 3C Statement pursuant to 42 U.S.C. § 262(*l*)(3)(C) ("Genentech's 3C Statement"). Genentech included responses to Amgen's non-infringement and invalidity statements for each of the patents addressed in Amgen's 3B Statement and maintained that ABP 980 will infringe at

least 18 Genentech patents. With its 3C Statement, Genentech proposed that Amgen agree that all 18 of these patents be included in a first-phase infringement action under § 262(*l*)(6).

33. On [redacted], and [redacted], Amgen produced additional documents regarding [redacted]. These supplemental productions still failed to fully describe Amgen's manufacturing process.

34. After Genentech served its 3C Statement, the parties initiated negotiations under § 262(*l*)(4). On May 23, 2018, Genentech and Amgen agreed that the 37 patents addressed in the exhibits to Genentech's 3C Statement shall be the subject of an action for patent infringement under § 262(*l*)(6).

35. In light of the parties' agreement, § 262(*l*)(6)(A) required Genentech to bring an action for patent infringement with respect to each of the 37 patents that were part of the parties' agreement. This action is Genentech's action pursuant to § 262(*l*)(6)(A).

36. On [redacted], Amgen purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it intends to commence commercial marketing of ABP 980 in the United States no earlier than 180 from [redacted] (i.e., [redacted]).

37. On June 21, 2018, Plaintiffs sued Amgen for infringement of all 37 patents that the parties agreed to litigate during their § 262(*l*)(4) negotiations. On July 19, 2018, Plaintiffs and Amgen stipulated to dismiss with prejudice all claims for infringement of U.S. Patent Nos. 6,242,177, 6,489,447, 6,586,206, 6,870,034, 7,449,184, 7,501,122, 8,044,017, 8,314,225, 8,357,301, 8,460,895, 8,691,232, 8,710,196, 8,771,988, 9,047,438, 9,080,183, 9,428,766, 9,487,809, 9,493,744, and 9,868,760 relating to ABP 980, subject to certain conditions.

AMGEN'S aBLA PRODUCT

38. Amgen has publicly stated that its aBLA product is biosimilar to Herceptin®. For example, Amgen has issued press releases claiming that ABP 980 is "a biosimilar candidate to Herceptin®" and "ABP 980 is a biosimilar candidate to trastuzumab,"[4] and it has announced the results of an Amgen study that purports to conclude that "[e]fficacy, safety and immunogenicity data support ABP 980 as a trastuzumab biosimilar."[5]

39. Given Amgen's claim of biosimilarity, Amgen's aBLA product must "utilize the same mechanism or mechanisms of action [as Herceptin®] for the condition or conditions of use prescribed, recommended, or suggested in the proposed labeling." 42 U.S.C. § 262(k)(2)(A)(i)(II).

40. Under 35 U.S.C. § 271(e)(2)(C), Amgen has committed a statutory act of patent infringement with respect to patents identified by Genentech under 42 U.S.C. § 262(*l*)(3), through the submission of its aBLA application for ABP 980.

GENENTECH'S ASSERTED PATENTS

41. Genentech has spent over two decades and significant resources developing Herceptin®, and the USPTO has awarded to Genentech numerous patents on innovations resulting from this massive undertaking. These patents cover the antibody trastuzumab, along with its manufacture and use.

42. Upon information and belief, Amgen's aBLA product will infringe at least the following patents, which Genentech has asserted in this lawsuit: U.S. Patent No. 6,331,415, U.S.

[4] http://www.amgen.com/media/news-releases/2017/07/amgen-and-allergan-submit-biosimilar-biologics-license-application-for-abp-980-to-us-food-and-drug-administration/

[5] https://www.amgen.com/media/news-releases/2017/09/amgen-and-allergan-present-phase-3-data-on-biosimilar-trastuzumab-candidate-abp-980-at-the-european-society-for-medical-oncology-2017-congress/

Patent No. 7,923,221, U.S. Patent No. 6,407,213, U.S. Patent No. 7,846,441, U.S. Patent No. 7,892,549, U.S. Patent No. 6,627,196, U.S. Patent No. 7,371,379, U.S. Patent No. 6,417,335, U.S. Patent No. 9,249,218, U.S. Patent No. 8,574,869, U.S. Patent No. 6,620,918, U.S. Patent No. 7,993,834, U.S. Patent No. 8,076,066, U.S. Patent No. 8,425,908, U.S. Patent No. 8,440,402, U.S. Patent No. 6,121,428, U.S. Patent No. 8,512,983, and U.S. Patent No. 9,714,293.

The Cabilly Patents

43. U.S. Patent Nos. 6,331,415 and 7,923,221 (collectively, the "Cabilly Patents") describe and claim a process for producing monoclonal antibodies, such as Herceptin®, from recombinant DNA. This effective and efficient process applies a novel co-expression technique to produce antibody heavy and light chains in a single host cell, and has given rise to an entire industry of therapeutic monoclonal antibodies.

44. U.S. Patent No. 6,331,415 ("the '415 patent"), titled "Methods of Producing Immunoglobulins, Vectors and Transformed Host Cells for Use Therein," was duly and legally issued by the Patent Office on December 18, 2001. A true and correct copy of the '415 patent is attached as Exhibit A. Genentech and the City of Hope are the owners by assignment of the '415 patent.

45. U.S. Patent No. 7,923,221 ("the '221 patent"), titled "Methods of Making Antibody Heavy and Light Chains Having Specificity for a Desired Antigen," was duly and legally issued by the Patent Office on April 12, 2011. A true and correct copy of the '221 patent is attached as Exhibit B. Genentech and the City of Hope are the owners by assignment of the '221 patent.

The ’213 Patent

46. U.S. Patent No. 6,407,213 (“the ’213 patent”) claims the Herceptin® antibody itself, along with other humanized monoclonal antibodies. The inventors of the ’213 patent discovered that by grafting the key parts of a mouse antibody onto a human antibody consensus sequence, they could create antibodies that were both tolerated by the immune system and effective to treat diseases like HER2-positive breast cancer. The techniques described in the ’213 patent allowed scientists to efficiently design antibodies for specific disease targets by modifying mouse antibodies produced in the laboratory in specific ways so that they are compatible with a human immune system.

47. The ’213 patent, titled “Method for Making Humanized Antibodies,” was duly and legally issued by the Patent Office on June 18, 2002. A true and correct copy of the ’213 patent is attached as Exhibit C. Genentech is the owner by assignment of the ’213 patent.

The Combination Chemotherapy Patents

48. U.S. Patent No. 7,846,441 (“the ’441 patent”), claims the administration of Herceptin® in combination with a chemotherapy agent known as a taxoid, in the absence of an anthracycline derivative (another chemotherapy agent) in an amount effective to extend time to disease progression without overall increase in severe adverse events. This specific method of treatment unexpectedly resulted in a significant improvement in patient outcomes. It nearly doubled the time until disease progression compared to treatment using a taxoid alone, and it also avoided the serious cardiotoxicity associated with Herceptin® in combination with anthracycline derivatives that unexpectedly presented during the Herceptin® clinical trials.

49. The ’441 patent, titled “Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on December 7, 2010. A true and correct copy of the ’441 patent is attached as Exhibit D. Genentech is the owner by assignment of the ’441 patent.

50. U.S. Patent No. 7,892,549 (“the ’549 patent”) is a continuation to the ’441 patent that claims a method of treating a patient with HER2-positive breast cancer by administering Herceptin® in combination with a taxoid and a further growth inhibitory agent or further therapeutic agent.

51. The ’549 patent, titled “Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on February 22, 2011. A true and correct copy of the ’549 patent is attached as Exhibit E. Genentech is the owner by assignment of the ’549 patent.

52. U.S. Patent No. 8,425,908 (“the ’908 patent”), claims priority to the same provisional application as the ’441 and ’549 patents. The ’908 patent claims a method of treating a patient with HER2-positive gastric cancer by administering Herceptin® in combination with chemotherapy and in the absence of an anthracycline derivative.

53. The ’908 patent, titled “Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on April 23, 2013. A true and correct copy of the ’908 patent is attached as Exhibit F. Genentech is the owner by assignment of the ’908 patent.

The Method of Administration Patents

54. U.S. Patent Nos. 6,627,196 and 7,371,379 (collectively, the “Method of Administration Patents”) generally cover the most common administration method for Herceptin®: an initial dose of 8 mg/kg, followed by 6 mg/kg doses once every three weeks. Herceptin® was initially approved for administration on a weekly regimen, but Genentech discovered that the drug could be dosed only once every three weeks without reducing safety or

effectiveness. The discovery of three-weekly dosing has had a marked impact on patients' quality of life by providing the same life-saving effects of Herceptin® while allowing patients to receive treatment less frequently.

55. U.S. Patent No. 6,627,196 ("'196 patent"), titled "Dosages for Treatment with Anti-ErbB2 Antibodies," was duly and legally issued by the Patent Office on September 30, 2003. A true and correct copy of the '196 patent is attached as Exhibit G. Genentech is the owner by assignment of the '196 patent.

56. U.S. Patent No. 7,371,379 ("the '379 patent"), titled "Dosages for Treatment with Anti-ErbB2 Antibodies," was duly and legally issued by the Patent Office on May 13, 2008. A true and correct copy of the '379 patent is attached as Exhibit H. Genentech is the owner by assignment of the '379 patent.

The Acidic Variants Patents

57. U.S. Patent Nos. 6,417,335 and 9,249,218 (collectively, the "Acidic Variants Patents") cover compositions with reduced amounts of more acidic structural variants of trastuzumab ("acidic variants") and chromatographic processes for removing these acidic variants during purification. Some trastuzumab acidic variants have lower potency than trastuzumab itself. The Acidic Variants Patents describe and claim chromatographic processes and compositions that ensure the Herceptin® drug product is uniformly pure and effective.

58. U.S. Patent No. 6,417,335 ("the '335 patent"), titled "Protein Purification," was duly and legally issued by the Patent Office on July 9, 2002. A true and correct copy of the '335 patent is attached as Exhibit I. Genentech is the owner by assignment of the '335 patent.

59. U.S. Patent No. 9,249,218 ("the '218 patent"), titled "Protein Purification," was duly and legally issued by the Patent Office on February 2, 2016. A true and correct copy of the '218 patent is attached as Exhibit J. Genentech is the owner by assignment of the '218 patent.

HER2 Diagnostic Patents

60. U.S. Patent Nos. 7,993,834, 8,076,066, and 8,440,402 claim novel techniques for identifying patients who might benefit from trastuzumab therapy using gene amplification techniques even where immunohistochemistry techniques suggest that the patient may not overexpress HER2.

61. U.S. Patent No. 7,993,834 ("the '834 patent"), titled "Detection of ErbB2 Gene Amplification to Increase the Likelihood of the Effectiveness of ErbB2 Antibody Breast Cancer Therapy," was duly and legally issued by the Patent Office on August 9, 2011. A true and correct copy of the '834 patent is attached as Exhibit K. Genentech is the owner by assignment of the '834 patent.

62. U.S. Patent No. 8,076,066 ("the '066 patent"), titled "Gene Detection Assay for Improving the Likelihood of an Effective Response to a HER2 Antibody Cancer Therapy," was duly and legally issued by the Patent Office on December 13, 2011. A true and correct copy of the '066 patent is attached as Exhibit L. Genentech is the owner by assignment of the '066 patent.

63. U.S. Patent No. 8,440,402 ("the '402 patent"), titled "Gene Detection Assay for Improving the Likelihood of an Effective Response to a HER2 Antibody Cancer Therapy," was duly and legally issued by the Patent Office on May 14, 2013. A true and correct copy of the '402 patent is attached as Exhibit M. Genentech is the owner by assignment of the '402 patent.

Cell Culture, Purification, and Antibody Manufacturing Patents

64. U.S. Patent Nos. 6,121,428, 6,620,918, 8,512,983, 8,574,869, and 9,714,293 claim novel techniques developed by Genentech relating to various aspects of cell culture, purification, and antibody purification.

65. U.S. Patent No. 6,121,428 ("the '428 patent"), titled "Protein Recovery," was duly and legally issued by the Patent Office on September 19, 2000. A true and correct copy of the '428 patent is attached as Exhibit N. Genentech is the owner by assignment of the '428 patent.

66. U.S. Patent No. 6,620,918 ("the '918 patent"), titled "Separation of Polypeptide Monomers," was duly and legally issued by the Patent Office on September 16, 2003. A true and correct copy of the '918 patent is attached as Exhibit O. Genentech is the owner by assignment of the '918 patent.

67. U.S. Patent No. 8,512,983 ("the '983 patent"), titled "Production of Proteins in Glutamine-Free Cell Culture Media," was duly and legally issued by the Patent Office on August 20, 2013. A true and correct copy of the '983 patent is attached as Exhibit P. Genentech is the owner by assignment of the '983 patent.

68. U.S. Patent No. 8,574,869 ("the '869 patent"), titled "Prevention of Disulfide Bond Reduction During Recombinant Production of Polypeptides," was duly and legally issued by the Patent Office on November 5, 2013. A true and correct copy of the '869 patent is attached as Exhibit Q. Genentech is the owner by assignment of the '869 patent.

69. U.S. Patent No. 9,714,293 ("the '293 patent"), titled "Production of Proteins in Glutamine-Free Cell Culture Media," was duly and legally issued by the Patent Office on July 25, 2017. A true and correct copy of the '293 patent is attached as Exhibit R. Genentech is the owner by assignment of the '293 patent.

COUNT I
INFRINGEMENT OF U.S. PATENT NO. 6,331,415

70. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

71. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Plaintiffs believe that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Plaintiffs if a person not licensed by Plaintiffs engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '415 patent. Genentech included the '415 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '415 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

72. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '415 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '415 patent under 35 U.S.C. § 271(e)(2)(C)(i).

73. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Plaintiffs reasonably believe that Amgen will infringe the '415 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence

commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

74. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Plaintiffs are entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '415 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

75. Amgen has knowledge of and is aware of the '415 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '415 patent is willful.

76. Plaintiffs will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '415 patent. Plaintiffs have no adequate remedy at law.

77. Plaintiffs are entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT II
INFRINGEMENT OF U.S. PATENT NO. 7,923,221

78. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

79. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Plaintiffs believe that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Plaintiffs if a person not licensed by Plaintiffs engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '221 patent.

Genentech included the '221 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '221 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

80. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '221 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '221 patent under 35 U.S.C. § 271(e)(2)(C)(i).

81. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Plaintiffs reasonably believe that Amgen will infringe the '221 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

82. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Plaintiffs are entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '221 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

83. Amgen has knowledge of and is aware of the '221 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this

Complaint. Amgen's infringement of the '221 patent is willful.

84. Plaintiffs will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '221 patent. Plaintiffs have no adequate remedy at law.

85. Plaintiffs are entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT III
INFRINGEMENT OF U.S. PATENT NO. 6,407,213

86. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

87. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '213 patent. Genentech included the '213 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '213 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

88. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '213 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '213 patent under 35 U.S.C. § 271(e)(2)(C)(i).

89. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '213 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

90. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '213 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

91. Amgen has knowledge of and is aware of the '213 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '213 patent is willful.

92. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '213 patent. Genentech has no adequate remedy at law.

93. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT IV
INFRINGEMENT OF U.S. PATENT NO. 7,846,441

94. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

95. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '441 patent. Genentech included the '441 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '441 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

96. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '441 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '441 patent under 35 U.S.C. § 271(e)(2)(C)(i).

97. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '441 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence

commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

98. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '441 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

99. Amgen has knowledge of and is aware of the '441 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '441 patent is willful.

100. By the filing of an aBLA that, on information and belief, includes a proposed package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '441 patent, either literally or under the doctrine of equivalents.

101. Upon information and belief, Amgen is aware, have knowledge, and/or are willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's proposed package insert and, therefore, will directly infringe at least one claim of the '441 patent, either literally or under the doctrine of equivalents.

102. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '441 patent, either literally or under the doctrine of equivalents, by at least Amgen's proposed package insert for the Amgen aBLA product.

103. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '441 patent. Genentech has no adequate remedy at law.

104. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT V
INFRINGEMENT OF U.S. PATENT NO. 7,892,549

105. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

106. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '549 patent. Genentech included the '549 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '549 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

107. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '549 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '549 patent under 35 U.S.C. § 271(e)(2)(C)(i).

108. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '549 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

109. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '549 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

110. Amgen has knowledge of and is aware of the '549 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '549 patent is willful.

111. By the filing of an aBLA that, on information and belief, includes a proposed package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '549 patent, either literally or under the doctrine of equivalents.

112. Upon information and belief, Amgen is aware, have knowledge, and/or are willfully blind to the fact that patients will administer and/or use and medical practitioners will

prescribe and/or administer the Amgen aBLA product according to Amgen's proposed package insert and, therefore, will directly infringe at least one claim of the '549 patent, either literally or under the doctrine of equivalents.

113. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '549 patent, either literally or under the doctrine of equivalents, by at least Amgen's proposed package insert for the Amgen aBLA product.

114. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '549 patent. Genentech has no adequate remedy at law.

115. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT VI
INFRINGEMENT OF U.S. PATENT NO. 8,425,908

116. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

117. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '908 patent. Genentech included the '908 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a

claim-by-claim basis, the factual and legal basis of its opinion that the '908 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

118. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '908 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '908 patent under 35 U.S.C. § 271(e)(2)(C)(i).

119. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '908 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

120. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '908 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

121. Amgen has knowledge of and is aware of the '908 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '908 patent is willful.

122. By the filing of an aBLA that, on information and belief, includes a proposed package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '908 patent, either literally or under the doctrine of equivalents.

123. Upon information and belief, Amgen is aware, have knowledge, and/or are willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's proposed package insert and, therefore, will directly infringe at least one claim of the '908 patent, either literally or under the doctrine of equivalents.

124. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '908 patent, either literally or under the doctrine of equivalents, by at least Amgen's proposed package insert for the Amgen aBLA product.

125. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '908 patent. Genentech has no adequate remedy at law.

126. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT VII
INFRINGEMENT OF U.S. PATENT NO. 6,627,196

127. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

128. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '196 patent. Genentech included the '196 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '196 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

129. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '196 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '196 patent under 35 U.S.C. § 271(e)(2)(C)(i).

130. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '196 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

131. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '196 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

132. Amgen has knowledge of and is aware of the '196 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '196 patent is willful.

133. By the filing of an aBLA that, on information and belief, includes a proposed package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '196 patent, either literally or under the doctrine of equivalents.

134. Upon information and belief, Amgen is aware, have knowledge, and/or are willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's proposed package insert and, therefore, will directly infringe at least one claim of the '196 patent, either literally or under the doctrine of equivalents.

135. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '196 patent, either literally or under the doctrine of equivalents, by at least Amgen's proposed package insert for the Amgen aBLA product.

136. Plaintiffs will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '196 patent. Plaintiffs have

no adequate remedy at law.

137. Plaintiffs are entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT VIII
INFRINGEMENT OF U.S. PATENT NO. 7,371,379

138. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

139. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '379 patent. Genentech included the '379 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '379 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

140. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '379 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '379 patent under 35 U.S.C. § 271(e)(2)(C)(i).

141. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '379 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its

activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

142. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '379 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

143. Amgen has knowledge of and is aware of the '379 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '379 patent is willful.

144. By the filing of an aBLA that, on information and belief, includes a proposed package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '379 patent, either literally or under the doctrine of equivalents.

145. Upon information and belief, Amgen is aware, have knowledge, and/or are willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's proposed package insert and, therefore, will directly infringe at least one claim of the '379 patent, either literally or under the doctrine of equivalents.

146. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '379 patent, either literally or under the doctrine of equivalents, by at least Amgen's proposed package insert for the Amgen aBLA product.

147. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '379 patent. Genentech has no adequate remedy at law.

148. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT IX
INFRINGEMENT OF U.S. PATENT NO. 6,417,335

149. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

150. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '335 patent. Genentech included the '335 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '335 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

151. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '335 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '335 patent under 35 U.S.C. § 271(e)(2)(C)(i).

152. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '335 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

153. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '335 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

154. Amgen has knowledge of and is aware of the '335 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '335 patent is willful.

155. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '335 patent. Genentech has no adequate remedy at law.

156. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT X
INFRINGEMENT OF U.S. PATENT NO. 9,249,218

157. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

158. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the ’218 patent. Genentech included the ’218 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the ’218 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

159. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the ’218 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the ’218 patent under 35 U.S.C. § 271(e)(2)(C)(i).

160. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the ’218 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the

use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

161. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '218 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

162. Amgen has knowledge of and is aware of the '218 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '218 patent is willful.

163. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '218 patent. Genentech has no adequate remedy at law.

164. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT XI
INFRINGEMENT OF U.S. PATENT NO. 7,993,834

165. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

166. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent

infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '834 patent. Genentech included the '834 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '834 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

167. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '834 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '834 patent under 35 U.S.C. § 271(e)(2)(C)(i).

168. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '834 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

169. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and

promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '834 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

170. Amgen has knowledge of and is aware of the '834 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '834 patent is willful.

171. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '834 patent. Genentech has no adequate remedy at law.

172. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT XII
INFRINGEMENT OF U.S. PATENT NO. 8,076,066

173. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

174. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '066 patent. Genentech included the '066 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '066 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

175. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '066 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '066 patent under 35 U.S.C. § 271(e)(2)(C)(i).

176. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '066 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

177. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '066 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

178. Amgen has knowledge of and is aware of the '066 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '066 patent is willful.

179. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '066 patent. Genentech has no adequate remedy at law.

180. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT XIII
INFRINGEMENT OF U.S. PATENT NO. 8,440,402

181. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

182. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '402 patent. Genentech included the '402 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '402 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

183. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '402 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '402 patent under 35 U.S.C. § 271(e)(2)(C)(i).

184. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '402 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the

use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

185. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '402 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

186. Amgen has knowledge of and is aware of the '402 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '402 patent is willful.

187. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '402 patent. Genentech has no adequate remedy at law.

188. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT XIV
INFRINGEMENT OF U.S. PATENT NO. 6,121,428

189. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

190. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent

infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '428 patent. Genentech included the '428 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '428 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

191. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '428 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '428 patent under 35 U.S.C. § 271(e)(2)(C)(i).

192. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen has infringed the '428 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement.

193. Amgen had knowledge of and was aware of the '428 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Amgen's infringement of the '428 patent was willful.

COUNT XV
INFRINGEMENT OF U.S. PATENT NO. 6,620,918

194. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

195. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '918 patent. Genentech included the '918 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '918 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

196. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '918 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '918 patent under 35 U.S.C. § 271(e)(2)(C)(i).

197. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '918 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

198. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '918 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

199. Amgen has knowledge of and is aware of the '918 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '918 patent is willful.

200. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '918 patent. Genentech has no adequate remedy at law.

201. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT XVI
INFRINGEMENT OF U.S. PATENT NO. 8,512,983

202. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

203. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '983 patent. Genentech included the '983 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a

claim-by-claim basis, the factual and legal basis of its opinion that the '983 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

204. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '983 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '983 patent under 35 U.S.C. § 271(e)(2)(C)(i).

205. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '983 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

206. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '983 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

207. Amgen has knowledge of and is aware of the '983 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '983 patent is willful.

208. Genentech will suffer irreparable injury for which damages are an inadequate

remedy unless Amgen is enjoined from infringing the claims of the '983 patent. Genentech has no adequate remedy at law.

209. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT XVII
INFRINGEMENT OF U.S. PATENT NO. 8,574,869

210. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

211. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '869 patent. Genentech included the '869 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '869 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

212. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '869 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '869 patent under 35 U.S.C. § 271(e)(2)(C)(i).

213. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will

infringe the '869 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262(*l*)(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

214. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '869 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

215. Amgen has knowledge of and is aware of the '869 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '869 patent is willful.

216. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '869 patent. Genentech has no adequate remedy at law.

217. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT XVIII
INFRINGEMENT OF U.S. PATENT NO. 9,714,293

218. Plaintiffs incorporate by reference paragraphs 1-69 as if fully set forth herein.

219. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '293 patent. Genentech included the '293 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '293 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C.§ 262(*l*)(3)(C).

220. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '293 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '293 patent under 35 U.S.C. § 271(e)(2)(C)(i).

221. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(*l*)(2), Genentech reasonably believes that Amgen will infringe the '293 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its proposed ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement is imminent because, among other things, Amgen has purported to notify Genentech pursuant to 42 U.S.C. § 262())(8)(A) that it may commence commercial marketing of ABP 980 in the United States as early as [redacted]. *See supra* ¶ 36.

222. Pursuant to 42 U.S.C. § 262(*l*)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's proposed ABP 980 drug product will infringe the '293 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

223. Amgen has knowledge of and is aware of the '293 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(*l*)(3)(A) and the filing of this Complaint. Amgen's infringement of the '293 patent is willful.

224. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '293 patent. Genentech has no adequate remedy at law.

225. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(*l*)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

PRAYER FOR RELIEF

WHEREFORE, Plaintiffs respectfully request that this Court enter judgment in their favor against Amgen and grant the following relief:

a. a judgment that Amgen has infringed or induced infringement of one or more claims of the asserted patents under 35 U.S.C. § 271(e)(2)(C);

b. a judgment that Amgen has infringed or will infringe, or has induced or will induce infringement, of one or more claims of the asserted patents by engaging in the manufacture, import, offer for sale, sale, or use within the United States of the Amgen aBLA product before the expirations of the asserted patents;

c. preliminary and/or permanent equitable relief, including but not limited to a preliminary and permanent injunction that enjoins Amgen, its officers, partners, agents, servants, employees, parents, subsidiaries, affiliate corporations, other related business entities, and all other persons acting in concert, participation, or in privity with Amgen and/or its successors or assigns from infringing the asserted patents, or contributing to or inducing anyone to do the same, by acts including the manufacture, use, offer to sell, sale, distribution, or importation of any current or future versions of a product that infringes, or the use or manufacturing of which infringes the asserted patents;

d. monetary damages in the event that Amgen imports, manufactures, or launches its biosimilar product and/or otherwise practices the patented inventions in the United States prior to the expiration of the asserted patents, including lost profits and/or a reasonable royalty, and an accounting and/or ongoing royalty for any post-judgment infringement;

e. a judgment that Amgen's infringement was willful and enhancement of any monetary damages pursuant to 35 U.S.C. § 284;

f. a declaration that this is an exceptional case and an award to Plaintiffs of their attorneys' fees, costs, and expenses pursuant to 35 U.S.C. § 271(e)(4) and 35 U.S.C. § 285; and

g. such other relief as this Court may deem just and proper.

Dated: July 19, 2018

Respectfully submitted,

/s/ Daniel M. Silver

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EXHIBIT A

US006331415B1

(12) **United States Patent**
Cabilly et al.

(10) **Patent No.: US 6,331,415 B1**
(45) **Date of Patent: Dec. 18, 2001**

(54) **METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN**

(75) Inventors: **Shmuel Cabilly**, Monrovia; **Herbert L. Heyneker**, Burlingame; **William E. Holmes**, Pacifica; **Arthur D. Riggs**, La Verne; **Ronald B. Wetzel**, San Francisco, all of CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **07/205,419**

(22) Filed: **Jun. 10, 1988**

Related U.S. Application Data

(63) Continuation of application No. 06/483,457, filed on Apr. 8, 1983, now Pat. No. 4,816,567.

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(57) **ABSTRACT**

The invention relates to processes for producing an immunoglobulin or an immunologically functional immunoglobulin fragment containing at least the variable domains of the immunoglobulin heavy and light chains. The processes can use one or more vectors which produce both the heavy and light chains or fragments thereof in a single cell. The invention also relates to the vectors used to produce the immunoglobulin or fragment, and to cells transformed with the vectors.

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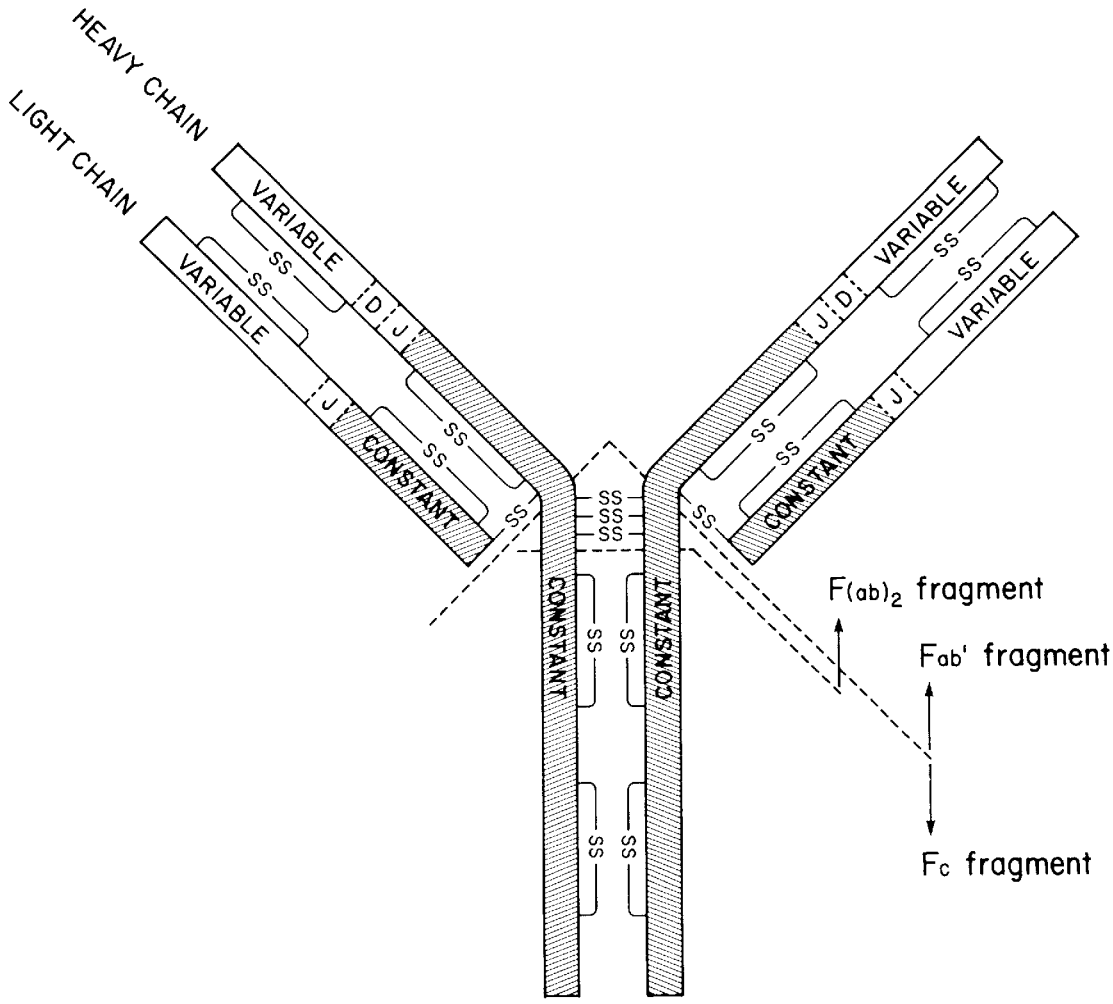
HEAVY CHAIN
LIGHT CHAIN
VARIABLE
D
J
CONSTANT
SS
F(ab)2 fragment
Fab' fragment
Fc fragment

Fig. 1.

```
                                                                                                                    haeIII
                                                  tth111                                  tth111        hphI       haeI
1       GTTGCTGTGG TTGTCTGGTG TTGAAGGAGA CATTGTGATG ACCCAGTCTC ACAAATTCAT GTCCACATCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG
        CAACGACACC AACAGACCAC AACTTCCTCT GTAACACTAC TGGGTCAGAG TGTTTAAGTA CAGGTGTAGT CATCCTCTGT CCCAGTCGTA GTGGACGTTC
                                                                                                      sfaNI

                                                                                                     scrFI
                           fnu4HI     scrFI              scrFI                                       nciI
                fokI       bbv        ecoRII             ecoRII                                       hpaII      hinfI
101     GCCAGTCAGG ATGTGGGTGC TGCTATAGCC TGGTATCAAC AGAAACCAGG ACAATCTCCT AAACTACTGA TTTACTGGGC ATCCACCCGG CACACTGGAG
        CGGTCAGTCC TACACCCACG ACGATATCGG ACCATAGTTG TCTTTGGTCC TGTTAGAGGA TTTGATGACT AAATGACCCG TAGGTGGGCC GTGTGACCTC
                                                                                     fokI    sfaNI

                                 xhoII
             sau3A                sau3A
             dpnI                 dpnI                   hphI                                                hincII
201     TCCCTGATCG CTTCACAGGC AGTGGATCTG GGACAGATTT CACTCTCACC ATTAGCAATG TGCAGTCTGA TGACTTGGCA GATTATTTCT GTCAACAATA
        AGGGACTAGC GAAGTGTCCG TCACCTAGAC CCTGTCTAAA GTGAGAGTGG TAATCGTTAC ACGTCAGACT ACTGAACCGT CTAATAAAGA CAGTTGTTAT

                                       sau96                          fnu4HI                                 hpaI
                   mnlI                avaII  aluI      aluI sfaNI    bbv                  mboII             hincII
301     TAGCGGGTAT CCTCTCACGT TCGGTGCTGG GACCAAGCTG GAGCTGAAAC GGGCTGATGC TGCACCAACT GTATCCATCT TCCCACCATC CAGTGAGCAG
        ATCGCCCATA GGAGAGTGCA AGCCACGACC CTGGTTCGAC CTCGACTTTG CCCGACTACG ACGTGGTTGA CATAGGTAGA AGGGTGGTAG GTCACTCGTC
                                                                                           fokI

                         mnlI
                   mnlI  ddeI                 xmnI                                    mboII                  acyI
401     TTAACATCTG GAGGTGCCTC AGTCGTGTGC TTCTTGAACA ACTTCTACCC CAAAGACATC AATGTCAAGT GGAAGATTGA TGGCAGTGAA CGACAAAATG
        AATTGTAGAC CTCCACGGAG TCAGCACACG AAGAACTTGT TGAAGATGGG GTTTCTGTAG TTACAGTTCA CCTTCTAACT ACCGTCACTT GCTGTTTTAC
```

Fig. 2A.

```
                                  sau3A
                                  dpnI                                            fnu4HI
         hgaI                   bclI                                              bbv        mnlI  hincII                                  aluI
501      GCGTCCTGAA CAGTTGGACT GATCAGGACA GCAAAGACAG CACCTACAGC ATGAGCAGCA CCCTCACGTT GACCAAGGAC GAGTATGAAC GACATAACAG
         CGCAGGACTT GTCAACCTGA CTAGTCCTGT CGTTTCTGTC GTGGATGTCG TACTCGTCGT GGGAGTGCAA CTGGTTCCTG CTCATACTTG CTGTATTGTC

                    mnlI                                                                                     sau96   hgaI
                      haeIII                                                                                     ddeI
                     haeI                    hphI             aluI                                           avaII   acyI
601      CTATACCTGT GAGGCCACTC ACAAGACATC AACTTCACCC ATTGTCAAGA GCTTCAACAG GAATGAGTGT TAGAGACAAA GGTCCTGAGA CGCCACCACC
         GATATGGACA CTCCGGTGAG TGTTCTGTAG TTGAAGTGGG TAACAGTTCT CGAAGTTGTC CTTACTCACA ATCTCTGTTT CCAGGACTCT GCGGTGGTGG

                                                                                                                 mnlI
         aluI     aluI            mboII    ddeI          mnlI                                 hgiA        mnlI        mnlI
701      AGCTCCCCAG CTCCATCCTA TCTTCCCTTC TAAGGTCTTG GAGGCTTCCC CACAAGCGAC CTACCACTGT TGCGGTGCTC CAAACCTCCT CCCCACCTCC
         TCGAGGGGTC GAGGTAGGAT AGAAGGGAAG ATTCCAGAAC CTCCGAAGGG GTGTTCGCTG GATGGTGACA ACGCCACGAG GTTTGGAGGA GGGGTGGAGG
             fokI

            mnlI
         mnlI   mnlI                                          xmnI                 hinfI
801      TTCTCCTCCT CCTCCCTTTC CTTGGCTTTT ATCATGCTAA TATTTGCAGA AAATATTCAA TAAAGTGAGT CTTTGCACTT GA
         AAGAGGAGGA GGAGGGAAAG GAACCGAAAA TAGTACGATT ATAAACGTCT TTTATAAGTT ATTTCACTCA GAAACGTGAA CT
```

nucleotides: 882

Fig. 2B.

```
     -9                                      1                                       10                                      20
    leu leu trp leu ser gly val glu gly asp ile val met thr gln ser his lys phe met ser thr ser val gly asp arg val ser
G   UUG CUG UGG UUG UCU GGU GUU GAA GGA GAC AUU GUG AUG ACC CAG UCU CAC AAA UUC AUG UCC ACA UCA GUA GGA GAC AGG GUC AGC

                                     30                                      40                                      50
ile thr cys lys ala ser gln asp val gly ala ala ile ala trp tyr gln gln lys pro gly gln ser pro lys leu leu ile tyr trp
AUC ACC UGC AAG GCC AGU CAG GAU GUG GGU GCU GCU AUA GCC UGG UAU CAA CAG AAA CCA GGA CAA UCU CCU AAA CUA CUG AUU UAC UGG

                                     60                                      70                                      80
ala ser thr arg his thr gly val pro asp arg phe thr gly ser gly ser gly thr asp phe thr leu thr ile ser asn val gln ser
GCA UCC ACC CGG CAC ACU GGA GUC CCU GAU CGC UUC ACA GGC AGU GGA UCU GGG ACA GAU UUC ACU CUC ACC AUU AGC AAU GUG CAG UCU

                                     90                                     100                                     110
asp asp leu ala asp tyr phe cys gln gln tyr ser gly tyr pro leu thr phe gly ala gly thr lys leu glu leu lys arg ala asp
GAU GAC UUG GCA GAU UAU UUC UGU CAA CAA UAU AGC GGG UAU CCU CUC ACG UUC GGU GCU GGG ACC AAG CUG GAG CUG AAA CGG GCU GAU

                                    120                                     130                                     140
ala ala pro thr val ser ile phe pro pro ser ser glu gln leu thr ser gly gly ala ser val val cys phe leu asn asn phe tyr
GCU GCA CCA ACU GUA UCC AUC UUC CCA CCA UCC AGU GAG CAG UUA ACA UCU GGA GGU GCC UCA GUC GUG UGC UUC UUG AAC AAC UUC UAC

                                    150                                     160                                     170
pro lys asp ile asn val lys trp lys ile asp gly ser glu arg gln asn gly val leu asn ser trp thr asp gln asp ser lys asp
CCC AAA GAC AUC AAU GUC AAG UGG AAG AUU GAU GGC AGU GAA CGA CAA AAU GGC GUC CUG AAC AGU UGG ACU GAU CAG GAC AGC AAA GAC

                                    180                                     190                                     200
ser thr tyr ser met ser ser thr leu thr leu thr lys asp glu tyr glu arg his asn ser tyr thr cys glu ala thr his lys thr
AGC ACC UAC AGC AUG AGC AGC ACC CUC ACG UUG ACC AAG GAC GAG UAU GAA CGA CAU AAC AGC UAU ACC UGU GAG GCC ACU CAC AAG ACA

                                    210             214
ser thr ser pro ile val lys ser phe asn arg asn glu cys AM
UCA ACU UCA CCC AUU GUC AAG AGC UUC AAC AGG AAU GAG UGU UAG AGACAAAGGUCCUGAGACGCCACCACCAGCUCCCCAGCUCCAUCCUAUCUUCCCUUCUAA

GGUCUUGGAGGCUUCCCCACAAGCGACCUACCACUGUUGCGGUGCUCCAAACCUCCUCCCCACCUCCUUCUCCUCCUCCUCCCUUUCCUUGGCUUUUAUCAUGCUAAUAUUUGCAGAAAA

UAUUCAAUAAAGUGAGUCUUUGCACUUGA
```

Fig. 3

```
                           sau96                                ddeI
         hinfI             avaII mnlI                              aluI                              ahaIII          sfaNI
1        GAGTCAGCAC TGAACACGGA CCCCTCACGA TGAACTTCGG GCTCAGCTTG ATTTACCTTG TCCTTGTTTT AAAAGTTGTC CAGTGTGAAG TGATGCTGGT
         CTCAGTCGTG ACTTGTGCCT GGGGAGTGCT ACTTGAAGCC CGAGTCGAAC TAAATGGAAC AGGAACAAAA TTTTCAACAG GTCACACTTC ACTACGACCA

                                      scrFI   sau96
                                         mnlI                         fnu4HI
          hinfI       hinfI           ecoRII  avaII                   bbv mnlI   hinfI
101      GGAGTCTGGG GGAGTCTTAA TGGAGCCTGG AGGGTCCCTG AAACTCTCCT GTGCAGCCTC TGGATTCACT TTCAGTAGAT ATGCCATGTC TTGGGTTCGC
         CCTCAGACCC CCTCAGAATT ACCTCGGACC TCCCAGGGAC TTTGAGAGGA CACGTCGGAG ACCTAAGTGA AAGTCATCTA TACGGTACAG AACCCAAGCG

              hpaII     mnlI                                                                                  hphI
           hinfI      mboII                                                                                hinfI
201      CAGACTCCGG AGAAGAGGCT GGAGTGGGTC GCAACCATTA GTAGTGGTGG TAGTTCACAC CTTCCATCCA GACAGTGTGA AGGGCGATTC ACCATCTCCA
         GTCTGAGGCC TCTTCTCCGA CCTCACCCAG CGTTGGTAAT CATCACCACC ATCAAGTGTG GAAGGTAGGT CTGTCACACT TCCCGCTAAG TGGTAGAGGT
                                                                   fokI

                                                                 mnlI     mnlI
                                     rsaI                      ddeI    ddeI        haeIII                    mnlI
301      GAGACAATGC CAAGAACACC CTGTACCTGC AAATGAGCAG TCTGAGGTCT GAGGACACGG CCATGTATTA CTGTGCAAGA CCCCCTCTTA TTTCGTTAGT
         CTCTGTTACG GTTCTTGTGG GACATGGACG TTTACTCGTC AGACTCCAGA CTCCTGTGCC GGTACATAAT GACACGTTCT GGGGGAGAAT AAAGCAATCA

                                                                                                                   xhoII
                                                                                                                scrFI
                                                                                                             sau96    sau3A
                                                          mnlI           mnlI                                   ecoRII
                                                           ddeI   hphI     ddeI                              haeIII   dpnI
401      AGCGGACTAT GCTATGGACT ACTGGGGTCA AGGAACCTCA GTCACCGTCT CCTCAGCCAA AACGACACCC CCATCTGTCT ATCCACTGGC CCCTGGATCT
         TCGCCTGATA CGATACCTGA TGACCCCAGT TCCTTGGAGT CAGTGGCAGA GGAGTCGGTT TTGCTGTGGG GGTAGACAGA TAGGTGACCG GGGACCTAGA
```

Fig. 4A.

```
                                    scrFI                                                      xhoII
                         ncoI   sfaNI    fokI                                                   sau3A
        fnu4HI                hphI  ecoRII    scrFI                                     scrFI        dpnI
        bbv                   bstEII          ecoRII                ddeI                ecoRII      bamHI
501     GCTGCCCAAA CTAACTCCAT GGTGACCCTG GGATGCCTGG TCAAGGGCTA TTTCCCTGAG CCAGTGACAG TGACCTGGAA CTCTGGATCC CTGTCCAGCG
        CGACGGGTTT GATTGAGGTA CCACTGGGAC CCTACGGACC AGTTCCCGAT AAAGGGACTC GGTCACTGTC ACTGGACCTT GAGACCTAGG GACAGGTCGC

                                                            fnu4HI                           sau96
                        pvuII                               bbv  ddeI                    mnlI
          hgiA           aluI    pstI        mnlI       ddeI   aluI             mnlI         haeIII           hphI
601     GTGTGCACAC CTTCCCAGCT GTCCTGCAGT CTGACCTCTA CACTCTGAGC AGCTCAGTGA CTGTCCCCTC CAGCCCTCGG CCCAGCGAGA CCGTCACCTG
        CACACGTGTG GAAGGGTCGA CAGGACGTCA GACTGGAGAT GTGAGACTCG TCGAGTCACT GACAGGGGAG GTCGGGAGCC GGGTCGCTCT GGCAGTGGAC

                     scrFI
                        haeIII
                     nciI     fnu4HI                           scrFI
               bglI   hpaII   bbv                              ecoRII                    ndeI  rsaI
701     CAACGTTGCC CACCCGGCCA GCAGCACCAA GGTGGACAAG AAAATTGTGC CCAGGGATTG TGGTTGTAAG CCTTGCATAT GTACAGTCCC AGAAGTATCA
        GTTGCAACGG GTGGGCCGGT CGTCGTGGTT CCACCTGTTC TTTTAACACG GGTCCCTAAC ACCAACATTC GGAACGTATA CATGTCAGGG TCTTCATAGT

                                                                   mstII                                     sau3A
                                                hphI           hinfI                                          dpnI  mnlI
            mboII  mboII               fokI hgiA                    ddeI                  accI           fokI    avaI
801     TCTGTCTTCA TCTTCCCCCC AAAGCCCAAG GATGTGCTCA CCATTACTCT GACTCCTAAG GTCACGTGTG TTGTGGTAGA CATCAGCAAG GATGATCCCG
        AGACAGAAGT AGAAGGGGGG TTTCGGGTTC CTACACGAGT GGTAATGAGA CTGAGGATTC CAGTGCACAC AACACCATCT GTAGTCGTTC CTACTAGGGC

                                                                      smaI
                                                                       scrFI
                                                                      scrFI
                                                                       nciI
                                                                      nciI
         sau96     pvuII                   mnlI          ddeI          hpaII
         avaII      aluI                      hgiA     aluI  hgaI     avaI  mnlI                        ddeI
901     AGGTCCAGTT CAGCTGGTTT GTAGATGATG TGGAGGTGCA CACAGCTCAG ACGCAACCCC GGGAGGAGCA GTTCAACAGC ACTTTCCGCT CAGTCAGTGA
        TCCAGGTCAA GTCGACCAAA CATCTACTAC ACCTCCACGT GTGTCGAGTC TGCGTTGGGG CCCTCCTCGT CAAGTTGTCG TGAAAGGCGA GTCAGTCACT
```

Fig. 4B.

```
                                                                         fnu4HI
                      scrFI                                              bbv
                      ecoRII                                   hincII      aluI               taqI
1001  ACTTCCCATC ATGCACCAGG ACTGGCTCAA TGGCAAGGAG TTCAAATGCA GGGTCAACAG TGCAGCTTTC CCTGCCCCCA TCGAGAAAAC CATCTCCAAA
      TGAAGGGTAG TACGTGGTCC TGACCGAGTT ACCGTTCCTC AAGTTTACGT CCCAGTTGTC ACGTCGAAAG GGACGGGGGT AGCTCTTTTG GTAGAGGTTT

                                                                      haeIII
                                                                     haeI
                                     rsaI           mnlI             balI
1101  ACCAAAGGCA GACCGAAGGC TCCACAGGTG TACACCATTC CACCTCCCAA GGAGCAGATG GCCAAGGATA AAGTCAGTCT GACCTGCATG ATAACAGACT
      TGGTTTCCGT CTGGCTTCCG AGGTGTCCAC ATGTGGTAAG GTGGAGGGTT CCTCGTCTAC CGGTTCCTAT TTCAGTCAGA CTGGACGTAC TATTGTCTGA

                                                  fnu4HI
      mboII   mboII                               bbv                         ddeI
1201  TCTTCCCTGA AGACATTACT GTGGAGTGGC AGTGGAATGG GCAGCCAGCG GAGAACTACA AGAACACTCA GCCCATCATG AACACGAATG GCTCTTACTT
      AGAAGGGACT TCTGTAATGA CACCTCACCG TCACCTTACC CGTCGGTCGC CTCTTGATGT TCTTGTGAGT CGGGTAGTAC TTGTGCTTAC CGAGAATGAA

                                                                                        sau96
                                                                                      mnlI                    mboII
       accI       aluI          mboII         mnlI              hphI                     haeIII               ddeI
1301  CGTCTACAGC AAGCTCAATG TGCAGAAGAG CAACTGGGAG GCAGGAAATA CTTTCACCTG CTCTGTGTTA CATGAGGGCC TGCACAACCA CCATACTGAG
      GCAGATGTCG TTCGAGTTAC ACGTCTTCTC GTTGACCCTC CGTCCTTTAT GAAAGTGGAC GAGACACAAT GTACTCCCGG ACGTGTTGGT GGTATGACTC

                         scrFI      sau3A                       sau96
           mnlI          ecoRII     dpnI                  mnlI  avaII      hinfI          mnlI     mnlI
1401  AAGAGCCTCT CCCACTCTCC TGGTAAATGA TCCCAGTGTC CTTGGAGCCC TCTGGTCCTA CAGGACTCTG ACACCTACCT CCACCCCTCC CTGTATAAAT
      TTCTCGGAGA GGGTGAGAGG ACCATTTACT AGGGTCACAG GAACCTCGGG AGACCAGGAT GTCCTGAGAC TGTGGATGGA GGTGGGGAGG GACATATTTA

1501  AAAGCACCCA GCACTGCCTT GGGAAAAA
      TTTCGTGGGT CGTGACGGAA CCCTTTTT
```

Fig. 4C.

-10 1
met asn phe gly leu ser leu ile tyr leu val leu val leu lys val val gln cys glu
GAGUCAGCACUGAACACGGACCCCUCACG AUG AAC UUC GGG CUC AGC UUG AUU UAC CUU GUC CUG GUU UUA AAA GUU GUC CAG UGU GAA

10 20 30
val met leu val glu ser gly gly val leu met glu pro gly gly ser leu lys leu ser cys ala ala ser gly phe thr phe ser arg
GUG AUG CUG GUG GAG UCU GGG GGA GUC UUA AUG GAG CCU GGA GGG UCC CUG AAA CUC UCC UGU GCA GCC UCU GGA UUC ACU UUC AGU AGA

40 50 60
tyr ala met ser trp val arg gln thr pro glu lys arg leu glu trp val ala thr ile ser ser gly **gly ser ser his leu pro ser**
UAU GCC AUG UCU UGG GUU CGC CAG ACU CCG GAG AAG AGG CUG GAG UGG GUC GCA ACC AUU AGU AGU GGU **GGU AGU UCA CAC CUU CCA UCC**

70 80 90
arg gln cys glu gly arg phe thr ile ser arg asp asn ala lys asn thr leu tyr leu gln met ser ser leu arg ser glu asp thr
AGA CAG UGU GAA GGG CGA UUC ACC AUC UCC AGA GAC AAU GCC AAG AAC ACC CUG UAC CUG CAA AUG AGC AGU CUG AGG UCU GAG GAC ACG

100 110 120
ala met tyr tyr cys ala arg pro pro leu ile ser leu val ala asp tyr ala met asp tyr trp gly gln gly thr ser val thr val
GCC AUG UAU UAC UGU GCA AGA CCC CCU CUU AUU UCG UUA GUA GCG GAC UAU GCU AUG GAC UAC UGG GGU CAA GGA ACC UCA GUC ACC GUC

130 140 150
ser ser ala lys thr thr pro pro ser val tyr pro leu ala pro gly ser ala ala gln thr asn ser met val thr leu gly cys leu
UCC UCA GCC AAA ACG ACA CCC CCA UCU GUC UAU CCA CUG GCC CCU GGA UCU GCU GCC CAA ACU AAC UCC AUG GUG ACC CUG GGA UGC CUG

160 170 180
val lys gly tyr phe pro glu pro val thr val thr trp asn ser gly ser leu ser ser gly val his thr phe pro ala val leu gln
GUC AAG GGC UAU UUC CCU GAG CCA GUG ACA GUG ACC UGG AAC UCU GGA UCC CUG UCC AGC GGU GUG CAC ACC UUC CCA GCU GUC CUG CAG

190 200 210
ser asp leu tyr thr leu ser ser ser val thr val pro ser ser pro arg pro ser glu thr val thr cys asn val ala his pro ala
UCU GAC CUC UAC ACU CUG AGC AGC UCA GUG ACU GUC CCC UCC AGC CCU CGG CCC AGC GAG ACC GUC ACC UGC AAC GUU GCC CAC CCG GCC

220 230 240
ser ser thr lys val asp lys lys ile val pro arg asp cys gly cys lys pro cys ile cys thr val pro glu val ser ser val phe
AGC AGC ACC AAG GUG GAC AAG AAA AUU GUG CCC AGG GAU UGU GGU UGU AAG CCU UGC AUA UGU ACA GUC CCA GAA GUA UCA UCU GUC UUC

Fig. 5A.

```
                                250                                     260                                     270
ile phe pro pro lys pro lys asp val leu thr ile thr leu thr pro lys val thr cys val val val asp ile ser lys asp asp pro
AUC UUC CCC CCA AAG CCC AAG GAU GUG CUC ACC AUU ACU CUG ACU CCU AAG GUC ACG UGU GUU GUG GUA GAC AUC AGC AAG GAU GAU CCC

                                280                                     290                                     300
glu val gln phe ser trp phe val asp asp val glu val his thr ala gln thr gln pro arg glu glu gln phe asn ser thr phe arg
GAG GUC CAG UUC AGC UGG UUU GUA GAU GAU GUG GAG GUG CAC ACA GCU CAG ACG CAA CCC CGG GAG GAG CAG UUC AAC AGC ACU UUC CGC

                                310                                     320                                     330
ser val ser glu leu pro ile met his gln asp trp leu asn gly lys glu phe lys cys arg val asn ser ala ala phe pro ala pro
UCA GUC AGU GAA CUU CCC AUC AUG CAC CAG GAC UGG CUC AAU GGC AAG GAG UUC AAA UGC AGG GUC AAC AGU GCA GCU UUC CCU GCC CCC

                                340                                     350                                     360
ile glu lys thr ile ser lys thr lys gly arg pro lys ala pro gln val tyr thr ile pro pro pro lys glu gln met ala lys asp
AUC GAG AAA ACC AUC UCC AAA ACC AAA GGC AGA CCG AAG GCU CCA CAG GUG UAC ACC AUU CCA CCU CCC AAG GAG CAG AUG GCC AAG GAU

                                370                                     380                                     390
lys val ser leu thr cys met ile thr asp phe phe pro glu asp ile thr val glu trp gln trp asn gly gln pro ala glu asn tyr
AAA GUC AGU CUG ACC UGC AUG AUA ACA GAC UUC UUC CCU GAA GAC AUU ACU GUG GAG UGG CAG UGG AAU GGG CAG CCA GCG GAG AAC UAC

                                400                                     410                                     420
lys asn thr gln pro ile met asn thr asn gly ser tyr phe val tyr ser lys leu asn val gln lys ser asn trp glu ala gly asn
AAG AAC ACU CAG CCC AUC AUG AAC ACG AAU GGC UCU UAC UUC GUC UAC AGC AAG CUC AAU GUG CAG AAG AGC AAC UGG GAG GCA GGA AAU

                                430                                     440                     447
thr phe thr cys ser val leu his glu gly leu his asn his his thr glu lys ser leu ser his ser pro gly lys OP
ACU UUC ACC UGC UCU GUG UUA CAU GAG GGC CUG CAC AAC CAC CAU ACU GAG AAG AGC CUC UCC CAC UCU CCU GGU AAA UGA UCCCAGUGUCCU

UGGAGCCCUCUGGUCCUACAGGACUCUGACACCUACCUCCACCCCUCCCUGUAUAAAUAAAGCACCCAGCACUGCCUUGGGAAAAA
```

Fig. 5B.

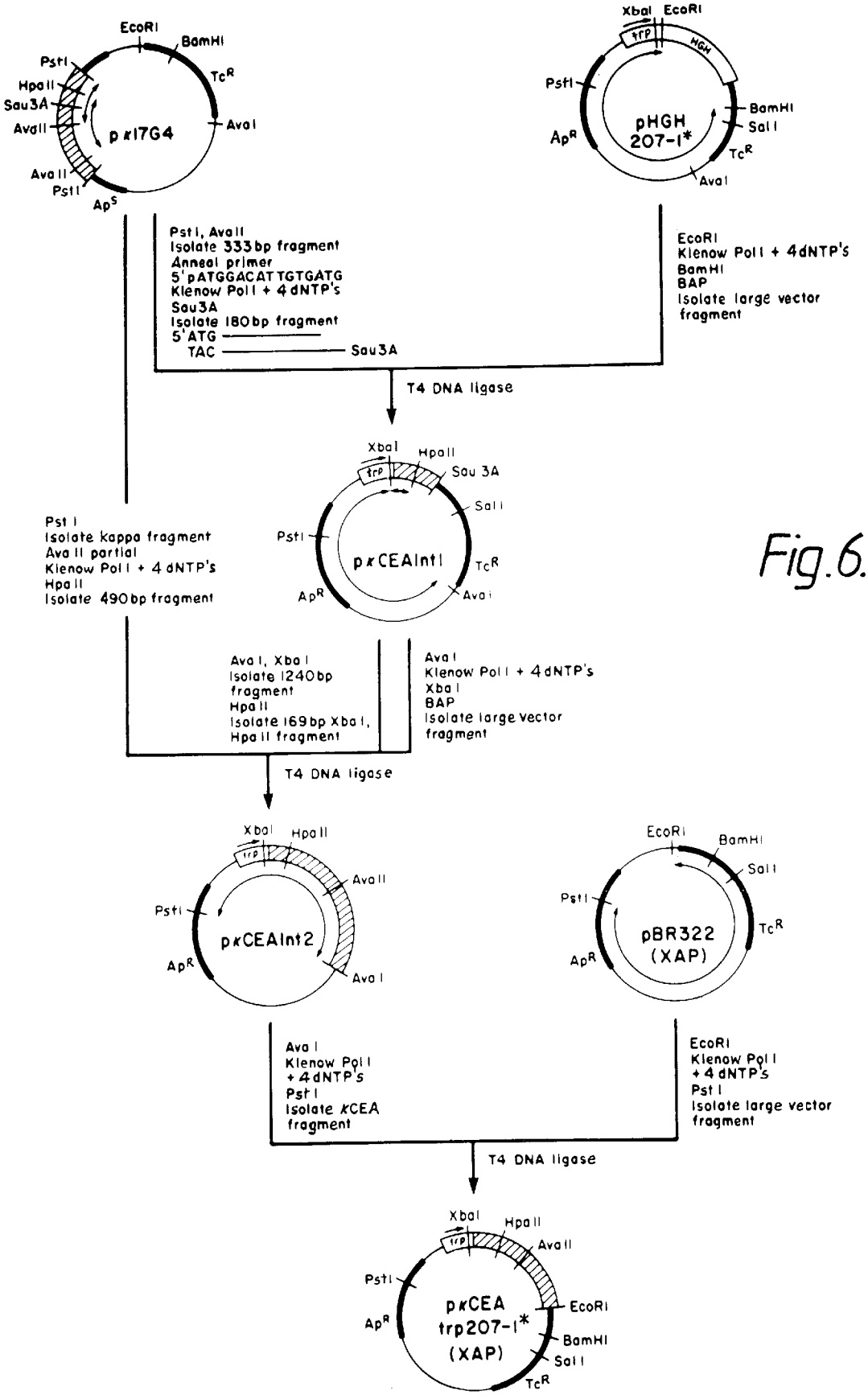
EcoRI
BamHI
TcR
AvaI
AvaII
PstI
HpaII
Sau3A
pκ17G4
ApS
XbaI
trp
HGH
pHGH 207-1*
ApR
SalI
PstI, AvaII
Isolate 333bp fragment
Anneal primer
5'pATGGACATTGTGATG
Klenow PolI + 4dNTP's
Sau3A
Isolate 180bp fragment
5'ATG
TAC
Sau3A
EcoRI
Klenow PolI + 4dNTP's
BamHI
BAP
Isolate large vector fragment
T4 DNA ligase
pκCEAInt1
Sau 3A
Pst I
Isolate kappa fragment
Ava II partial
Klenow PolI + 4 dNTP's
Hpa II
Isolate 490bp fragment
Ava I, Xba I
Isolate 1240bp fragment
Hpa II
Isolate 169bp Xba I, Hpa II fragment
Ava I
Klenow PolI + 4dNTP's
Xba I
BAP
Isolate large vector fragment
T4 DNA ligase
pκCEAInt2
pBR322 (XAP)
Ava I
Klenow PolI + 4dNTP's
Pst I
Isolate κCEA fragment
EcoRI
Klenow PolI + 4 dNTP's
Pst I
Isolate large vector fragment
T4 DNA ligase
pκCEA trp207-1* (XAP)

Fig. 6.

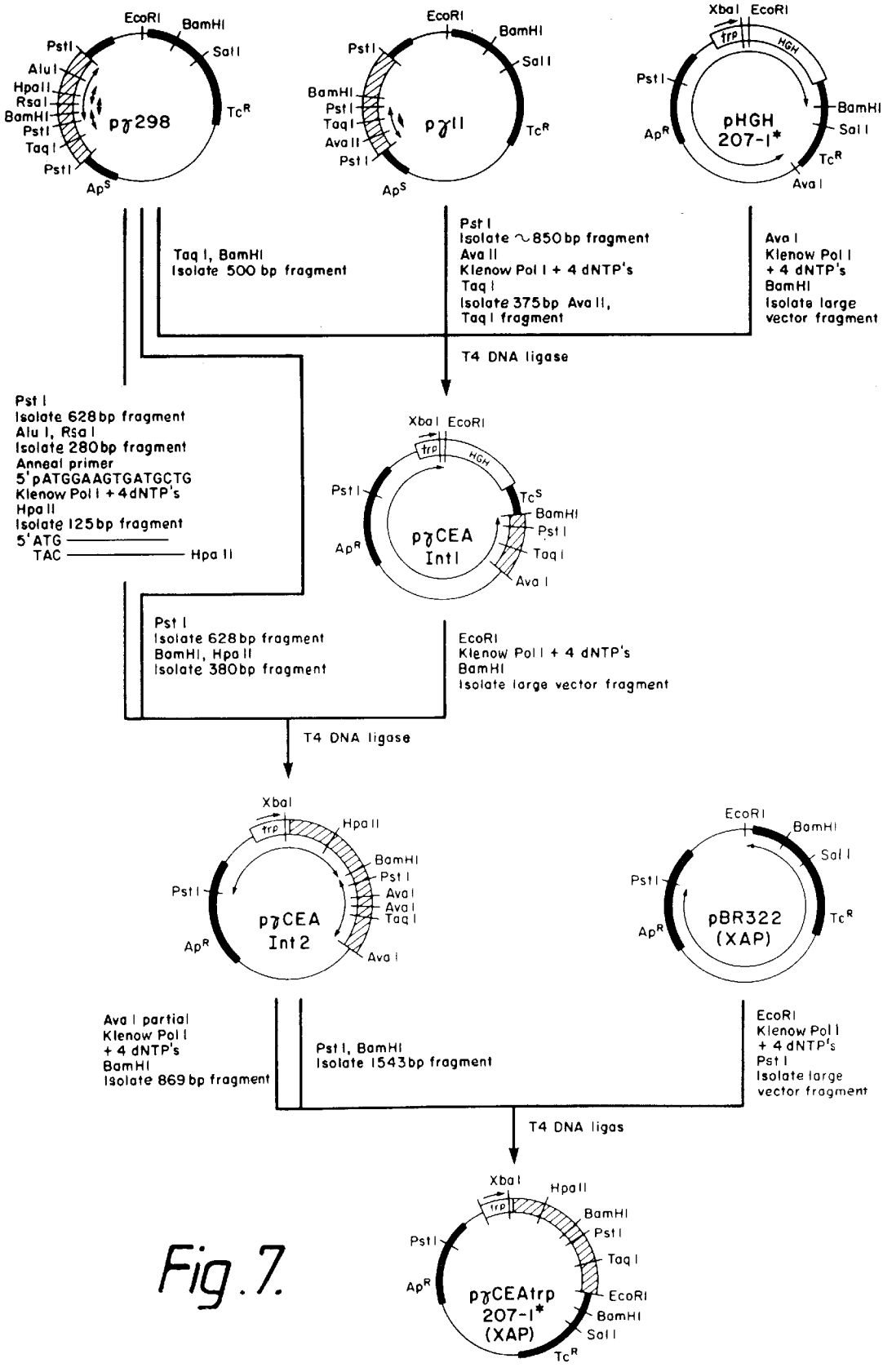
EcoRI
BamHI
Sal I
Tc^R
pγ298
Pst I
Alu I
Hpa II
Rsa I
BamHI
Pst I
Taq I
Pst I
Ap^S
pγII
Taq I
Ava II
Xba I
trp
HGH
pHGH 207-1*
Ap^R
Ava I
Taq I, BamHI
Isolate 500 bp fragment
Pst I
Isolate ~850 bp fragment
Ava II
Klenow Pol I + 4 dNTP's
Taq I
Isolate 375 bp Ava II, Taq I fragment
Ava I
Klenow Pol I + 4 dNTP's
BamHI
Isolate large vector fragment
T4 DNA ligase
Pst I
Isolate 628 bp fragment
Alu I, Rsa I
Isolate 280 bp fragment
Anneal primer
5'pATGGAAGTGATGCTG
Klenow Pol I + 4 dNTP's
Hpa II
Isolate 125 bp fragment
5'ATG
TAC
Hpa II
pγCEA Int1
Tc^S
Pst I
Ava I
Pst I
Isolate 628 bp fragment
BamHI, Hpa II
Isolate 380 bp fragment
EcoRI
Klenow Pol I + 4 dNTP's
BamHI
Isolate large vector fragment
T4 DNA ligase
Xba I
Hpa II
pγCEA Int2
Ava I
Ava I
Taq I
pBR322 (XAP)
Ava I partial
Klenow Pol I
+ 4 dNTP's
BamHI
Isolate 869 bp fragment
Pst I, BamHI
Isolate 1543 bp fragment
EcoRI
Klenow Pol I
+ 4 dNTP's
Pst I
Isolate large vector fragment
T4 DNA ligas
pγCEAtrp 207-1* (XAP)
EcoRI

Fig. 7.

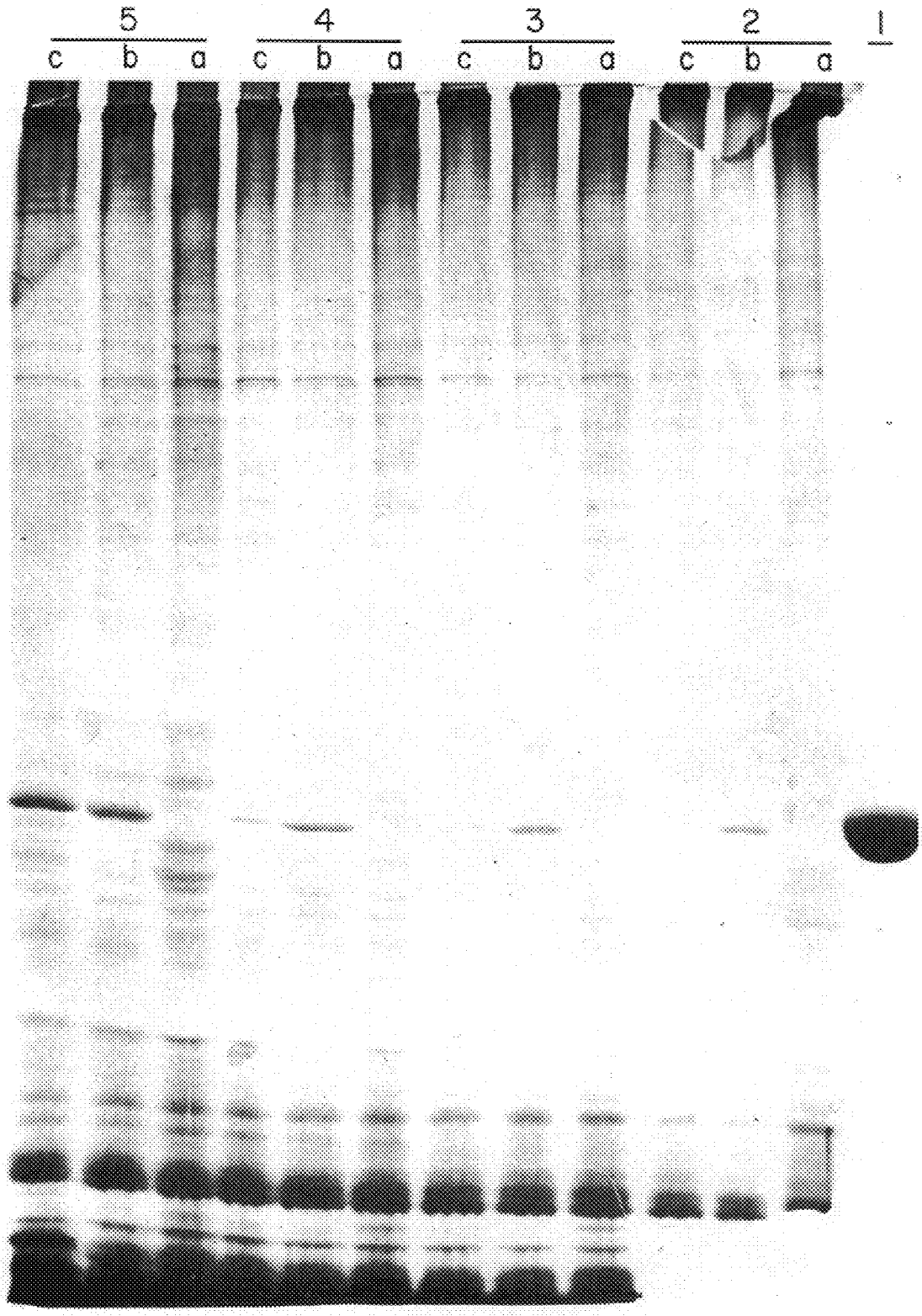

Fig. 8A.

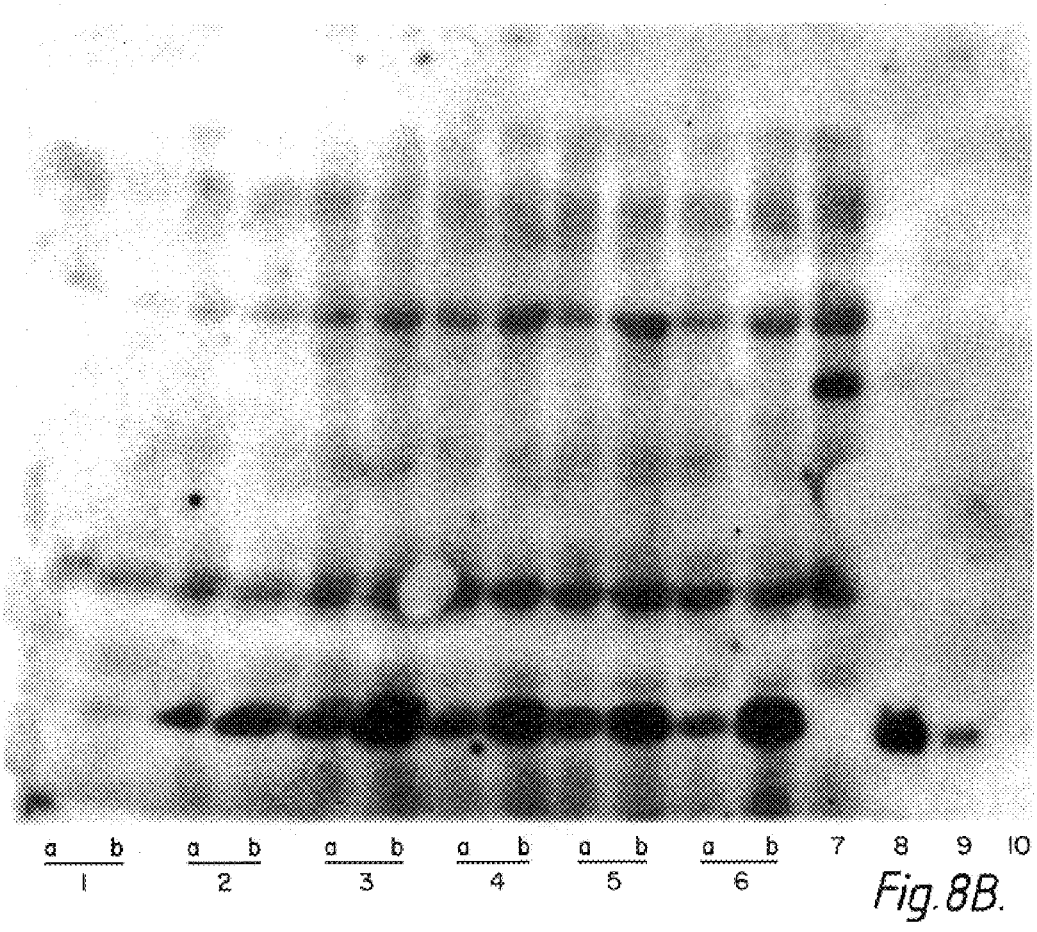

Fig. 8B.

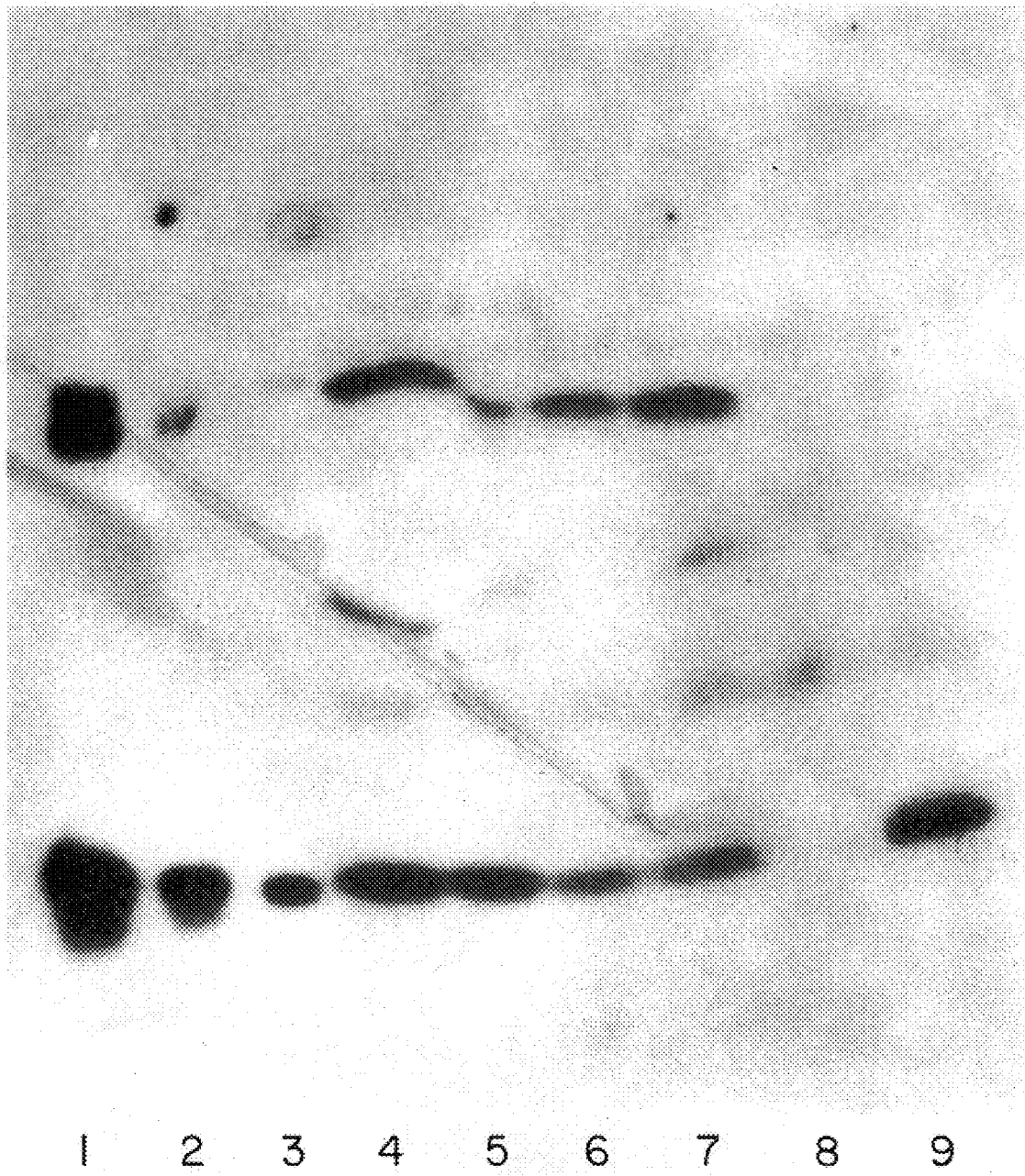

Fig. 8C.

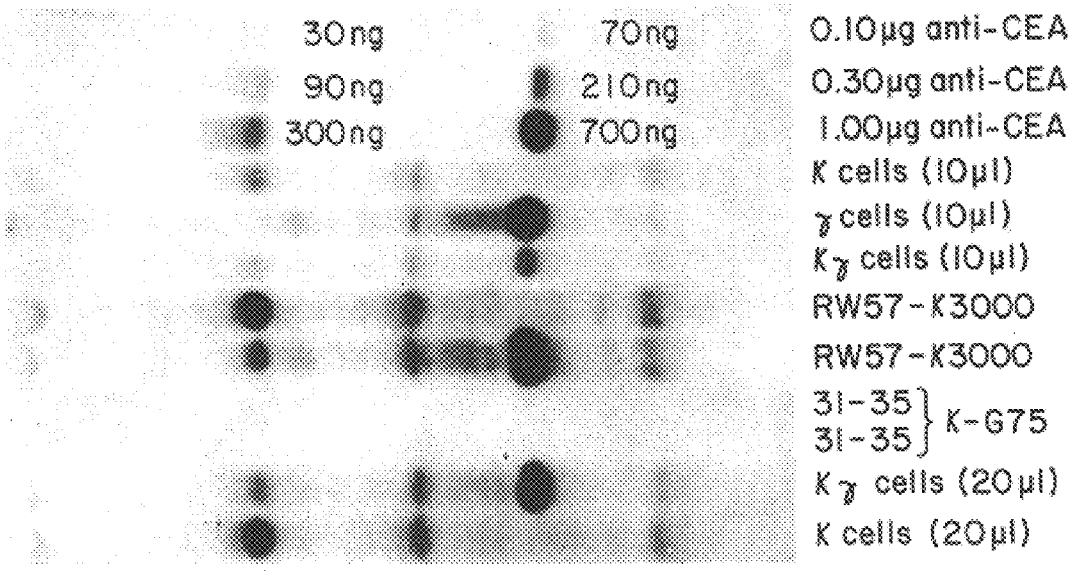
30 ng
90 ng
300 ng
70 ng
210 ng
700 ng
0.10 µg anti-CEA
0.30 µg anti-CEA
1.00 µg anti-CEA
K cells (10 µl)
γ cells (10 µl)
Kγ cells (10 µl)
RW57-K3000
RW57-K3000
31-35
31-35
K-675
Kγ cells (20 µl)
K cells (20 µl)

Fig. 9.

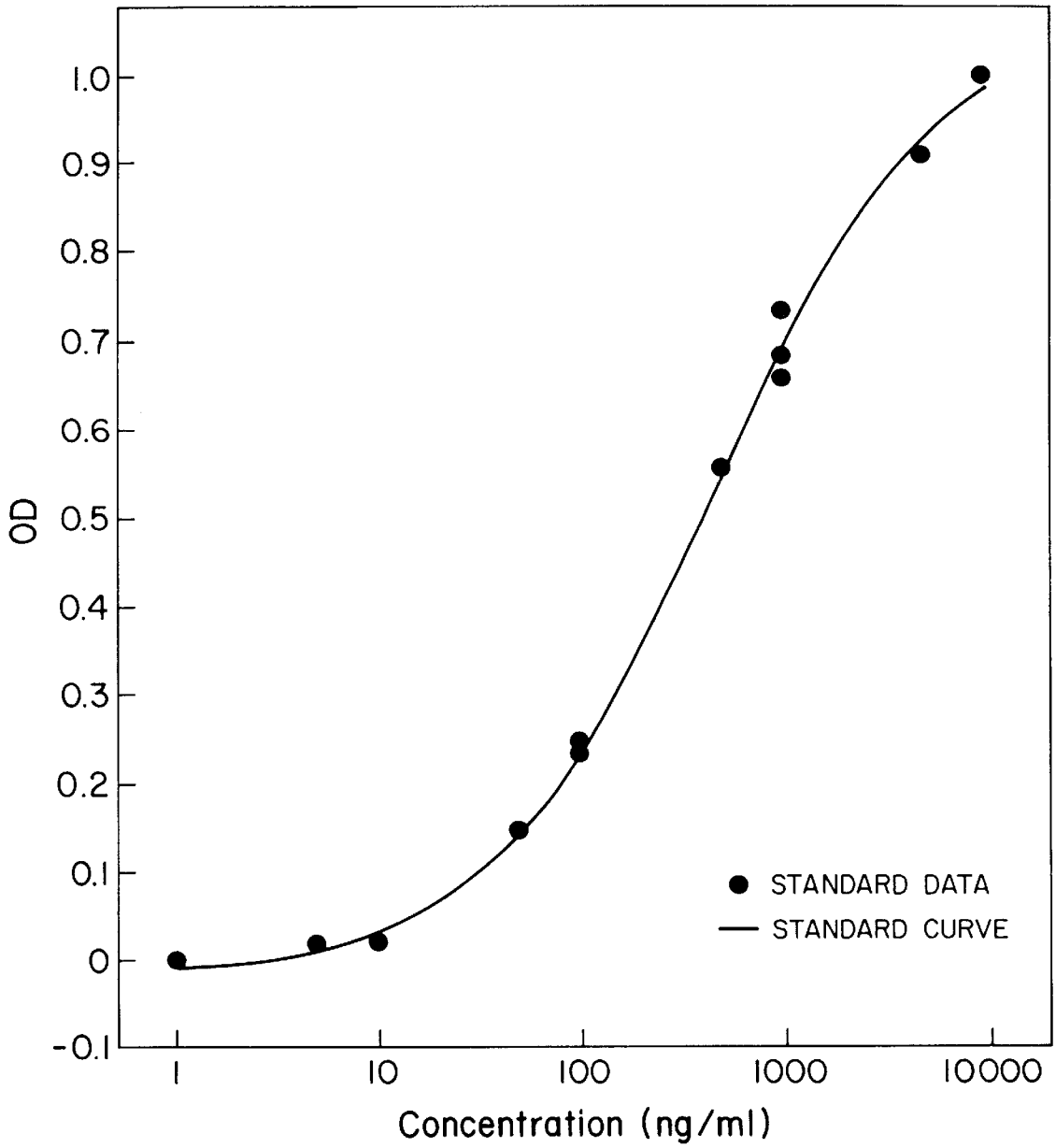
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
OD
1
10
100
1000
10000
Concentration (ng/ml)
STANDARD DATA
STANDARD CURVE

Fig. 10.

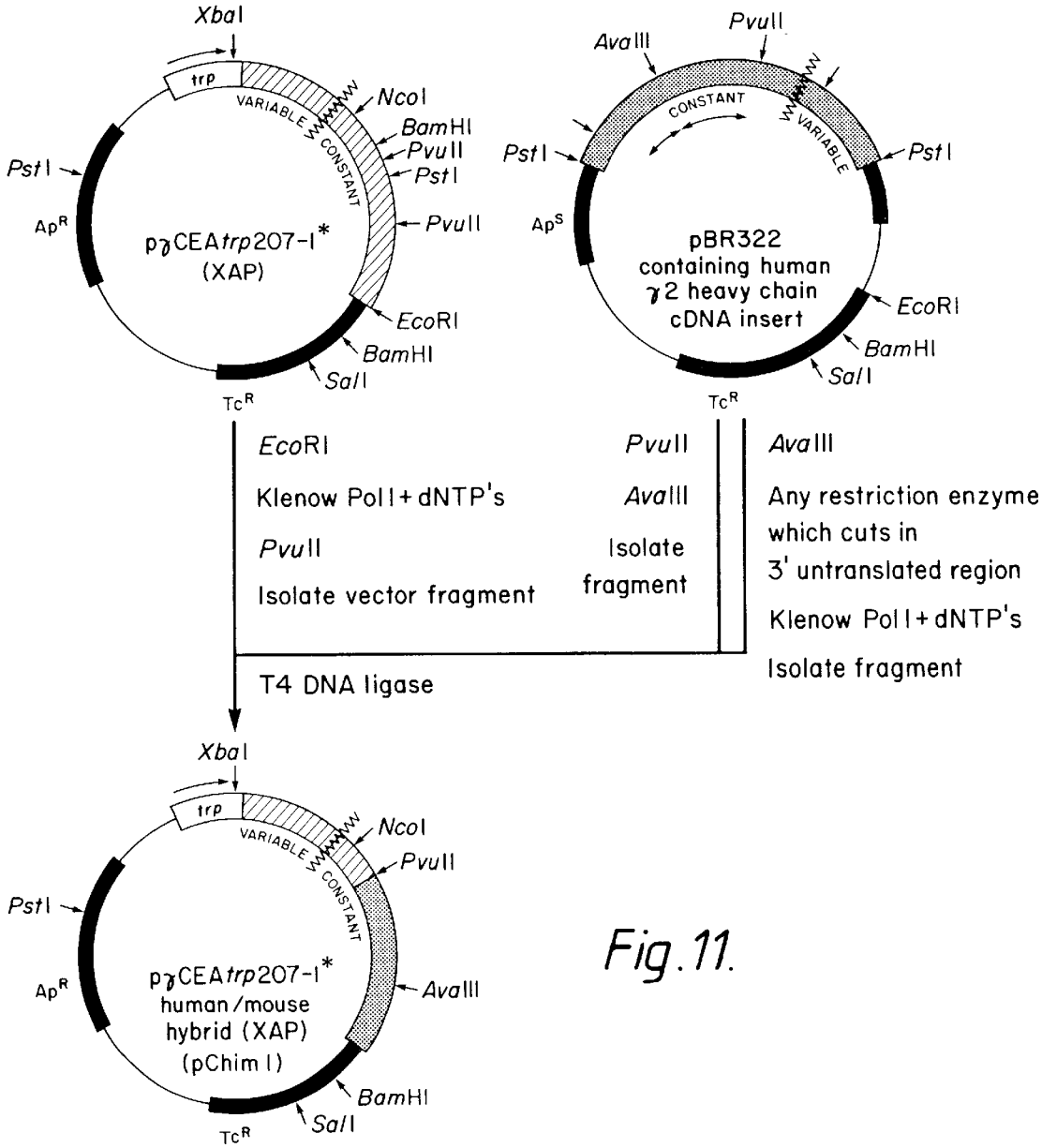
XbaI
trp
VARIABLE
CONSTANT
NcoI
BamHI
PvuII
PstI
PvuII
EcoRI
BamHI
SalI
PstI
ApR
pγCEAtrp207-1*
(XAP)
TcR
AvaIII
PvuII
CONSTANT
VARIABLE
PstI
PstI
ApS
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
TcR
EcoRI
Klenow PolI + dNTP's
PvuII
Isolate vector fragment
PvuII
AvaIII
Isolate
fragment
AvaIII
Any restriction enzyme
which cuts in
3' untranslated region
Klenow PolI + dNTP's
Isolate fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
NcoI
PvuII
PstI
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim I)
AvaIII
BamHI
SalI
TcR

Fig. 11.

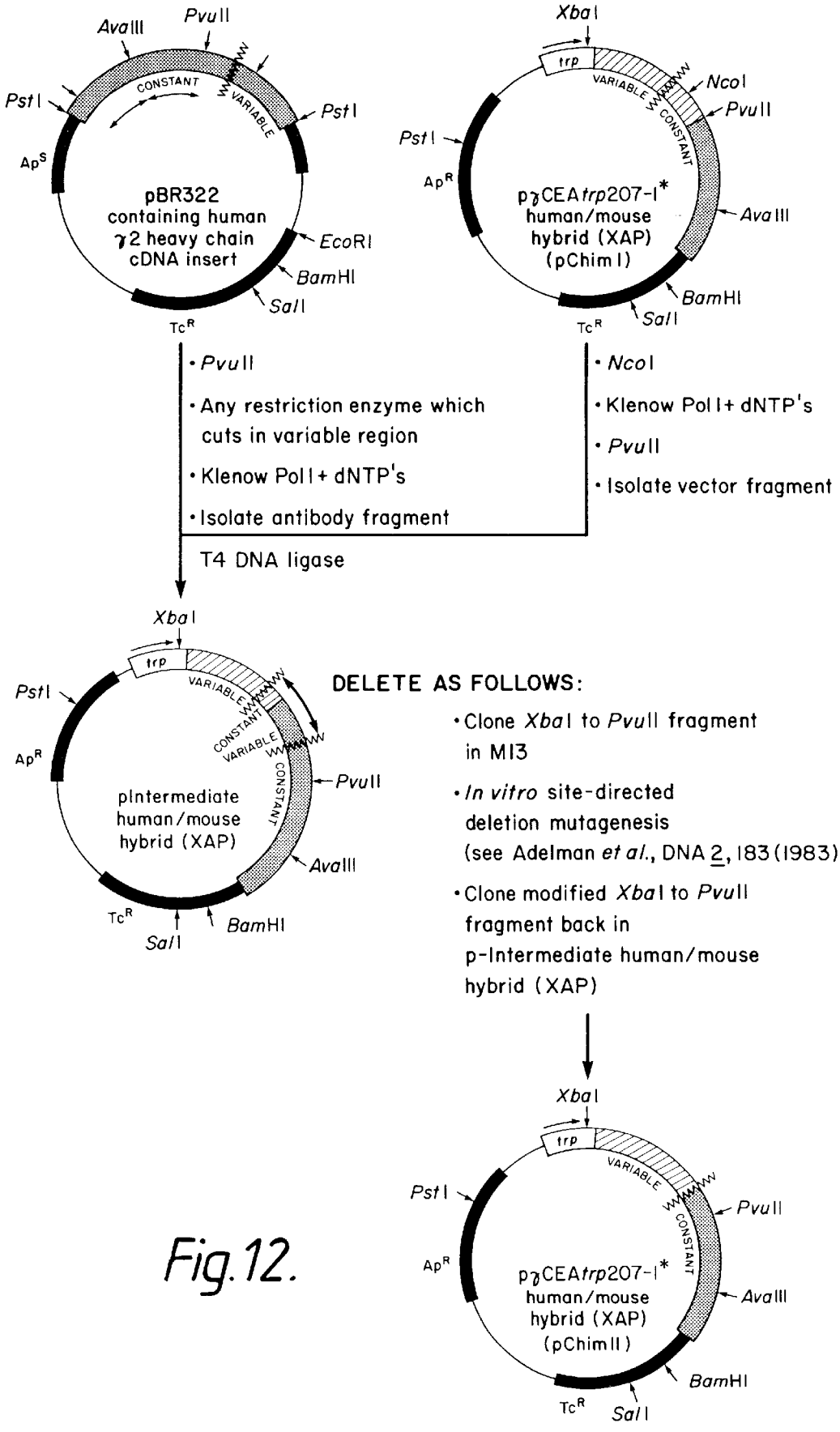
AvaIII
PvuII
PstI
CONSTANT
VARIABLE
PstI
ApS
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
TcR
XbaI
trp
VARIABLE
NcoI
PvuII
PstI
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim1)
AvaIII
BamHI
SalI
TcR
• PvuII
• Any restriction enzyme which cuts in variable region
• Klenow Pol I + dNTP's
• Isolate antibody fragment
• NcoI
• Klenow Pol I + dNTP's
• PvuII
• Isolate vector fragment
T4 DNA ligase
XbaI
trp
VARIABLE
PstI
ApR
CONSTANT
VARIABLE
CONSTANT
PvuII
pIntermediate
human/mouse
hybrid (XAP)
AvaIII
TcR
SalI
BamHI
DELETE AS FOLLOWS:
• Clone XbaI to PvuII fragment in M13
• In vitro site-directed deletion mutagenesis (see Adelman et al., DNA 2, 183 (1983))
• Clone modified XbaI to PvuII fragment back in p-Intermediate human/mouse hybrid (XAP)
XbaI
trp
VARIABLE
PstI
CONSTANT
PvuII
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim11)
AvaIII
BamHI
TcR
SalI

Fig. 12.

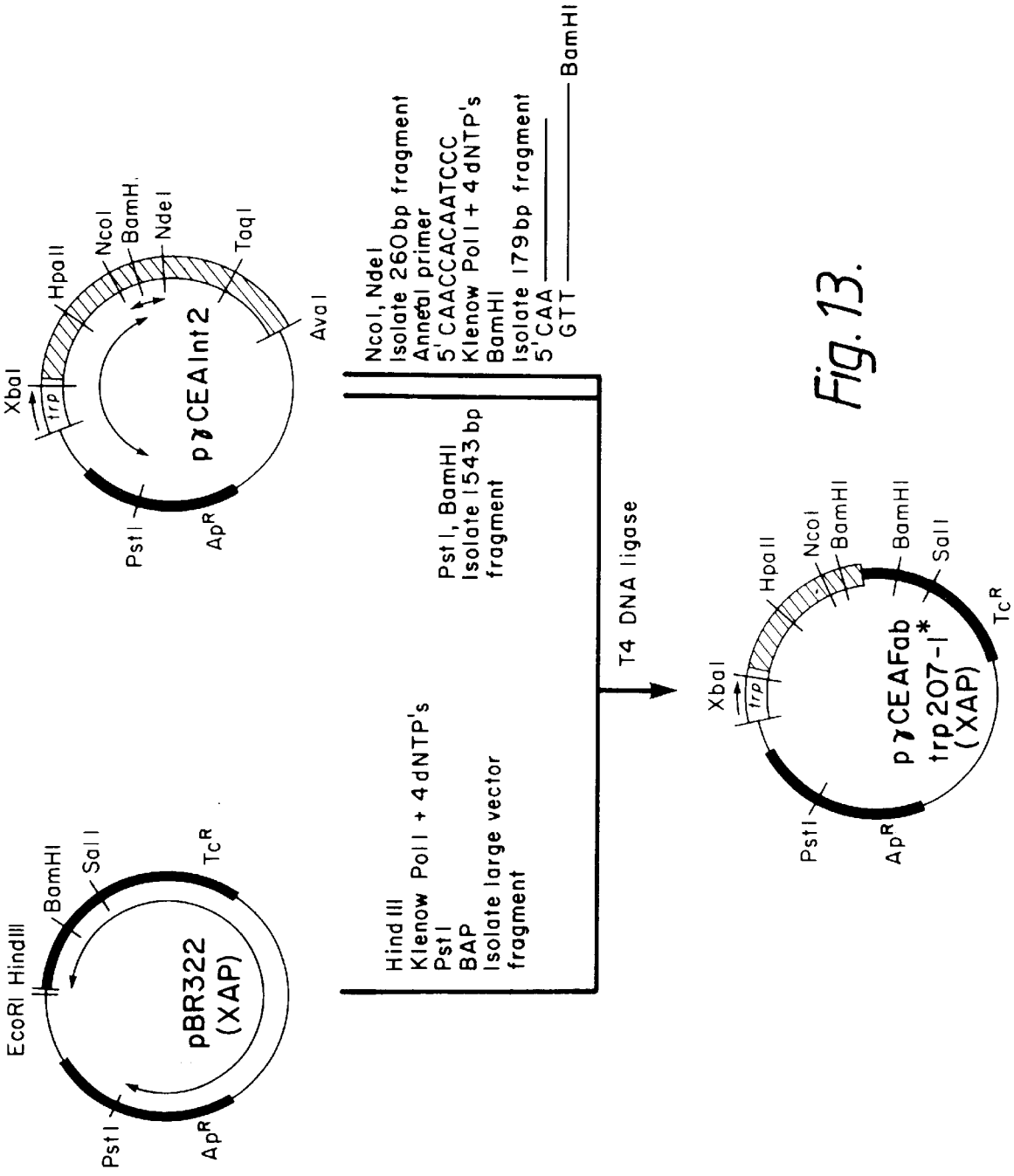
pγCEAInt2
XbaI
trp
HpaII
NcoI
BamH.
NdeI
TaqI
AvaI
PstI
ApR
NcoI, NdeI
Isolate 260bp fragment
Annetal primer
5' CAACCACAATCCC
Klenow PolI + 4 dNTP's
BamHI
Isolate 179 bp fragment
5'CAA
GTT
BamHI
PstI, BamHI
Isolate 1543 bp
fragment
HindIII
Klenow PolI + 4 dNTP's
PstI
BAP
Isolate large vector
fragment
T4 DNA ligase
pBR322
(XAP)
EcoRI HindIII
BamHI
SalI
TcR
PstI
ApR
pγCEAFab
trp207-1*
(XAP)
XbaI
trp
HpaII
NcoI
BamHI
BamHI
SalI
TcR
PstI
ApR

Fig. 13.

METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 06/483,457, filed Apr. 8, 1983, now U.S. Pat. No. 4,816,567, issued Mar. 28, 1989.

BACKGROUND OF THE INVENTION

This invention relates to the field of immunoglobulin production and to modification of naturally occurring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce both immunoglobulins which are analogous to those normally found in vertebrate systems and to take advantage of these gene modification techniques to construct chimeric or other modified forms.

A. Immunoglobulins and Antibodies

Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicited it.

Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

A.1 Source and Utility

Two major sources of vertebrate antibodies are presently utilized—generation in situ by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made in situ as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci*, 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The in situ immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B-cells—hence in situ generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur. J. Immunol.*, 6: 511 (1976)). In this process, splenocytes

or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed we segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci.* (*USA*), 77: 5429 (1980)); human-murine hybridomas (Schlom, J. , et al. (ibid) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized in vitro by transformation with viral DNA.

Polyclonal, or, much more preferably monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. These cells contain additional materials, notably nucleic acid fragments, but protein fragments as well, which are capable of enhancing, causing, or mediating carcinogic responses. Second, hybridoma lines producing monoclonal antibodies tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al. *Proc. Natl. Acad. Sci* (*USA*) 77: 2197 (1980); Morrison, S. L., *J. Immunol.* 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., *Biochemistry*, 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not suffer from the

foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

Even those immunoglobulins which lack the specificity of antibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in proteins replacement therapy for globulin related anemia. In this context, an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of canceling out specificity by manipulating the four chains of the tetramer separately.

A.2 General Structure Characteristics

The basic immunoglobin structural unit in vertebrate systems is now well understood (Edelman, G. M., *Ann. N.Y. Acad. Sci*., 190: 5 (1971)). The units are composed of two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000–70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket-the heavy-chains starting at the mouth of the Y and continuing through the divergent region as shown in FIG. 1. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class"of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified-as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occurring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

The remaining portions of the chain are referred to as constant regions and within a particular class do not to vary with the specificity of the antibody (i.e., the antigen eliciting it),

As stated above, there are five known major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding to γ, μ, α, δ, and ϵ heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E. A., *Structural Concepts in Immunology and Immunochemistry*, 2nd Ed., p. 413–436, Holt, Rinehart Winston (1976)), and other cellular responses (Andrews, D. W., et al., *Clinical Immunobiology* pp 1–18, W. B. Sanders (1980); Kohl, S., et al., *Immunology*, 48: 187 (1983)); while the variable region determines the antigen with which it will react.

B. Recombinant DNA Technology

Recombinant DNA technology has reached sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformants. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment.

The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems.

SUMMARY OF THE INVENTION

The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. These antibodies and NSIs can be readily prepared in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras of variants which draw their homology from species which differ from each other. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell. Thus, there are a number of "types" of immunoglobulins encompassed by the invention.

First, immunoglobulins, particularly antibodies, are produced using recombinant techniques which mimic the amino acid sequence of naturally occurring antibodies produced by either mammalian B cells in situ, or by B cells fused with suitable immortalizing tumor lines, i.e., hybridomas.

Second, the methods of this invention produce, and the invention is directed to, immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. Such reassembly is particularly useful in producing "hybrid" antibodies capable of binding more than one antigen; and in producing "composite" immunoglobulins wherein heavy and light chains of different origins essentially damp out specificity. Third, by genetic manipulation, "chimeric" antibodies can be formed wherein, for example, the variable regions correspond to the amino acid sequence from one mammalian model system, whereas the constant region mimics the amino acid sequence of another. Again, the derivation of these two mimicked sequences may be from different species. Fourth, also by genetic manipulation, "altered" antibodies with improved specificity and other characteristics can be formed.

Two other types of immunoglobulin-like moieties may be produced: "univalent" antibodies, which are useful as homing carriers to target tissues, and "Fab proteins" which include only the "Fab" region of an immunoglobulin molecule i.e., the branches of the "Y". These univalent antibodies and Fab fragments may also be "mammalian" i.e., mimic mammalian amino acid sequences; novel assemblies of mammalian chains, or chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions, thereof, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

In other aspects, the invention is directed to DNA which encodes the aforementioned NSIs, antibodies, and portions thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, the invention is directed to methods of producing these NSIs and antibodies, and the DNA sequences, plasmids, and transformed cells intermediate to them.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a representation of the general structure of immunoglobulins.

FIGS. **2**A–B shows the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

FIG. **3** shows the coding sequence of the fragment shown in FIG. **2**, along with the corresponding amino acid sequence.

FIGS. **4**A–C shows the combined detailed sequence of the cDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

FIGS. **5**A–B shows the corresponding amino acid sequence encoded by the fragment in FIG. **4**.

FIGS. **6** and **7** outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively.

FIGS. **8**A, **8**B, and **8**C show the results of sizing gels run on, extracts of *E. coli* expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

FIG. **9** shows the results of western blots of extracts of cells transformed as those in FIGS. **8**.

FIG. **10** shows a standard curve for ELISA assay of anti CEA activity.

FIGS. **11** and **12** show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain.

FIG. **13** shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

DETAILED DESCRIPTION

A. Definitions

As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of FIG. **1**, with or without covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity—i.e., those which are not antibodies.

"Mammalian antibodies" refers to antibodies wherein the amino acid sequences of the chains are homologous with those sequences found in antibodies produced by mammalian systems, either in situ, or in hybridomas. These antibodies mimic antibodies which are otherwise capable of being generated, although in impure form, in these traditional systems.

"Hybrid antibodies" refers to antibodies wherein chains are separately homologous with referenced mammalian antibody chains and represent novel assemblies of them, so that two different antigens are precipitable by the tetramer. In hybrid antibodies, one pair of heavy and light chain is homologous to antibodies raised against one antigen, while the other pair of heavy and light chain is homologous to those raised against another antigen. This results in the property of "divalence" i.e., ability to bind two antigens simultaneously. Such hybrids may, of course, also be formed using chimeric chains, as set forth below.

"Composite" immunoglobulins means those wherein the heavy and light chains mimic those of different species origins or specificities, and the resultant is thus likely to be a non-specific immunoglobulin (NSI), i.e.—lacking in antibody character.

"Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals while the constant portions are homologous to the sequences in antibodies derived from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non-human source.

However, the definition is not limited to this particular example. It includes any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, differing antigen responses, or differing species of origin and whether or not the fusion point is At the variable/constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher

specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation or to make other improvements in properties possessed by a particular constant region.

"Altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a mammalian or other vertebrate antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the constant region. Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics. The antibody can also be engineered so as to aid the specific delivery of a toxic agent according to the "magic bullet" concept. Alterations, can be made by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques (Dalbadie-McFarland, et al *Proc. Natl. Acad. Sci.* (*USA*), 79:6.409 (1982)).

"Univalent antibodies" refers to aggregations which comprise a heavy chain/light chain dimer bound to the Fc (or stem) region of a second heavy chain. Such antibodies are specific for antigen but have the additional desirable property of targeting tissues with specific antigenic surfaces, without causing its antigenic effectiveness to be impaired—i.e., there is no antigenic modulation. This phenomenon and the property of univalent antibodies in this regard is set forth in Glennie, M. J., et al., *Nature*, 295: 712 (1982). Univalent antibodies have heretofore been formed by proteolysis.

"Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as $F(ab)_2$), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, as have univalent ones, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack the "effector" Fc: portion they cannot effect, for example, lysis of the target cell by macrophages.

"Fab protein" has similar subsets according to the definition of the present invention as does the general term "antibodies" or "immunoglobulins". Thus, "mammalian" Fab protein, "hybrid" Fab protein "chimeric" Fab and "altered" Fab protein are defined analogously to the corresponding definitions set forth in the previous paragraphs for the various types of antibodies.

Individual heavy or light chains may of course be "mammalian", "chimeric" or "altered" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence—i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

B. Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In-general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F^-, λ^-, prototrophic, ATTC No. 27325), bacilli such as *Bacillus subtilus*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various Pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 (1977)). PBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial

plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems Chang et al, *Nature* 275: 615 (1978); Itakura, et al, *Science* 198: 1056 (1977); (Goeddel et al, *Nature* 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic kids Res.*, 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized., and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, Cell 20: 269 (1980)).

In addition to prokaryates, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb et al, *Nature*, 282: 39 (1979); Kingsman et al, *Gene*, 7: 141 (1979); Tschemper, et al, *Gene*, 10: 157 (1980)) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85: 12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.*, 255: 2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.*, 7: 149 (1968); Holland, et al, *Biochemistry*, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (Holland, ibid.). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers, et al, *Nature*, 273: 113 (1978)) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those host cells, vectors and expression systems exemplified.

C. Methods Employed

C.1 Transformation

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci.* (*USA*) 69: 2110 (1972).

C.2 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 μg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 μl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of *E. coli* DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, *Nucleic Acids Res.*, 8: 4057 (1980) incorporated herein by reference.

For ligation, approximately equimolar amounts of the desired components, suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.5 μg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

In the examples described below correct ligations for plasmid construction are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.,* 9:309 (1981) or by the method of Maxam, et al, *Methods in Enzymology,* 65:499 (1980).

D. Outline of Procedures

D.1 Mammalian Antibodies

The first type of antibody which forms a part of this invention, and is prepared by the methods thereof, is "mammalian antibody"-one wherein the heavy and light chains mimic the amino acid sequences of an antibody otherwise produced by a mature mammalian B lymphocyte either in situ or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA. The poly-A mRNA may, further, be fractionated to obtain sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli,* is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP^{32}. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. patent application Ser. Nos. 307,473; 291,892; and 305,657 (EPO Publ. Nos. 0036776; 0048970 and 0051873) have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

In the present invention, the gene coding for the light chain and that coding for the heaving chain are recovered separately by the procedures outlined above. Thus then may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such conditions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circumstances, in yeast and bacterial strains, can be secreted into the medium (yeast and gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures. Tissue culture cells as hosts also appear, in general, to permit reasonably facile recovery of heterologous proteins.

When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished in vitro as described below, or might be possible in vivo in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

D.2 Chain Recombination Techniques

The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins, Fab regions, and univalent antibodies. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield

native structure and activity (Freedman, R. B., et al. In *Enzymology of Post Translational Modification of Proteins,* I: 157–212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by disulfide bonds are more difficult to reconstruct in vitro after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R. E., et al., In *Peptides: Proceedings of the Seventh Annual American Peptide Symposium* (Rich, D. H. and Gross. E., eds.) 721–728, Pierce Chemical Co., Rockford, Ill. (1981)).

Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the inter-chain disulfides (Edelman, G. M., et al., *Proc. Natl. Acad. Sci. (USA)* 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of ~50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., *Proc. Natl. Acad. Sci. (USA)* 52: 1099 (1964); Whitney, P. L., et al., *Proc. Natl. Acad. Sci. (USA)* 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding pathway (see discussion in Freedman, M. H., et al., *J. Biol. Chem.* 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (ibid).

A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolysis, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (supra)). Oxidative sulfitolysis is a mild disulfide cleavage reaction (Means, G. E., et al., *Chemical Modification of Proteins,* Holden-Day, San Francisco (1971)) which is sometimes more gentle than reduction, and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur via thiol-disulfide interchange. In the present invention the heavy and light chain S-sulfonates generated by oxidative sulfitolysis were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Ser. No. 452,187, filed Dec. 22, 1982 (EPO Appln. No. 83.307840.5), incorporated herein by reference.

D.3 Variants Permitted by Recombinant Technology

Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of mammalian antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered mammalian immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, human-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

The present invention permits a more controlled assembly of desired chains, either by mixing the desired chains in vitro, or by transforming the same culture with the coding sequences for the desired chains.

D.4 Composite Immunoglobulins

The foregoing procedure, which describes in detail the recombinant production of mammalian antibodies is employed with some modifications to construct the remaining types of antibodies or NSIs encompassed by the present invention. To prepare the particular embodiment of composite non-specific immunoglobulin wherein the homology of the chains corresponds to the sequences of immunoglobulins of different specificities, it is of course, only necessary to prepare the heavy and light chains in separate cultures and reassemble them as desired.

For example, in order to make an anti-CEA light chain/antihepatitis heavy chain composite antibody, a suitable source for the mRNA used as a template for the light chain clone would comprise, for instance, the anti CEA producing cell line of paragraph E.1. The mRNA corresponding to heavy chain would be derived from B cells raised in response to hepatitis infection or from hybridoma in which the B cell was of this origin. It is clear that such composites can be assembled using the methods of the invention almost at will, and are limited only by available sources of mRNA suitable for use as templates for the respective chains. All other features of the process are similar to those described above.

D.5 Hybrid Antibodies

Hybrid antibodies are particularly useful as they are capable of simultaneous reaction with more than one antigen. Pairs of heavy and light chains corresponding to chains

of antibodies for different antigens, such as those set forth in paragraph D.4 are prepared in four separate cultures, thus preventing premature assembly of the tetramer. Subsequent mixing of the four separately prepared peptides then permits assembly into the desired tetramers. While random aggregation may lead to the formation of considerable undesired product, that portion of the product in which homologous light and heavy chains are bound to each other and mismatched to another pair gives the desired hybrid antibody.

D.6 Chimeric Antibodies

For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph C.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

For example, in a particularly preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and closed from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.7 Altered Antibodies

Altered antibodies present, in essence, an extension of chimeric ones. Again, the techniques of D.1 and D.2 are applicable; however, rather than splicing portions of the chain(s), suitable amino acid alterations, deletions or additions are made using available techniques such as mutagenesis (supra). For example, genes which encode antibodies having diminished complement fixation properties, or which have enhanced metal binding capacities are prepared using such techniques. The latter type may, for example, take advantage of the known gene sequence encoding metallothionein II (Karin, M., et al., *Nature,* 299: 797 (1982)). The chelating properties of this molecular fragment are useful in carrying heavy metals to tumor sites as an aid in tumor imaging (Scheinberg, D. A., et al., *Science,* 215: 19 (1982).

D.8 Univalent Antibodies

In another preferred embodiment, antibodies are formed which comprise one heavy and light chain pair coupled with the Fc region of a third (heavy) chain. These antibodies have a particularly useful property. They can, like ordinary antibodies, be used to target antigenic surfaces of tissues, such as tumors, but, unlike ordinary antibodies, they do not cause the antigenic surfaces of the target tissue to retreat and become non-receptive. Ordinary antibody use results in aggregation and subsequent inactivation, for several hours, of such surface antigens.

The method of construction of univalent antibodies is a straightforward application of the invention. The gene for heavy chain of the desired Fc region is cleaved by restriction enzymes, and only that portion coding for the desired Fc region expressed. This portion is then bound using the technique of D.2 to separately produced heavy chain the desired pairs separated from heavy/heavy and Fc/Fc combinations, and separately produced light chain added. Pre-binding of the two heavy chain portions thus diminishes the probability of formation of ordinary antibody.

D.9 Fab Protein

Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that the portion of the heavy chain coding for the amino terminal **220** amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

The invention has been described above in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti CEA antibody components, i.e. for a "mammalian antibody". Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of mammalian, composite, hybrid and chimeric immunoglobulins, and Fab proteins and univalent antibodies. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

The examples set forth below are included for illustrative purposes and do not limit the scope of the invention.

E.1 Construction of Expression Vectors for Murine anti-CEA Antibody Chains and Peptide Synthesis

Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., *J. Exp. Med.,* 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T. R., et al., *Cancer Res.* 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the $Ig\gamma_1$ class, CEA.66-E3, has been prepared as described by Wagener, C, et al., *J. Immunol.* (in press) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAse (Podell, D. N., et al., BBRC 81: 176 (1978)), and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C. water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a linear gradient from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H_2O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SDS gels by silver staining. The first two peaks were pure light chain (MW 25,000 daltons), the third peak showed a (7:3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J. E., *Methods in Enzymology,* 79: 31 (1981), with an NH_2-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa

chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

Total RNA from CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology,* 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM $MgCl_2$. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform: isoamyl alcohol 25:1 at 4° C. The aqueous phase was made 0.2 M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA was purified from total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l, Acad. Sci. (USA),* 69: 1408 (19672), 142 μg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of *E. coli* Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

5 μg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., *Nature* 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No. 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

The 14mer, 5' GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mouse MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5' GACCAGGCATCCCAG 3', complementary to a coding sequence located 72 basepairs 3' of the variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

Both probes were synthesized by the phosphotriester method described in German Offenlegungschrift 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 μl of 60 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 15 mM beta-mercaptoethanol, and 100 μCi (γ-^{32}P) ATP (Amersham, 5000 Ci/mMole). 5 units of T4 polynucleotide kinase were added and the reduction was allowed to proceed at 37° C. for 30 minutes and terminated by addition of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431–3, Cold Spring Harbor, Lab., Cold Spring Harbor, N.Y. (1972))+5 μg/ml tetracycline and stored at −20° C. after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 μg/ml tetracycline. After ~10 hours growth at 37° C. the colony filters were transferred to agar plates containing LB+5 μg/ml tetracycline and 12.5 μg/ml chloramphenicol and reincubated overnight at 37° C. The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci. (USA)* 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5 N NaOH, 1.5 M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3 M NaCl, 0.5 M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2XSSC, and subsequently baked for 2 hours in an 80° C. vacuum oven. The filters were prehybridized for ~2 hours at room temperature in 0.9 M NaCl, 1X Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1 M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 μg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using ~40×10^6 cpm of either the kinased kappa or gamma probe described above.

After extensive washing at 37° C. in 6X SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 16–24 hours at −80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith, *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). FIG. **2** shows the nucleotide sequence of the cDNA insert of pK17G4 and FIG. **3** shows the gene sequence with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain, deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acids 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides 374–388 (FIG. **2**).

E.1.6 Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region. Selected plasmid DNAs were digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and NcoI. The C-terminal region of the cDNA insert of pγ11 was sequence and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

FIG. **4** presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.,* 65: 560 (1980)) and FIG. **5** includes the translated sequence.

The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosolated protein (MW 52,258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 and pγ11 hybridized to nucleotides 528–542 (FIG. **4**).

E.1.7 Construction of a Plasmid for Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCEAtrp207-1*

FIG. **6** illustrated the construction of pKCEAtrp207-1*

First, an intermediate plasmid pHGH207-1*, having a single trp promoter, was prepared as follows:

The plasmid pHGH 207 (described in U.S. patent application Ser. No. 307,473, filed Oct. 1, 1981 (EPO Publn. No. 0036776)) has a double lac promoter followed by the trp promoter, flanked by EcoR I sites and was used to prepare pHGH207-1. pHGH207 was digested with BamH 1, followed by partial digestion with EcoR I. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoR I- BamH I fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet^R Amp^R colonies were isolated, and most of them contained pHGH207-1. pHGH207-1* which lacks the EcoR1 site between the amp^R gene and the trp promoter, was obtained by partial digestion of pHGH207-1 with EcoR I, filling in the ends with Klenow and dNTPs, and religation.

5 μg of pHGH207-1* was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 μl reaction containing 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C. for 1 hour, followed by extraction with phenol/$CHCl_3$ and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/$CHCl_3$ extraction and ethanol precipitation.

The DNA was resuspended in 50 μl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30' at 65° followed by phenol/$CHCl_3$ extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence was prepared as follows: 7 μg of pH17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolated by 6 percent gel electrophoresis, and electroelution. After phenol/$CHCl_3$ extraction, ethanol precipitation and resuspension in water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer was synthesized by the phosphotriester method G. O. 2,644,432 (supra) and has the following sequence:

```
Met Asp Ile Val Met
5' ATG GAC ATT GTT ATG 3'
```

The 5' methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5' end with 10 units T4 DNA kinase in 20 μl reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20 μl of the phosphorylated primer, heated to 95° C. for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C. this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.

100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20 μl of 20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 2.5 mM ATP and 1 unit of T4 DNA ligase. After overnight ligation at 14° C. the reaction was transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequence analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1 (FIG. **6**).

The remainder of the coding sequence of the kappa light chain gene was prepared as follows:

The Pst I cDNA insert fragment from 7 μg of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686 basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isolated and purified after gel electrophoresis.

10 μg of pKCEAInt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment, and

digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reaction and incubated overnight at 14°, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was designated pKCEAInt2.

Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared as described in U.S. application Ser. No. 452,227, filed Dec. 22, 1982; from pBR322 by deletion of the AvaI-PvuII fragment followed by ligation.)

The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis, and one plasmid DNA demonstrated the proper construction, and was designated pKCEAtrp207-I*.

E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1*

FIG. **7** illustrates the construction of pγCEAtrp207-1*. This plasmid was constructed in two parts beginning with construction of the C-terminal region of the gamma 1 gene.

5 μg of plasmid pHGH207-1* was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol/$CHCl_3$, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution.

~5 μg of pγ11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified, digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment (fragment B) was isolated and purified by gel electrophoresis and electroelution.

9 μg of pγ298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).

Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20 μl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pγCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (FIG. **5**).

To obtain the N-terminal sequences, 30 μg of pγ298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer

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      met glu val met leu
5' ATG GAA GTG ATG CTG 3'
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was synthesized by the phosphotriester method (supra).

The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blunt-end to Hpa II DNA fragment (fragment D) was purified from the gel.

A second aliquot of pγ298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I and Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.

~5 μg of pγCEAIntI was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.

In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 μl reaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pγCEAInt2.

The expression plasmid, pγCEAtrp207-I* used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pγCEAInt2.

pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pγCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pγCEAInt2 with Pst I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pγCEAInt2 with Ava I, blunt ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.

The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pγCEAtrp207-1*.

E.1.9 Production of Immunoglobulin Chains by *E. coli*

E. coli strain W3110 (ATTC No. 27325) was transformed with pγCEAtrp207-1* or pKCEAtrp207-1* using standard techniques.

To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1*, pKCEAtrp207-1*Δ, which had been modified by cleaving a Pst I-Pvu I fragment from the amp^R gene and religating. Cells transformed with pKCEAtrp207-1*Δ are thus sensitive to ampicillin but still resistant to tetracycline. Successful transformants were retransformed using pγCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1*Δ and pγCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 μg/ml tetracycline, and induced with indoleacrylic acid (IAA) when the OD 550 reads 0.5. The induced cells were grown at 37° C. during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1 M β-mercaptoethanol and boiled for 5 minutes. A 10×volume of acetone was added and the cells kept at 22° C. for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell, P. H., *J. Biol. Chem.,* 250: 4007 (1975)); boiled 3 minutes, recentrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman, D. et al., *Science* 211: 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

Cells transformed with pγCEAtrp207-1* showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1* showed the proper molecular weight band for light chain as identified by Western blot; double transformed cells showed bands for both heavy and light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in FIGS. **8**A, **8**B, and **8**C.

FIG. **8**A shows results developed by silver stain from cells transformed with pγCEAtrp207-1*. Lane 1 is monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b–5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a–5a are corresponding untransformed controls; Lanes 2c–5c are corresponding uninduced transformants.

FIG. **8**B shows results developed by Western blot from cells transformed with pKCEAtrp207-1*. Lanes 1b–6b are extracts from induced cell immediately, 1 hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a–6a corresponding uninduced controls. Lane 7 is an extract from a pγCEAtrp207-1* control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

FIG. **8**C shows results developed by Western blot from four colonies of double transformed cells 24 hours after IAA addition (lanes 4–7). Lanes 1–3 are varying amounts of monoclonal gamma chain controls, lanes 8 and 9 are untransformed and pγCEAtrp207-1* transformed cell extracts, respectively.

In another quantitative assay, frozen, transformed *E. coli* cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS)/β-mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using ^{125}I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in FIG. **9**. The figure shows that the *E. coli* products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in *E. coli*. Heavy chain from mammalian cells is expected to be slightly heavier than *E. coli* material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

	(Per gram of cells)
E. coli (W3110/pγCEAtrp207-1*)	5 mg γ
E. coli (W3110/pKCEAtrp207-1*)	1.5 mg K
E. coli (W3110/pKCEAtrp207-1*A, pγCEAInt2)	0.5 mg K, 1.0 mg γ

E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

In order to obtain heavy and light chain preparations for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

E. coli (W3110/pγCEAtrp207-1*) were inoculated into 500 ml LB medium containing 5 μg/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transferred to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 μg/ml tetracycline. Additional glucose was added during growth and at OD 550=20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50 μg/ml. The cells were fed additional glucose to a final OD 550=40, achieved approximately 6 hours from the IAA addition.

E. coli (W3110) cells transformed with pKCEA trp 207-1* and double transformed (with pKCEAtrp207-1*Δ and pγCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after IAA addition at harvest was 25–30.

The cells were then harvested by centrifugation, and frozen.

E.2 Assay Method for Reconstituted Antibody

Anti-CEA activity was determined by ELISA as a criterion for successful reconstitution. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubating 100 μl of 2–5 μg CEA/ml solution in 0.1M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 μl of 0.5 percent BSA in PBS for 2 hours at 37° C., followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in FIG. **10**), was run, which consisted of 50 μl samples of 10 μg, 5 μg, 1 μg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng anti-CEA/ml in 0.5 percent BSA in PBS, plus 50 μl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37° C.

The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 μl of an enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37° C. for 90 minutes. The plates were washed 4 times with PBS before adding the substrate, 100 μl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37° C. for color development.

The A_{450} of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A_{450} data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples' concentration were calculated based on the A_{450} data.

E.3 Reconstitution of Recombinant Antibody and Assay

Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 30,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PMSF, and used immediately or stored frozen at −80° C.; frozen lysates were never thawed more than once.

The S-sulfonate of *E. coli* produced anti-CEA heavy chain (γ) was prepared as follows: Recombinant *E. coli* cells transformed with pγCEAtrp207-1* which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1 mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of γ-$SS0_3$.

650 μl of cell lysate from cells of various *E. coli* strains producing various IgG chains, was added to 500 mg urea. To this was added β-mercaptoethanol to 20 mM, Tris-HCl, pH 8.5 to 50 mM and EDTA to 1 mM, and in some experiments, γ-$SS0_3$ was added to 0.1 mg/ml. After standing at 25° for 30–90 mins., the reaction mixtures were dialyzed at 4° against a buffer composed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10 mM glycine ethyl ester, 5 mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N_2-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16–48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1 mM PMSF and dialysis continued another 16–24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in x ng/ml anti-CEA. Also shown are the reconstitution efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of γ and K chains in the reaction mixtures.

	ng/ml anti-CEA	Percent recombination
E. coli W3110 producing IFN-αA (control)	0	—
E. coli (W3110/pKCEAtrp207-1*)	108	—
E. coli (W3110/pKCEAtrp207-1*), plus γ-SSO_3	848	0.33
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEAInt2)	1580	0.76
Hybridoma anti-CEA K-SSO_3 and γ-SSO_3	540	0.40

E.4 Preparation of Chimeric Antibody

FIGS. **11** and **12** show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human γ-2 constant region.

A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA library obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACTCGACACAA 3 ' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., *Cell,* 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., *Proc. Natl. Acad. Sci.* (*USA*), 79: 1984 (1982) incorporated herein by reference).

As shown in FIG. **11**, two fragments are obtained from this cloned human gamma 2 plasmid (pγ2). The first fragment is formed by digestion with PvuII followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the pγ2 with any restriction enzyme which cleaves in the 3' untranslated region of γ2, as deduced from the nucleotide sequence, filling in the Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 6 percent PAGE. (The choice of a two step, two fragment composition to supply the PvuII-3' untranslated fragment provides a cleaner path to product due to the proximity of the AvaIII site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pγCEA207-1* is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

The location and DNA sequence surrounding the PvuII site in the mouse gamma-1 gene are identical to the location and DNA sequence surrounding the PvuII site in the human gamma-2 gene.

The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when transformed into *E. coli,* but one wherein the change from mouse to human does not take place at the variable to constant junction.

FIG. **12** shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human γ-2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described pγ2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and isolating the short fragment which comprises the junction between variable and constant regions of this chain.

Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., *Nucleic Acids Res.* 9: 309 (1981), followed by in vitro site directed deletion mutagenesis as described by Adelman, et al., *DNA,* in press (1983) which is incorporated

herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form pChim2. This plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable/human constant chimeric heavy chain.

In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than γ chain, the expression plasmid for chimeric light chain is prepared.

The foregoing two plasmids are then double transformed into *E. coli* W3110, the cells grown and the chains reconstituted as set forth in paragraph E.1–E.3 supra.

E.5 Preparation of Altered Murine Anti-CEA Antibody

E.5.1 Construction of Plasmid Vectors for Direct Expression of Altered Murine Anti-CEA Heavy Chain Gene

The cysteine residues, and the resultant disulfide bonds in the region of amino acids 216–230 in the constant region of murine anti-CEA heavy chain are suspected to be important for complement fixation (Klein, et al., *Proc. Natl. Acad. Sci., (USA)*, 78: 524 (1981)) but not for the antigen binding property of the resulting antibody. To decrease the probability of incorrect disulfide bond formation during reconstitution according to the process of the invention herein, the nucleotides encoding the amino acid residues 226–232 which includes codons for three cysteines, are deleted as follows:

A "deleter" deoxyoligonucelotide, 5' CTAACACCATGTCAGGGT is used to delete the relevant portions of the gene from pγCEAtrp207-1* by the procedure of Wallace, et al., *Science,* 209: 1396 (1980) or of Adelman, et al., *DNA* 2, 183 (1983). Briefly, the "deleter" deoxyoligonucelotide is annealed with denatured pγCEAtrp207-1* DNA, and primer repair synthesis carried out in vitro, followed by screening by hybridization of presumptive deletion clones with P^{32} labelled deleter sequence.

E.5.2 Production of Cysteine Deficient Altered Antibody

The plasmid prepared in E.5.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains, and the altered antibody reconstituted as described in E.1.10.

E.6 Preparation of Fab

E.6.1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene pγCEAFabtrp207-1*

FIG. **13** presents the construction of pγCEAFabtrp207-1*. 5 μg of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment I, was recovered using 6 percent PAGE followed by electroelution.

5 μg of pγCEAtrp207-1* was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a termination codon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 μg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (FIG. **4**) which has the following sequence:

```
AspCysGlyStop
5' GGGATTGTGGTTG  3'
```

The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

E.6.2 Production of Fab Protein

The plasmid prepared in E.6.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

What is claimed is:

1. A process for producing an immunoglobulin molecule or an immunologically functional immunoglobulin fragment comprising at least the variable domains of the immunoglobulin heavy and light chains, in a single host cell, comprising the steps of:

(i) transforming said single host cell with a first DNA sequence encoding at least the variable domain of the immunoglobulin heavy chain and a second DNA sequence encoding at least the variable domain of the immunoglobulin light chain, and

(ii) independently expressing said first DNA sequence and said second DNA sequence so that said immunoglobulin heavy and light chains are produced as separate molecules in said transformed single host cell.

2. The process according to claim **1** wherein said first and second DNA sequences are present in different vectors.

3. The process according to claim **1** wherein said first and second DNA sequences are present in a single vector.

4. A process according to claim **3** wherein the vector is a plasmid.

5. The process according to claim **4** wherein the plasmid is pBR322.

6. The process according to claim **1** wherein the host cell is a bacterium or yeast.

7. The process according to claim **6** wherein the host cell is *E. coli* or *S. cerevisiae.*

8. A process according to claim **7** wherein the host cell is *E. coli* strain X1776 (ATCC No. 31537).

9. A process according to claim **1** wherein the immunoglobulin heavy and light chains are expressed in the host cell and secreted therefrom as an immunologically functional immunoglobulin molecule or immunoglobulin fragment.

10. A process according to claim **1** wherein the immunoglobulin heavy and light chains are produced in insoluble form and are solubilized and allowed to refold in solution to form an immunologically functional immunoglobulin molecule or immunoglobulin fragment.

11. A process according to claim **1** wherein the DNA sequences code for the complete immunoglobulin heavy and light chains.

12. The process according to claim **1** wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from the same source as the variable domain to which it is attached.

13. The process according to claim **1** wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from a species or class different from that from which the variable domain to which it is attached is derived.

14. The process according to claim **1** wherein said first and second DNA sequences are derived from one or more monoclonal antibody producing hybridomas.

15. A vector comprising a first DNA sequence encoding at least a variable domain of an immunoglobulin heavy chain and a second DNA sequence encoding at least a variable domain of an immunoglobulin light chain wherein said first DNA sequence and said second DNA sequence are located in said vector at different insertion sites.

16. A vector according to claim **15** which is a plasmid.

17. A host cell transformed with a vector according to claim **15**.

18. A transformed host cell comprising at least two vectors, at least one of said vectors comprising a DNA sequence encoding at least a variable domain of an immunoglobulin heavy chain and at least another one of said vectors comprising a DNA sequence encoding at least the variable domain of an immunoglobulin light chain.

19. The process of claim **1** wherein the host cell is a mammalian cell.

20. The transformed host cell of claim **18** wherein the host cell is a mammalian cell.

21. A method comprising

a) preparing a DNA sequence consisting essentially of DNA encoding an immunoglobulin consisting of an immunoglobulin heavy chain and light chain or Fab region, said immunoglobulin having specificity for a particular known antigen;

b) inserting the DNA sequence of step a) into a replicable expression vector operably linked to a suitable promoter;

c) transforming a prokaryotic or eukaryotic microbial host cell culture with the vector of step b);

d) culturing the host cell; and

e) recovering the immunoglobulin from the host cell culture, said immunoglobulin being capable of binding to a known antigen.

22. The method of claim **21** wherein the heavy and light chain are the heavy and light chains of anti-CEA antibody.

23. The method of claim **21** wherein the heavy chain is of the gamma family.

24. The method of claim **21** wherein the light chain is of the kappa family.

25. The method of claim **21** wherein the vector contains DNA encoding both a heavy chain and a light chain.

26. The method of claim **21** wherein the host cell is *E. coli* or yeast.

27. The method of claim **26** wherein the heavy chain and light chains or Fab region are deposited within the cells as insoluble particles.

28. The method of claim **27** wherein the heavy and light chains are recovered from the particles by cell lysis followed by solubilization in denaturant.

29. The method of claim **21** wherein the heavy and light chains are secreted into the medium.

30. The method of claim **21** wherein the host cell is a gram negative bacterium and the heavy and light chains are secreted into the periplasmic space of the host cell bacterium.

31. The method of claim **21** further comprising recovering both heavy and light chain and reconstituting light chain and heavy chain to form an immunoglobulin having specific affinity for a particular known antigen.

32. The insoluble particles of heavy chain and light chains or Fab region produced by the method of claim **27**.

33. A process for producing an immunoglobulin molecule or an immunologically functional immunoglobulin fragment comprising at least the variable domains of the immunoglobulin heavy and light chains, in a single host cell, comprising:

independently expressing a first DNA sequence encoding at least the variable domain of the immunoglobulin heavy chain and a second DNA sequence encoding at least the variable domain of the immunoglobulin light chain so that said immunoglobulin heavy and light chains are produced as separate molecules in said single host cell transformed with said first and second DNA sequences.

34. The process of claim **9**, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

35. The process of claim **10**, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

36. The process of claim **33**, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,331,415 B1
DATED : December 18, 2001
INVENTOR(S) : Shmuel Cabilly et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [73], Assignee, please insert -- City of Hope, Duarte, CA (US) --.

Column 19,
Line 56, please change "BamH 1" to -- BamH I --.

Signed and Sealed this

Twenty-fifth Day of June, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

US006331415C1

(12) EX PARTE REEXAMINATION CERTIFICATE (6829th)

United States Patent

Cabilly et al.

(10) Number: US 6,331,415 C1

(45) Certificate Issued: May 19, 2009

(54) **METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN**

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(51) **Int. Cl.**

C12N 15/13	(2006.01)
C12N 15/00	(2006.01)
C12N 15/63	(2006.01)

(52) **U.S. Cl.** **435/69.6**; 435/252.1; 435/252.3; 435/252.33; 435/254.11; 435/254.2; 435/254.21; 435/69.7; 435/70.21; 435/71.2; 435/71.1; 435/70.1; 435/320.1; 435/455; 435/483; 435/485; 435/471; 435/69.1

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

The invention relates to processes for producing an immunoglobulin or an immunologically functional immunoglobulin fragment containing at least the variable domains of the immunoglobulin heavy and light chains. The processes can use one or more vectors which produce both the heavy and light chains or fragments thereof in a single cell. The invention also relates to the vectors used to produce the immunoglobulin or fragment, and to cells transformed with the vectors.

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Deposition transcript of Herbert Heyneker (Oct. 28, 1997), with exhibits.
Deposition transcript of William E. Holmes (Oct. 19, 2007), with exhibits.
Deposition transcript of Arthur D. Riggs, M.D. (Nov. 16, 2007).
Deposition transcript of Ronald Burnell Wetzel (Nov. 29, 2007), with exhibits.
Deposition transcript of Ian M. Armitage (Oct. 16, 2007), with exhibits.
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EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

The patentability of claims **1**–**20** and **33**–**36** is confirmed.

Claims **21**, **27** and **32** are determined to be patentable as amended.

Claims **22**–**26** and **28**–**31**, dependent on an amended claim, are determined to be patentable.

21. A method comprising

a) preparing a *first* DNA sequence **[**consisting essentially of DNA**]** encoding an immunoglobulin **[**consisting of

an immunoglobulin**]** heavy chain and *a second DNA sequence encoding an immunoglobulin* light chain **[**or Fab region, said immunoglobulin having specificity for a particular known antigen**]**;

b) inserting the DNA **[**sequence**]** *sequences* of step a) into a replicable expression vector *wherein each sequence is* operably linked to a suitable promoter;

c) transforming a prokaryotic or eukaryotic microbial host cell culture with the vector of step b);

d) culturing the host cell *so that said immunoglobulin heavy and light chains are produced as separate molecules in said transformed host cell*; and

e) recovering the immunoglobulin from the host cell culture, said immunoglobulin being capable of binding to a known antigen.

27. The method of claim **26** wherein the heavy chain and light **[**chains or Fab region**]** *chain* are deposited within the cells as insoluble particles.

32. The insoluble particles of heavy chain and light chains **[**or Fab region**]** produced by the method of claim **27**.

* * * * *

EXHIBIT B

US007923221B1

(12) **United States Patent**
Cabilly et al.

(10) **Patent No.: US 7,923,221 B1**
(45) **Date of Patent: *Apr. 12, 2011**

(54) **METHODS OF MAKING ANTIBODY HEAVY AND LIGHT CHAINS HAVING SPECIFICITY FOR A DESIRED ANTIGEN**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **08/422,187**

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(58) **Field of Classification Search** 435/69.6, 435/69.1, 325, 252.3; 530/387.3
See application file for complete search history.

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(57) **ABSTRACT**

The invention relates to processes for producing an immunoglobulin or an immunologically functional immunoglobulin fragment containing at least the variable domains of the immunoglobulin heavy and light chains. The processes can use one or more vectors which produce both the heavy and light chains or fragments thereof in a single cell. The invention also relates to the vectors used to produce the immunoglobulin or fragment, and to cells transformed with the vectors.

47 Claims, 19 Drawing Sheets

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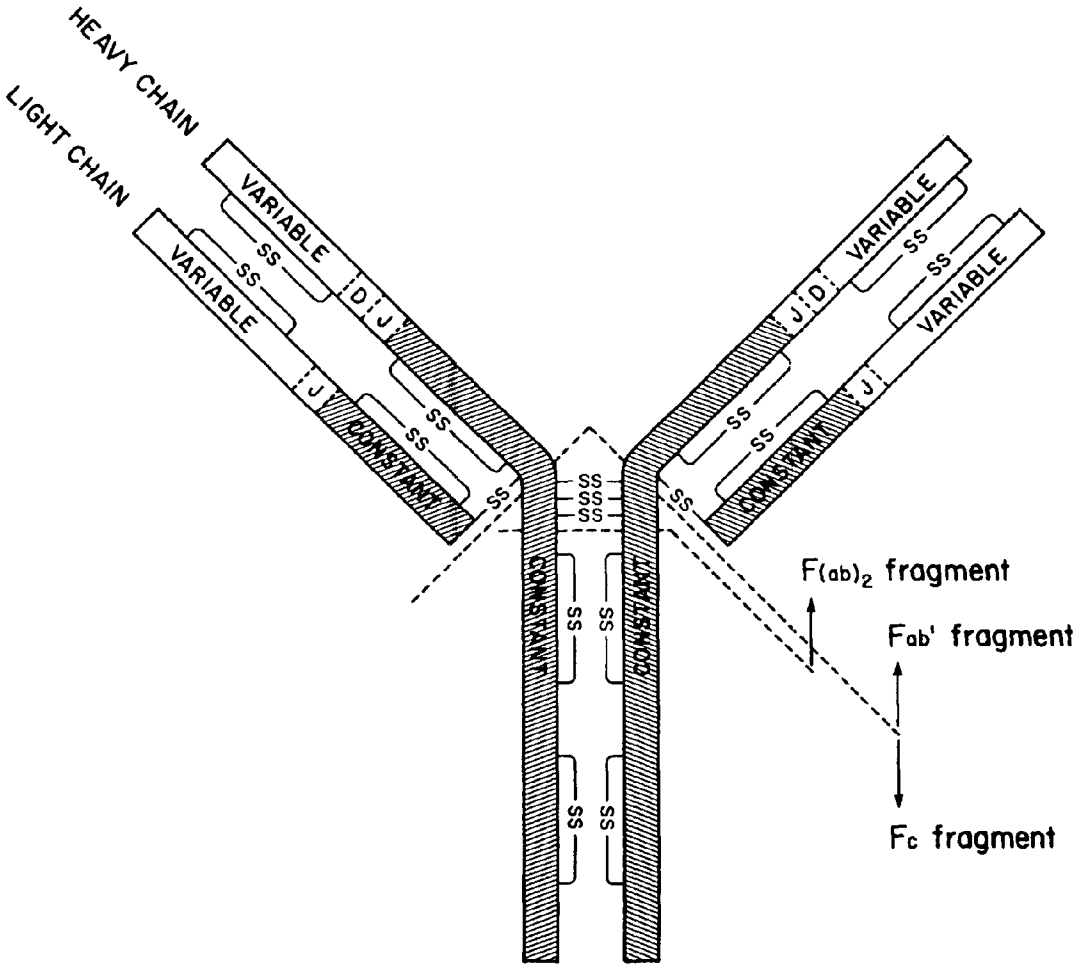
HEAVY CHAIN
LIGHT CHAIN
VARIABLE
CONSTANT
D
J
SS
F(ab)2 fragment
Fab' fragment
Fc fragment

Fig 1

tth111
hphI
haeIII
haeI
GTTGCTGTGG TTGTCTGGTG TTGAAGGAGA CATTGTGATG ACCCAGTCTC ACAAATTCAT GTCCACATCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG
CAACGACACC AACAGACCAC AACTTCCTCT GTAACACTAC TGGGTCAGAG TGTTTAAGTA CAGGTGTAGT CATCCTCTGT CCCAGTCGTA GTGGACGTTC
sfaNI
scrFI
nciI
hpaII
hinfI
fnu4HI
bbv
scrFI
ecoRII
fokI
GCCAGTCAGG ATGTGGGTGC TGCTATAGCC TGGTATCAAC AGAAACCAGG ACAATCTCCT AAACTACTGA TTTACTGGGC ATCCACCCGG CACACTGGAG
CGGTCAGTCC TACACCCACG ACGATATCGG ACCATAGTTG TCTTTGGTCC TGTTAGAGGA TTTGATGACT AAATGACCCG TAGGTGGGCC GTGTGACCTC
fokI
sfaNI
xhoII
sau3A
dpnI
hphI
hincII
sau3A
dpnI
TCCCTGATCG CTTCACAGGC AGTGGATCTG GGACAGATTT CACTCTCACC ATTAGCAATG TGCAGTCTGA TGACTTGGCA GATTATTTCT GTCAACAATA
AGGGACTAGC GAAGTGTCCG TCACCTAGAC CCTGTCTAAA GTGAGAGTGG TAATCGTTAC ACGTCAGACT ACTGAACCGT CTAATAAAGA CAGTTGTTAT
fnu4HI
bbv
hpaI
hincII
sau96
aval1
aluI
aluI
sfaNI
mboII
mnlI
TAGCGGGTAT CCTCTCACGT TCGGTGCTGG GACCAAGCTG GAGCTGAAAC GGGCTGATGC TGCACCAACT GTATCCATCT TCCCACCATC CAGTGAGCAG
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fokI
mnlI
ddeI
xmnI
mboII
acyI
mnlI
TTAACATCTG GAGGTGCCTC AGTCGTGTGC TTCTTGAACA ACTTCTACCC CAAAGACATC AATGTCAAGT GGAAGATTGA TGGCAGTGAA CGACAAAATG
AATTGTAGAC CTCCACGGAG TCAGCACACG AAGAACTTGT TGAAGATGGG GTTTCTGTAG TTACAGTTCA CCTTCTAACT ACCGTCACTT GCTGTTTTAC

Fig. 2A.

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                                sau3A
                                dpnI                                           fnu4HI
          hgaI               bclI                                              bbv        mnlI   hincII                                 aluI
501       GCGTCCTGAA CAGTTGGACT GATCAGGACA GCAAAGACAG CACCTACAGC ATGAGCAGCA CCCTCACGTT GACCAAGGAC GAGTATGAAC GACATAACAG
          CGCAGGACTT GTCAACCTGA CTAGTCCTGT CGTTTCTGTC GTGGATGTCG TACTCGTCGT GGGAGTGCAA CTGGTTCCTG CTCATACTTG CTGTATTGTC

                     mnlI                                                                                   sau96     hgaI
                       haeIII                                                                                   ddeI
                      haeI                      hphI                aluI                                    avaII     acyI
601       CTATACCTGT GAGGCCACTC ACAAGACATC AACTTCACCC ATTGTCAAGA GCTTCAACAG GAATGAGTGT TAGAGACAAA GGTCCTGAGA CGCCACCACC
          GATATGGACA CTCCGGTGAG TGTTCTGTAG TTGAAGTGGG TAACAGTTCT CGAAGTTGTC CTTACTCACA ATCTCTGTTT CCAGGACTCT GCGGTGGTGG

                                                                                                                mnlI
          aluI     aluI              mboII     ddeI            mnlI                                  hgiA     mnlI          mnlI
701       AGCTCCCCAG CTCCATCCTA TCTTCCCTTC TAAGGTCTTG GAGGCTTCCC CACAAGCGAC CTACCACTGT TGCGGTGCTC CAAACCTCCT CCCCACCTCC
          TCGAGGGGTC GAGGTAGGAT AGAAGGGAAG ATTCCAGAAC CTCCGAAGGG GTGTTCGCTG GATGGTGACA ACGCCACGAG GTTTGGAGGA GGGGTGGAGG
              fokI

                  mnlI
              mnlI   mnlI                                          xmnI                 hinfI
801       TTCTCCTCCT CCTCCCTTTC CTTGGCTTTT ATCATGCTAA TATTTGCAGA AAATATTCAA TAAAGTGAGT CTTTGCACTT GA
          AAGAGGAGGA GGAGGGAAAG GAACCGAAAA TAGTACGATT ATAAACGTCT TTTATAAGTT ATTTCACTCA GAAACGTGAA CT
```

nucleotides: 882

Fig. 2B.

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    -9                                1                                       10                                      20
    leu leu trp leu ser gly val glu gly asp ile val met thr gln ser his lys phe met ser thr ser val gly asp arg val ser
G   UUG CUG UGG UUG UCU GGU GUU GAA GGA GAC AUU GUG AUG ACC CAG UCU CAC AAA UUC AUG UCC ACA UCA GUA GGA GAC AGG GUC AGC

                                       30                                      40                                      50
ile thr cys lys ala ser gln asp val gly ala ala ile ala trp tyr gln gln lys pro gly gln ser pro lys leu leu ile tyr trp
AUC ACC UGC AAG GCC AGU CAG GAU GUG GGU GCU GCU AUA GCC UGG UAU CAA CAG AAA CCA GGA CAA UCU CCU AAA CUA CUG AUU UAC UGG

                                       60                                      70                                      80
ala ser thr arg his thr gly val pro asp arg phe thr gly ser gly ser gly thr asp phe thr leu thr ile ser asn val gln ser
GCA UCC ACC CGG CAC ACU GGA GUC CCU GAU CGC UUC ACA GGC AGU GGA UCU GGG ACA GAU UUC ACU CUC ACC AUU AGC AAU GUG CAG UCU

                                       90                                     100                                     110
asp asp leu ala asp tyr phe cys gln gln tyr ser gly tyr pro leu thr phe gly ala gly thr lys leu glu leu lys arg ala asp
GAU GAC UUG GCA GAU UAU UUC UGU CAA CAA UAU AGC GGG UAU CCU CUC ACG UUC GGU GCU GGG ACC AAG CUG GAG CUG AAA CGG GCU GAU

                                      120                                     130                                     140
ala ala pro thr val ser ile phe pro pro ser ser glu gln leu thr ser gly gly ala ser val val cys phe leu asn asn phe tyr
GCU GCA CCA ACU GUA UCC AUC UUC CCA CCA UCC AGU GAG CAG UUA ACA UCU GGA GGU GCC UCA GUC GUG UGC UUC UUG AAC AAC UUC UAC

                                      150                                     160                                     170
pro lys asp ile asn val lys trp lys ile asp gly ser glu arg gln asn gly val leu asn ser trp thr asp gln asp ser lys asp
CCC AAA GAC AUC AAU GUC AAG UGG AAG AUU GAU GGC AGU GAA CGA CAA AAU GGC GUC CUG AAC AGU UGG ACU GAU CAG GAC AGC AAA GAC

                                      180                                     190                                     200
ser thr tyr ser met ser ser thr leu thr leu thr lys asp glu tyr glu arg his asn ser tyr thr cys glu ala thr his lys thr
AGC ACC UAC AGC AUG AGC AGC ACC CUC ACG UUG ACC AAG GAC GAG UAU GAA CGA CAU AAC AGC UAU ACC UGU GAG GCC ACU CAC AAG ACA

                                      210             214
ser thr ser pro ile val lys ser phe asn arg asn glu cys AM
UCA ACU UCA CCC AUU GUC AAG AGC UUC AAC AGG AAU GAG UGU UAG AGACAAAGGUCCUGAGACGCCACCACCAGCUCCCCAGCUCCAUCCUAUCUUCCCUUCUAA

GGUCUUGGAGGCUUCCCCACAAGCGACCUACCACUGUUGCGGUGCUCCAAACCUCCUCCCCACCUCCUUCUCCUCCUCCUCCCUUUCCUUGGCUUUUAUCAUGCUAAUAUUUGCAGAAAA

UAUUCAAUAAAGUGAGUCUUUGCACUUGA
```

Fig. 3

```
                          sau96                      ddeI
        hinfI             avaII mnlI                    aluI                     ahaIII           sfaNI
1       GAGTCAGCAC TGAACACGGA CCCCTCACGA TGAACTTCGG GCTCAGCTTG ATTTACCTTG TCCTTGTTTT AAAAGTTGTC CAGTGTGAAG TGATGCTGGT
        CTCAGTCGTG ACTTGTGCCT GGGGAGTGCT ACTTGAAGCC CGAGTCGAAC TAAATGGAAC AGGAACAAAA TTTTCAACAG GTCACACTTC ACTACGACCA

                                   scrFI   sau96
                                       mnlI                      fnu4HI
         hinfI      hinfI          ecoRII  avaII                 bbv mnlI   hinfI
101     GGAGTCTGGG GGAGTCTTAA TGGAGCCTGG AGGGTCCCTG AAACTCTCCT GTGCAGCCTC TGGATTCACT TTCAGTAGAT ATGCCATGTC TTGGGTTCGC
        CCTCAGACCC CCTCAGAATT ACCTCGGACC TCCCAGGGAC TTTGAGAGGA CACGTCGGAG ACCTAAGTGA AAGTCATCTA TACGGTACAG AACCCAAGCG

              hpaII    mnlI                                                                             hphI
          hinfI     mboII                                                                            hinfI
201     CAGACTCCGG AGAAGAGGCT GGAGTGGGTC GCAACCATTA GTAGTGGTGG TAGTTCACAC CTTCCATCCA GACAGTGTGA AGGGCGATTC ACCATCTCCA
        GTCTGAGGCC TCTTCTCCGA CCTCACCCAG CGTTGGTAAT CATCACCACC ATCAAGTGTG GAAGGTAGGT CTGTCACACT TCCCGCTAAG TGGTAGAGGT
                                                                    fokI

                                                       mnlI    mnlI
                                rsaI                 ddeI   ddeI       haeIII                      mnlI
301     GAGACAATGC CAAGAACACC CTGTACCTGC AAATGAGCAG TCTGAGGTCT GAGGACACGG CCATGTATTA CTGTGCAAGA CCCCCTCTTA TTTCGTTAGT
        CTCTGTTACG GTTCTTGTGG GACATGGACG TTTACTCGTC AGACTCCAGA CTCCTGTGCC GGTACATAAT GACACGTTCT GGGGGAGAAT AAAGCAATCA

                                                                                                               xhoII
                                                                                                            scrFI
                                                                                                       sau96    sau3A
                                              mnlI             mnlI                                         ecoRII
                                               ddeI  hphI       ddeI                                   haeIII   dpnI
401     AGCGGACTAT GCTATGGACT ACTGGGGTCA AGGAACCTCA GTCACCGTCT CCTCAGCCAA AACGACACCC CCATCTGTCT ATCCACTGGC CCCTGGATCT
        TCGCCTGATA CGATACCTGA TGACCCCAGT TCCTTGGAGT CAGTGGCAGA GGAGTCGGTT TTGCTGTGGG GGTAGACAGA TAGGTGACCG GGGACCTAGA
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Fig. 4A.

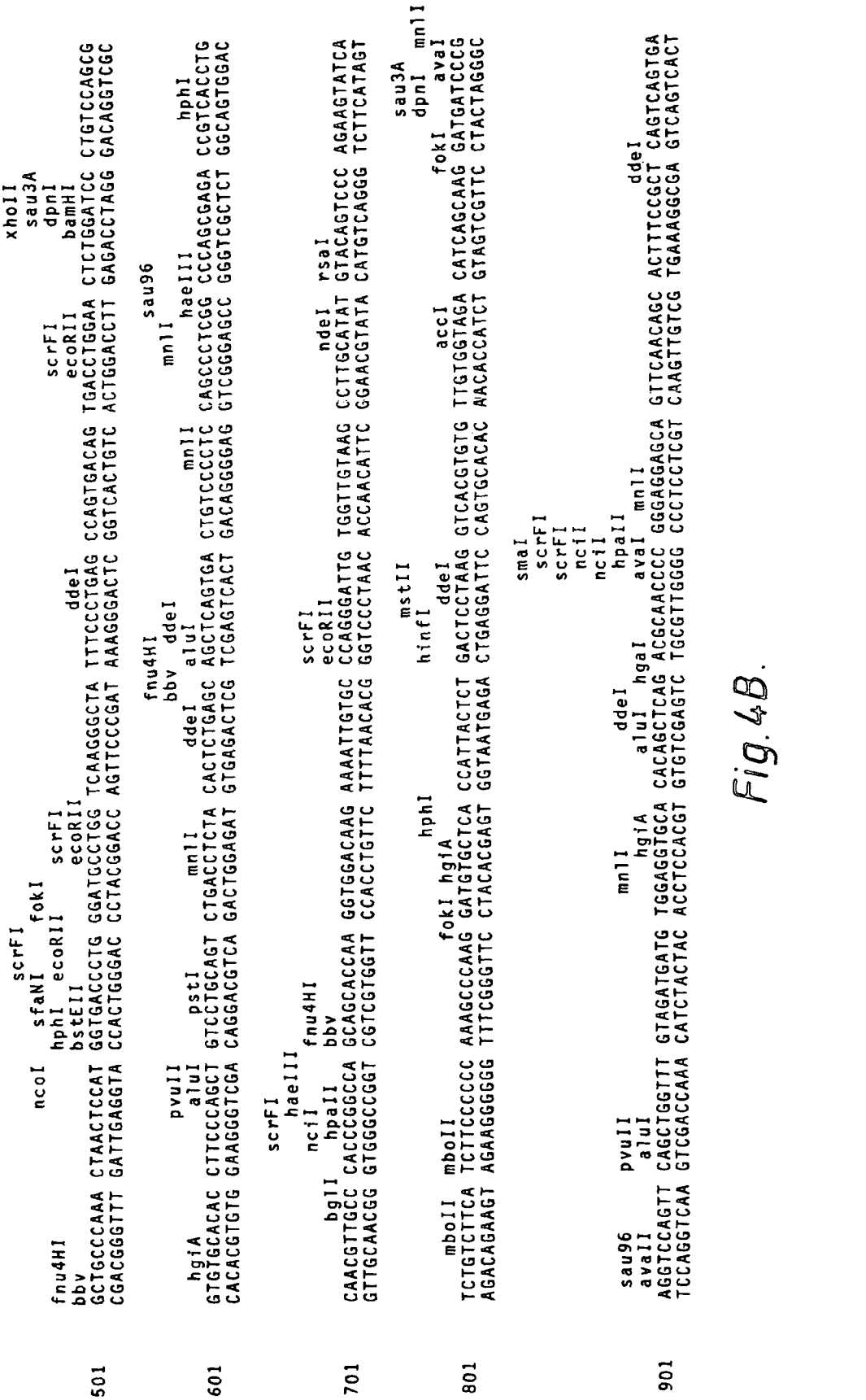
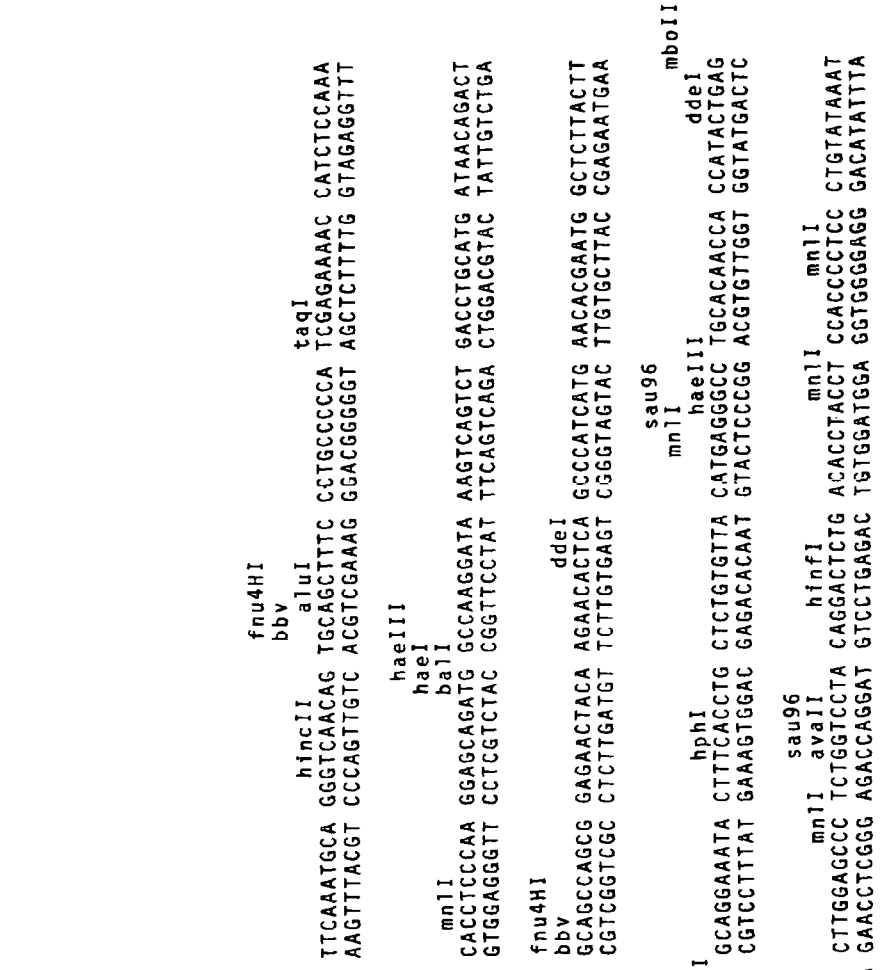


Fig. 4B.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bbv
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ecoRII
hincII
aluI
taqI
haeIII
haeI
ball
rsaI
mnlI
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bbv
ddeI
sau96
mnlI
haeIII
mboII
mboII
mboII
mnlI
hphI
ddeI
accI
aluI
mboII
mnlI
sau96
scrFI
sau3A
mnlI
avaII
hinfI
mnlI
mnlI
ecoRII
dpnI
1001
ACTTCCCATC ATGCACCAGG ACTGGCTCAA TGGCAAGGAG TTCAAATGCA GGGTCAACAG TGCAGCTTTC CCTGCCCCCA TCGAGAAAAC CATCTCCAAA
TGAAGGGTAG TACGTGGTCC TGACCGAGTT ACCGTTCCTC AAGTTTACGT CCCAGTTGTC ACGTCGAAAG GGACGGGGGT AGCTCTTTTG GTAGAGGTTT
1101
ACCAAAGGCA GACCGAAGGC TCCACAGGTG TACACCATTC CACCTCCCAA GGAGCAGATG GCCAAGGATA AAGTCAGTCT GACCTGCATG ATAACAGACT
TGGTTTCCGT CTGGCTTCCG AGGTGTCCAC ATGTGGTAAG GTGGAGGGTT CCTCGTCTAC CGGTTCCTAT TTCAGTCAGA CTGGACGTAC TATTGTCTGA
1201
TCTTCCCTGA AGACATTACT GTGGAGTGGC AGTGGAATGG GCAGCCAGCG GAGAACTACA AGAACACTCA GCCCATCATG AACACGAATG GCTCTTACTT
AGAAGGGACT TCTGTAATGA CACCTCACCG TCACCTTACC CGTCGGTCGC CTCTTGATGT TCTTGTGAGT CGGGTAGTAC TTGTGCTTAC CGAGAATGAA
1301
CGTCTACAGC AAGCTCAATG TGCAGAAGAG CAACTGGGAG GCAGGAAATA CTTTCACCTG CTCTGTGTTA CATGAGGGCC TGCACAACCA CCATACTGAG
GCAGATGTCG TTCGAGTTAC ACGTCTTCTC GTTGACCCTC CGTCCTTTAT GAAAGTGGAC GAGACACAAT GTACTCCCGG ACGTGTTGGT GGTATGACTC
1401
AAGAGCCTCT CCCACTCTCC TGGTAAATGA TCCCAGTGTC CTTGGAGCCC TCTGGTCCTA CAGGACTCTG ACACCTACCT CCACCCCTCC CTGTATAAAT
TTCTCGGAGA GGGTGAGAGG ACCATTTACT AGGGTCACAG GAACCTCGGG AGACCAGGAT GTCCTGAGAC TGTGGATGGA GGTGGGGAGG GACATATTTA
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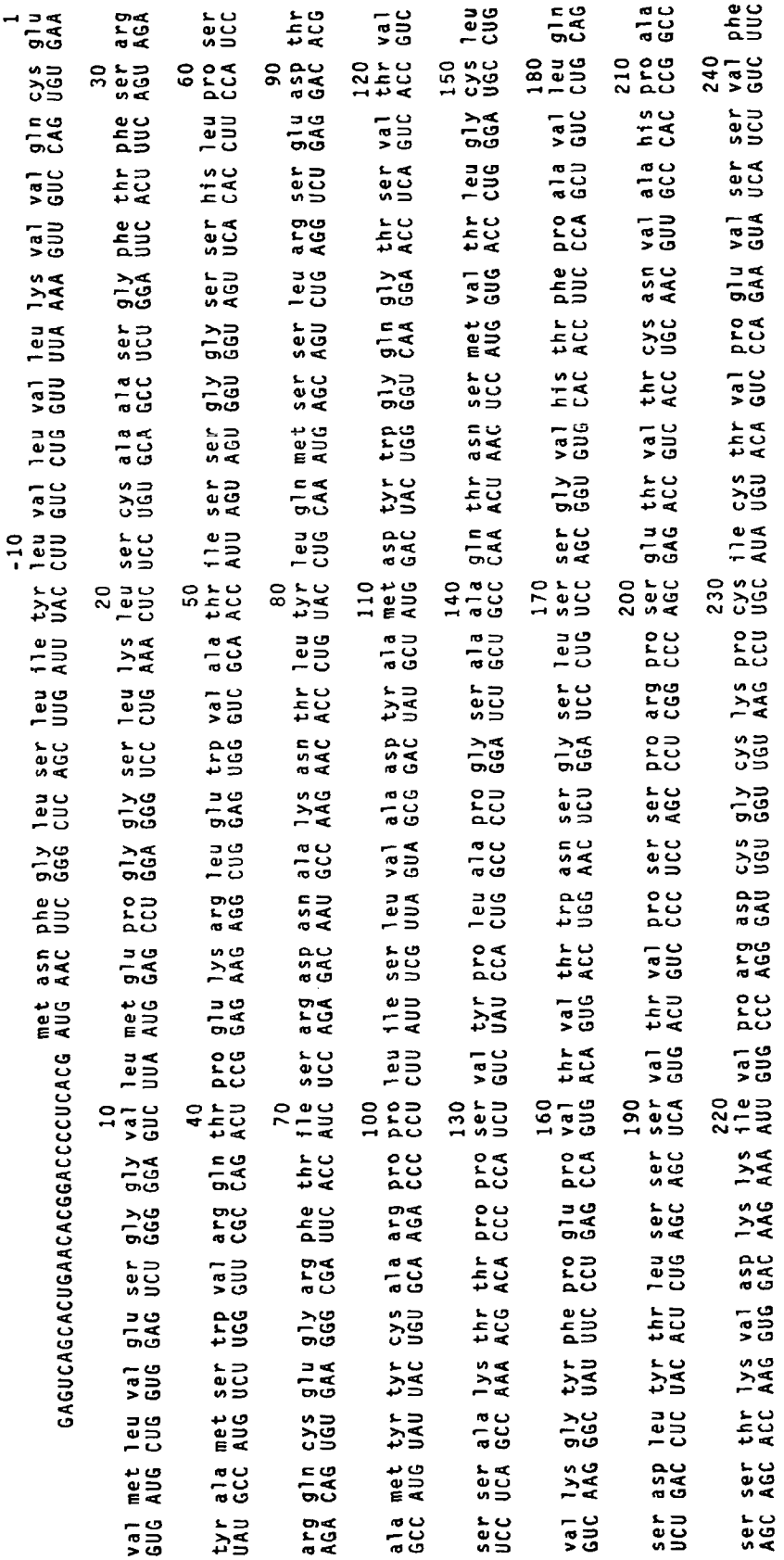
TTTCGTGGGT CGTGACGGAA CCCTTTTT

Fig. 4C.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-10 1
val met leu val glu ser gly gly val leu met glu pro gly gly ser leu lys leu ser cys ala ala ser gly phe thr phe ser arg
GUG AUG CUG GUG GAG UCU GGG GGA GUC UUA AUG GAG CCU GGA GGG UCC CUG AAA CUC UCC UGU GCA GCC UCU GGA UUC ACU UUC AGU AGA
10 20 30
tyr ala met ser trp val arg gln thr pro glu lys arg leu glu trp val ala thr ile ser ser gly gly ser ser his leu pro ser
UAU GCC AUG UCU UGG GUU CGC CAG ACU CCG GAG AAG AGG CUG GAG UGG GUC GCA ACC AUU AGU AGU GGU UCA CAC CUU CCA UCC
40 50 60
arg gln cys glu gly arg phe thr ile ser arg asp asn ala lys asn thr leu tyr leu gln met ser ser leu arg ser glu asp thr
AGA CAG UGU GAA GGG CGA UUC ACC AUC UCC AGA GAC AAU GCC AAG AAC ACC CUG UAC CUG CAA AUG AGC AGU CUG AGG UCU GAG GAC ACG
70 80 90
ala met tyr tyr cys ala arg pro pro leu ile ser leu val ala asp tyr ala met asp tyr trp gly gln gly thr ser val thr val
GCC AUG UAU UAC UGU GCA AGA CCC CCU CUU AUU UCG UUA GUA GCG GAC UAU GCU AUG GAC UAC UGG GGU CAA GGA ACC UCA GUC ACC GUC
100 110 120
ser ser ala lys thr thr pro pro ser val tyr pro leu ala pro gly ser ala ala gln thr asn ser met val thr leu gly cys leu
UCC UCA GCC AAA ACG ACA CCC CCA UCU GUC UAU CCA CUG GCC CCU GGA UCU GCU GCC CAA ACU AAC UCC AUG GUG ACC CUG GGA UGC CUG
130 140 150
val lys gly tyr phe pro glu pro val thr val thr trp asn ser gly ser leu ser ser gly val his thr phe pro ala val leu gln
GUC AAG GGC UAU UUC CCU GAG CCA GUG ACA GUG ACC UGG AAC UCU GGA UCC CUG UCC AGC GGU GUG CAC ACC UUC CCA GCU GUC CUG CAG
160 170 180
ser asp leu tyr thr leu ser ser ser val thr val pro ser ser pro arg pro ser glu thr val thr cys asn val ala his pro ala
UCU GAC CUC UAC ACU CUG AGC AGC UCA GUG ACU GUC CCC UCC AGC CCU CGG CCC AGC GAG ACC GUC ACC UGC AAC GUG GCC CAC CCG GCC
190 200 210
ser ser thr lys val asp lys lys ile val pro arg asp cys gly cys lys pro cys ile cys thr val pro glu val ser ser val phe
AGC AGC ACC AAG GUG GAC AAG AAA AUU GUG CCC AGG GAU UGU GGU UGU AAG CCU UGC AUA UGU ACA GUC CCA GAA GUA UCA UCU GUC UUC
220 230 240

Fig. 5A.

```
                                250                                                 260                                                 270
ile phe pro pro lys pro lys asp val leu thr ile thr leu thr pro lys val thr cys val val val asp ile ser lys asp asp pro
AUC UUC CCC CCA AAG CCC AAG GAU GUG CUC ACC AUU ACU CUG ACU CCU AAG GUC ACG UGU GUU GUG GUA GAC AUC AGC AAG GAU GAU CCC

                                280                                                 290                                                 300
glu val gln phe ser trp phe val asp asp val glu val his thr ala gln thr gln pro arg glu glu gln phe asn ser thr phe arg
GAG GUC CAG UUC AGC UGG UUU GUA GAU GAU GUG GAG GUG CAC ACA GCU CAG ACG CAA CCC CGG GAG GAG CAG UUC AAC AGC ACU UUC CGC

                                310                                                 320                                                 330
ser val ser glu leu pro ile met his gln asp trp leu asn gly lys glu phe lys cys arg val asn ser ala ala phe pro ala pro
UCA GUC AGU GAA CUU CCC AUC AUG CAC CAG GAC UGG CUC AAU GGC AAG GAG UUC AAA UGC AGG GUC AAC AGU GCA GCU UUC CCU GCC CCC

                                340                                                 350                                                 360
ile glu lys thr ile ser lys thr lys gly arg pro lys ala pro gln val tyr thr ile pro pro pro lys glu gln met ala lys asp
AUC GAG AAA ACC AUC UCC AAA ACC AAA GGC AGA CCG AAG GCU CCA CAG GUG UAC ACC AUU CCA CCU CCC AAG GAG CAG AUG GCC AAG GAU

                                370                                                 380                                                 390
lys val ser leu thr cys met ile thr asp phe phe pro glu asp ile thr val glu trp gln trp asn gly gln pro ala glu asn tyr
AAA GUC AGU CUG ACC UGC AUG AUA ACA GAC UUC UUC CCU GAA GAC AUU ACU GUG GAG UGG CAG UGG AAU GGG CAG CCA GCG GAG AAC UAC

                                400                                                 410                                                 420
lys asn thr gln pro ile met asn thr asn gly ser tyr phe val tyr ser lys leu asn val gln lys ser asn trp glu ala gly asn
AAG AAC ACU CAG CCC AUC AUG AAC ACG AAU GGC UCU UAC UUC GUC UAC AGC AAG CUC AAU GUG CAG AAG AGC AAC UGG GAG GCA GGA AAU

                                430                                                 440                             447
thr phe thr cys ser val leu his glu gly leu his asn his his thr glu lys ser leu ser his ser pro gly lys OP
ACU UUC ACC UGC UCU GUG UUA CAU GAG GGC CUG CAC AAC CAC CAU ACU GAG AAG AGC CUC UCC CAC UCU CCU GGU AAA UGA UCCCAGUGUCCU

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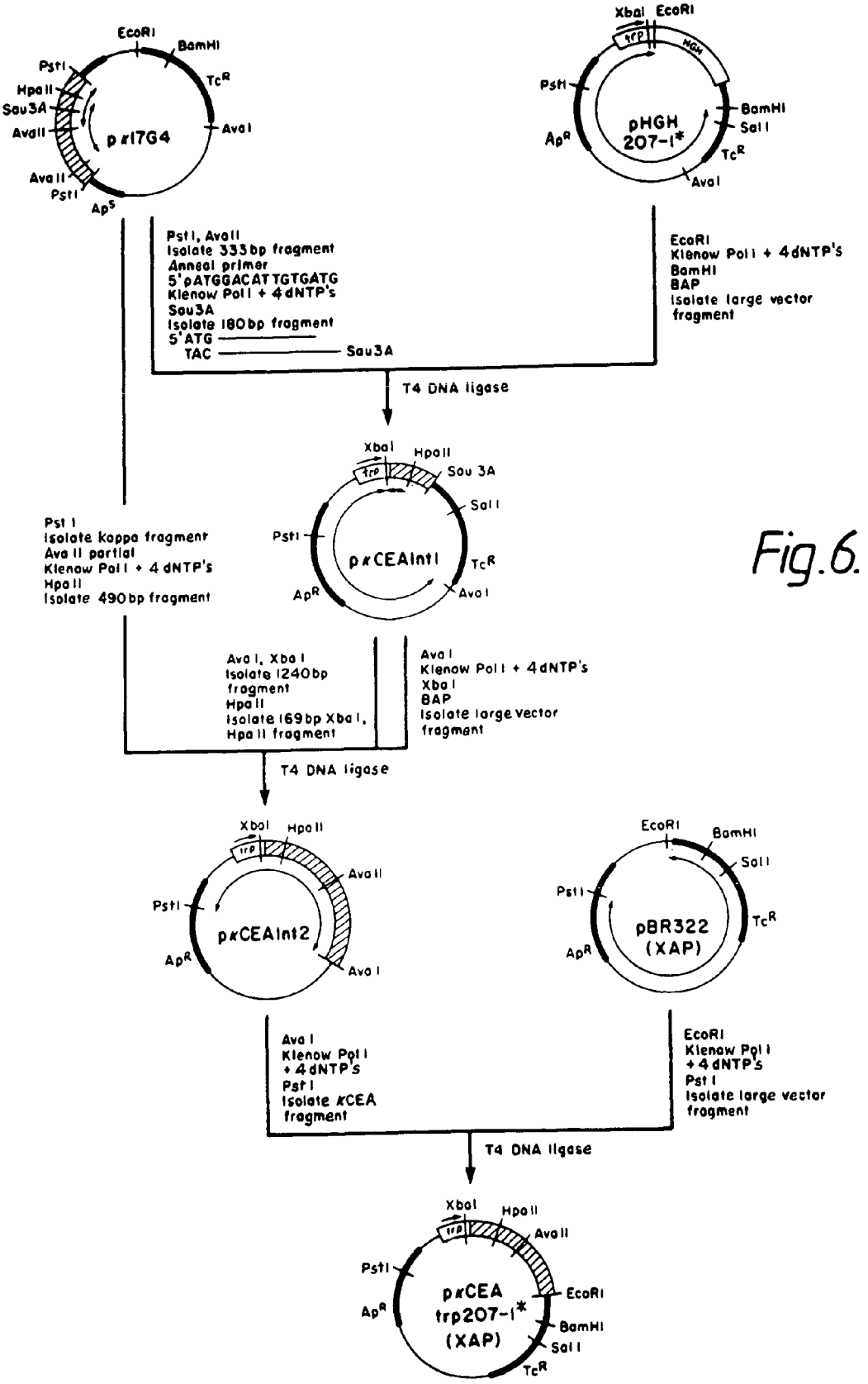
Fig. 5B.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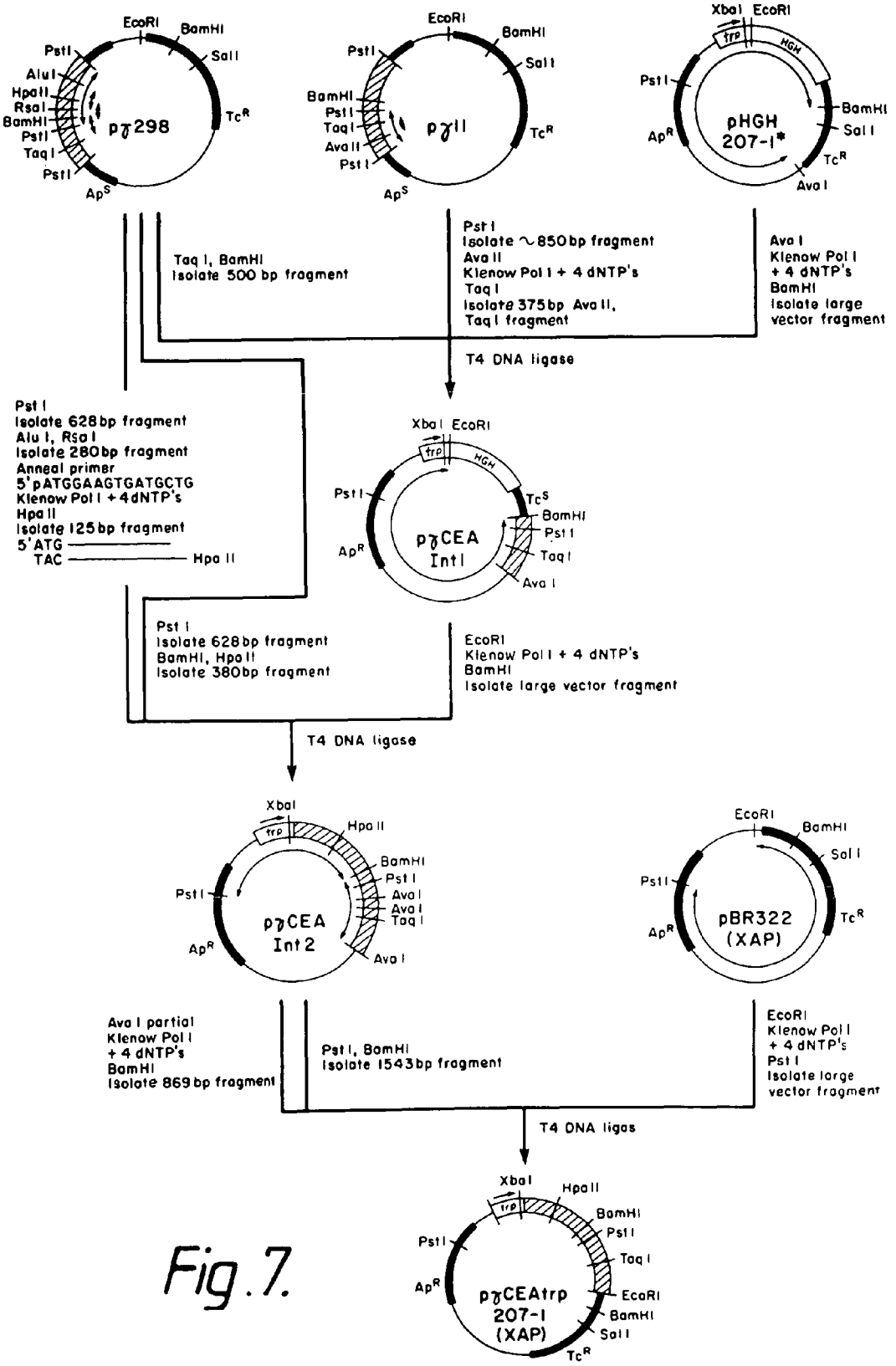
BamHI
PstI
HpaII
Sau3A
AvaII
TcR
AvaI
pκ17G4
AvaII
PstI
ApS
XbaI
EcoRI
trp
hGH
PstI
pHGH
207-1*
BamHI
SalI
ApR
TcR
AvaI
PstI, AvaII
Isolate 333bp fragment
Anneal primer
5'pATGGACATTGTGATG
Klenow PolI + 4dNTP's
Sau3A
Isolate 180bp fragment
5'ATG
TAC
Sau3A
EcoRI
Klenow PolI + 4dNTP's
BamHI
BAP
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
trp
Sau 3A
SalI
PstI
pκCEAint1
TcR
ApR
AvaI
PstI
Isolate kappa fragment
AvaII partial
Klenow PolI + 4dNTP's
HpaII
Isolate 490bp fragment
AvaI, XbaI
Isolate 1240bp fragment
HpaII
Isolate 169bp XbaI, HpaII fragment
AvaI
Klenow PolI + 4dNTP's
XbaI
BAP
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
trp
AvaII
PstI
pκCEAint2
ApR
AvaI
EcoRI
BamHI
SalI
PstI
pBR322
(XAP)
TcR
ApR
AvaI
Klenow PolI
+4dNTP's
PstI
Isolate κCEA fragment
EcoRI
Klenow PolI
+4dNTP's
PstI
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
trp
AvaII
PstI
pκCEA
trp207-1*
(XAP)
EcoRI
ApR
BamHI
SalI
TcR

Fig. 6.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BamHI
Sal I
PstI
Alu I
Hpa II
Rsa I
BamHI
Pst I
Taq I
Pst I
pγ298
Tc^R
Ap^S
Taq I, BamHI
Isolate 500 bp fragment
pγII
Ava II
Pst I
Pst I
Isolate ~850bp fragment
Ava II
Klenow Pol I + 4 dNTP's
Taq I
Isolate 375bp Ava II,
Taq I fragment
Xba I
trp
HGH
pHGH
207-1*
Ap^R
Tc^R
Ava I
Ava I
Klenow Pol I
+ 4 dNTP's
BamHI
Isolate large
vector fragment
T4 DNA ligase
Pst I
Isolate 628bp fragment
Alu I, Rsa I
Isolate 280bp fragment
Anneal primer
5'pATGGAAGTGATGCTG
Klenow Pol I + 4dNTP's
Hpa II
Isolate 125bp fragment
5'ATG
TAC
Hpa II
pγCEA
Int I
Tc^S
Ap^R
Pst I
Taq I
Ava I
Pst I
Isolate 628bp fragment
BamHI, Hpa II
Isolate 380bp fragment
EcoRI
Klenow Pol I + 4 dNTP's
BamHI
Isolate large vector fragment
T4 DNA ligase
Xba I
trp
Hpa II
BamHI
Pst I
Ava I
Ava I
Taq I
Ava I
pγCEA
Int 2
Ap^R
Pst I
pBR322
(XAP)
Ap^R
Tc^R
Ava I partial
Klenow Pol I
+ 4 dNTP's
BamHI
Isolate 869 bp fragment
Pst I, BamHI
Isolate 1543bp fragment
EcoRI
Klenow Pol I
+ 4 dNTP's
Pst I
Isolate large
vector fragment
T4 DNA ligas
Xba I
trp
Hpa II
BamHI
Pst I
Taq I
EcoRI
BamHI
Sal I
pγCEAtrp
207-1
(XAP)
Ap^R
Tc^R

Fig. 7.

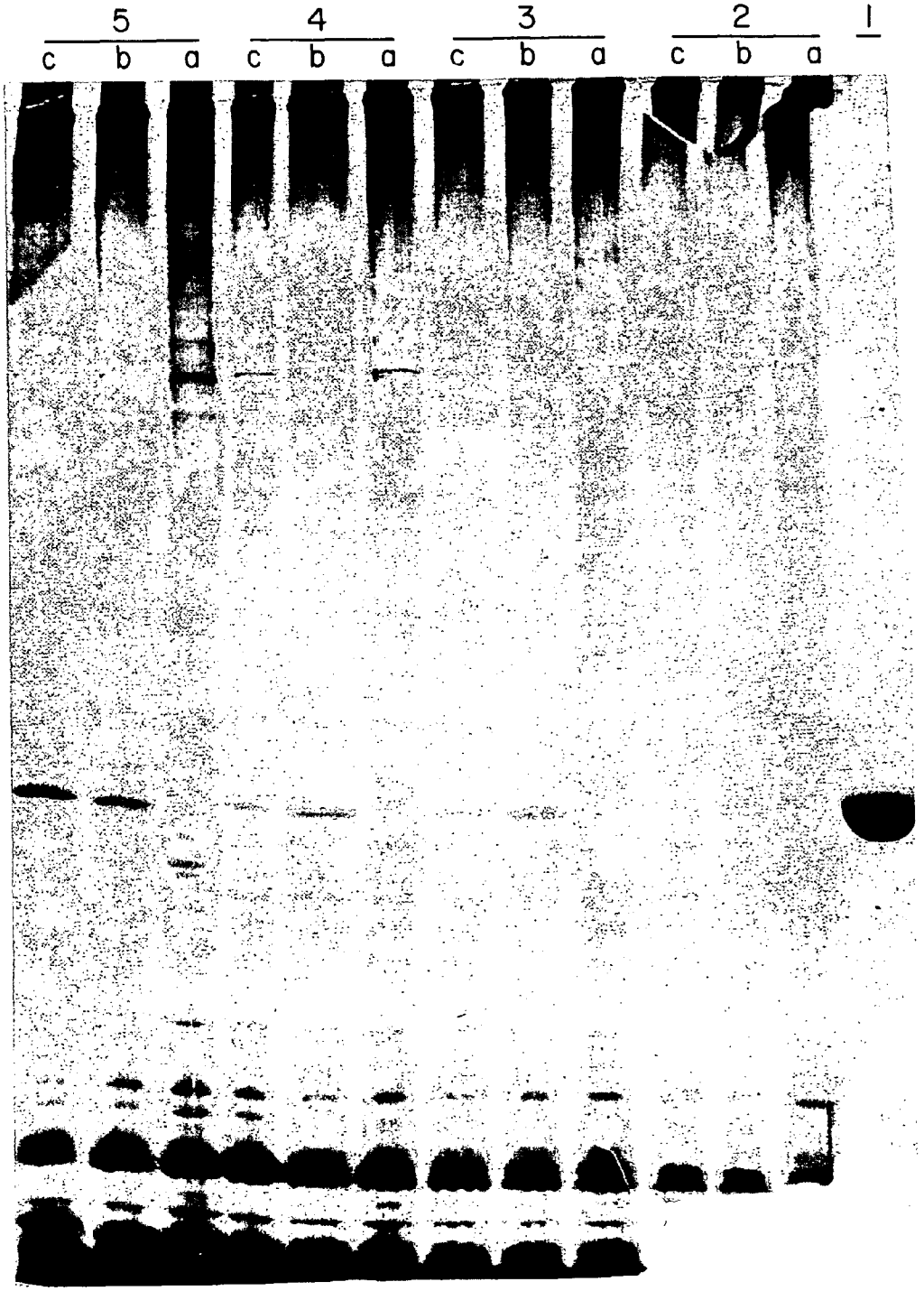


Fig. 8A.

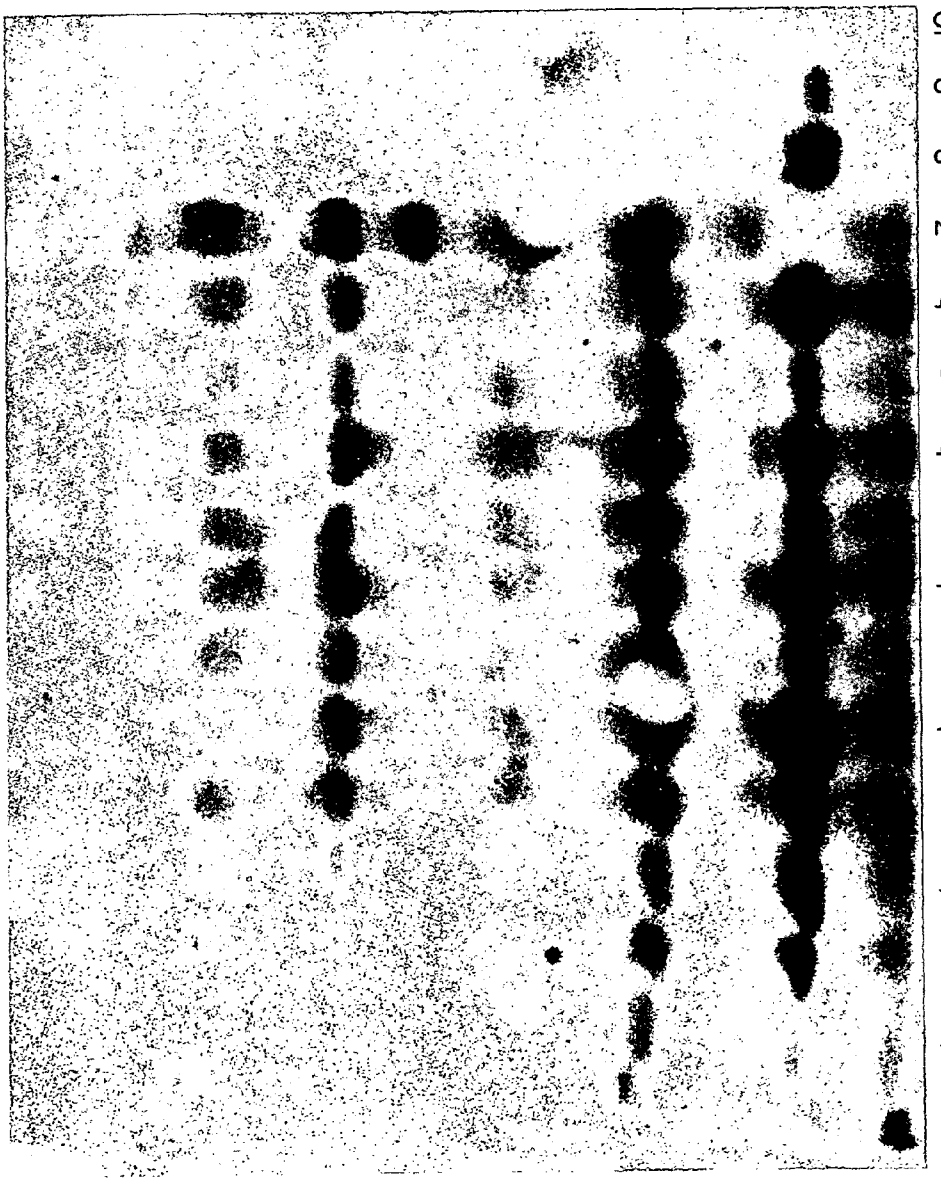


Fig.8B.

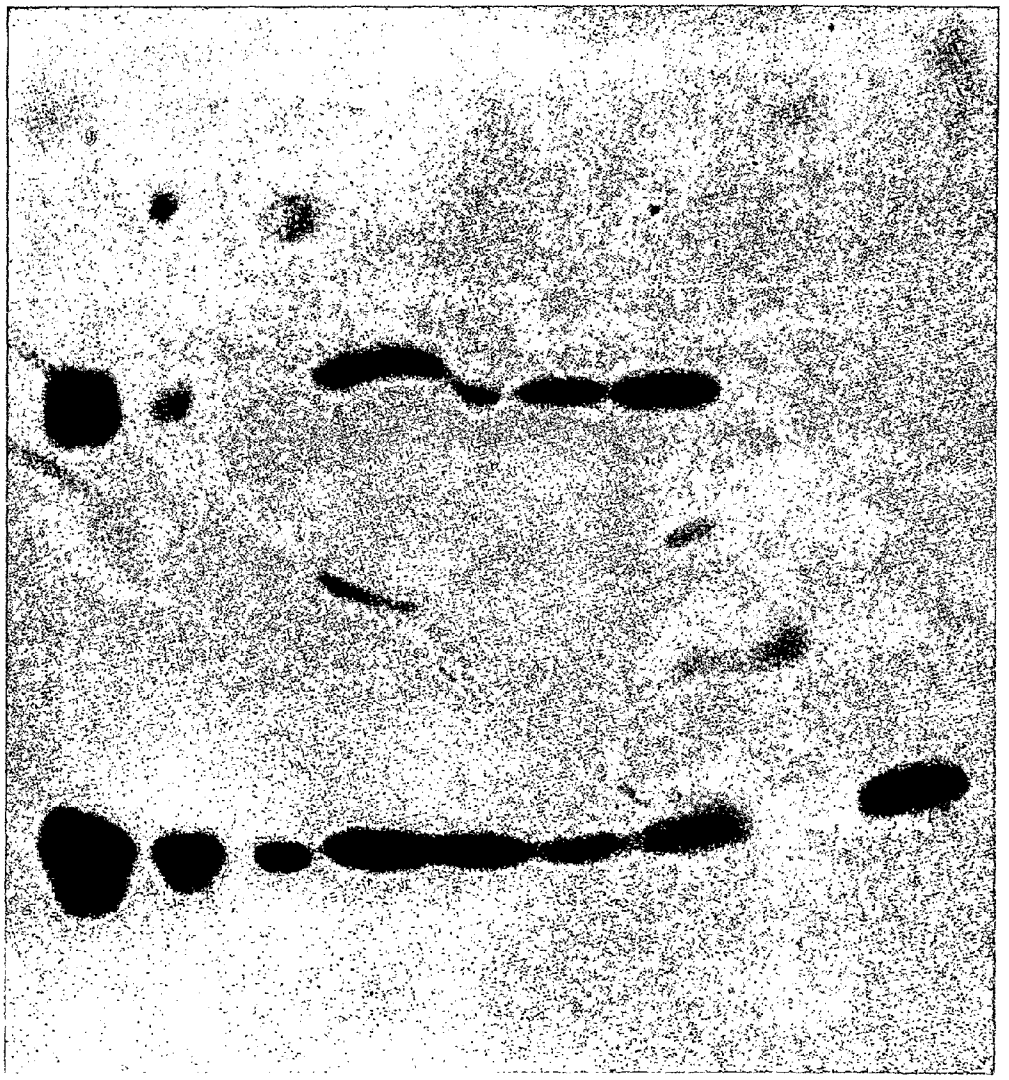
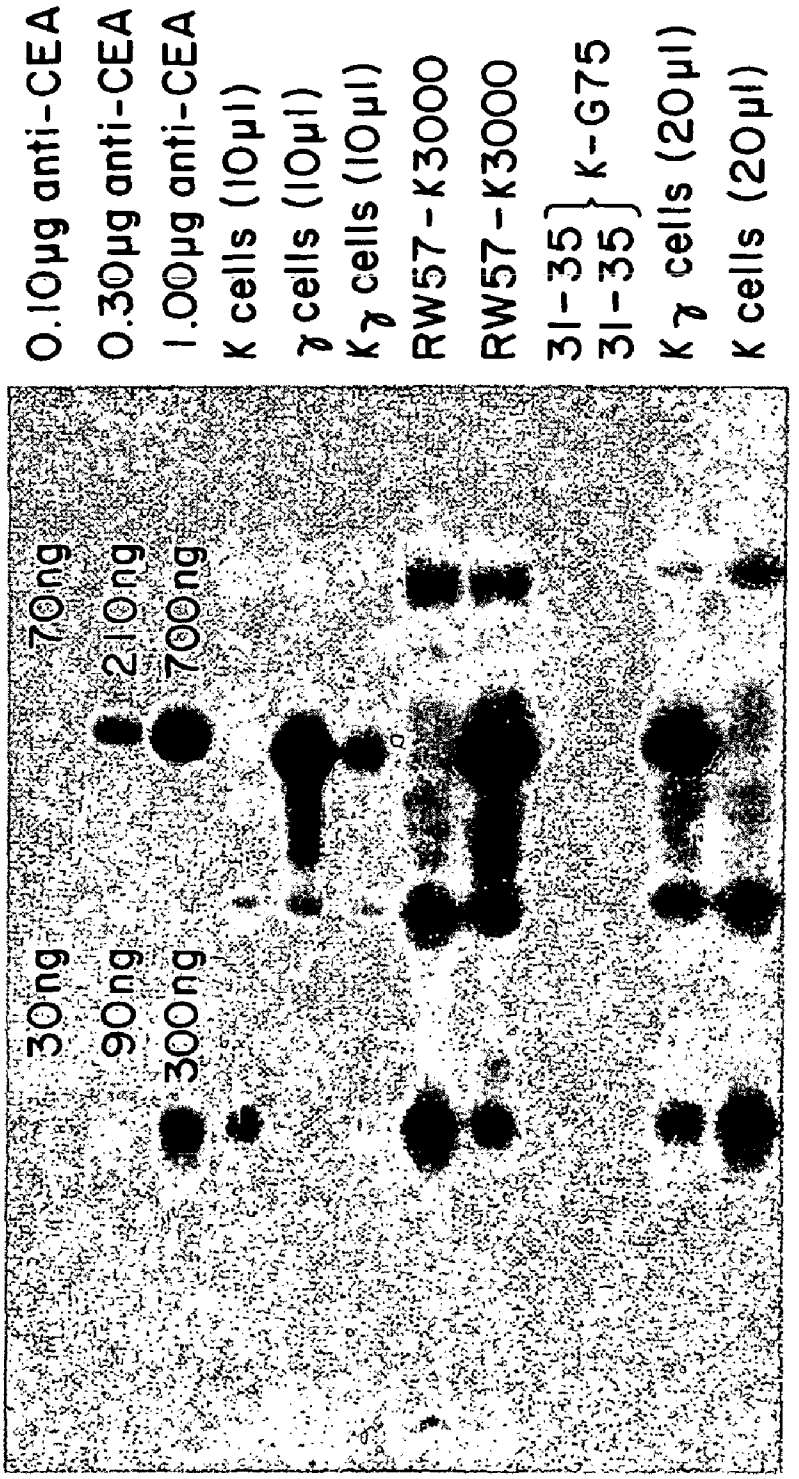


Fig. 8C.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0.30µg anti-CEA
1.00µg anti-CEA
K cells (10µl)
γ cells (10µl)
Kγ cells (10µl)
RW57-K3000
RW57-K3000
31-35
31-35
K-G75
Kγ cells (20µl)
K cells (20µl)
70ng
210ng
700ng
30ng
90ng
300ng

Fig. 9.

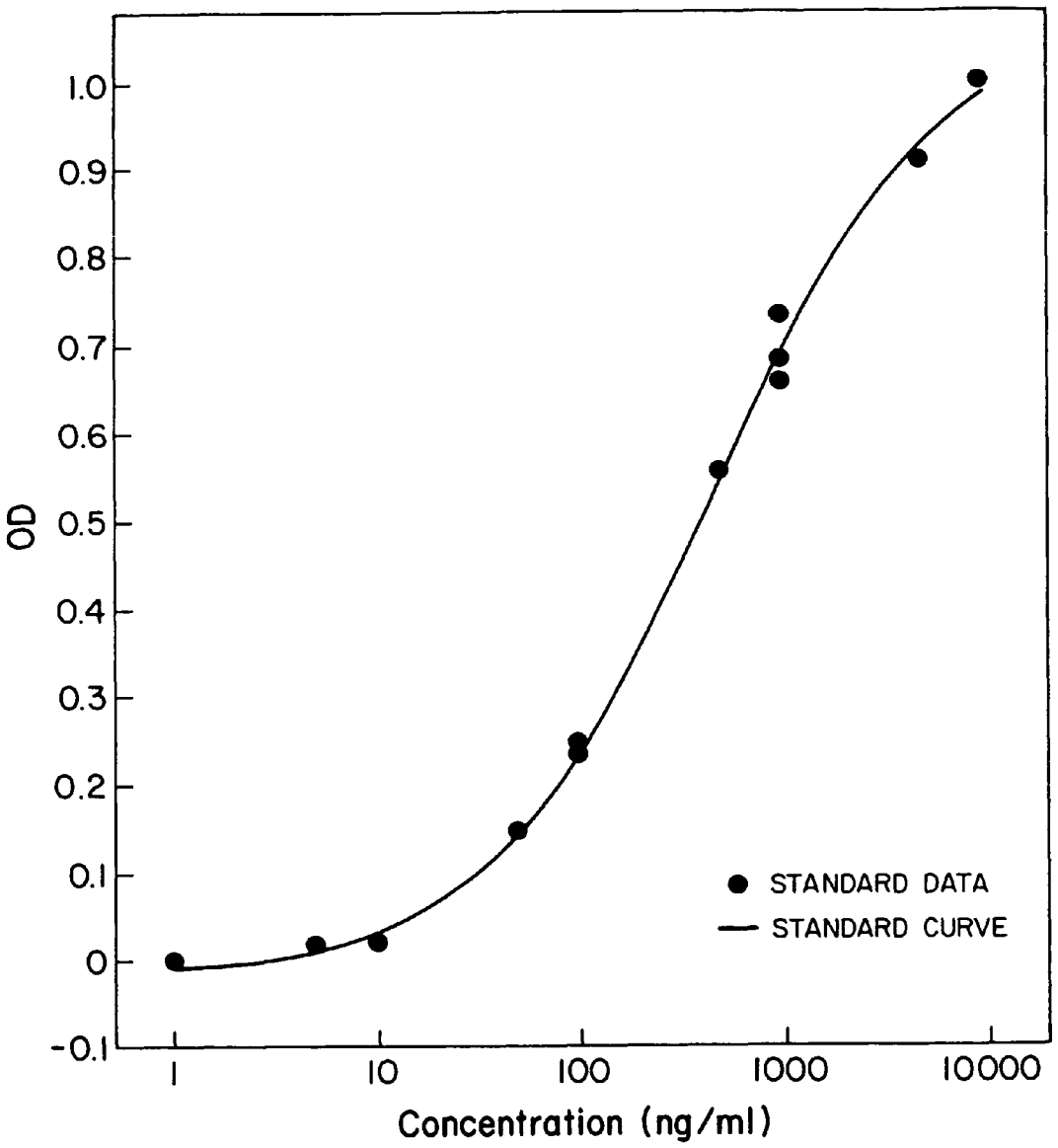
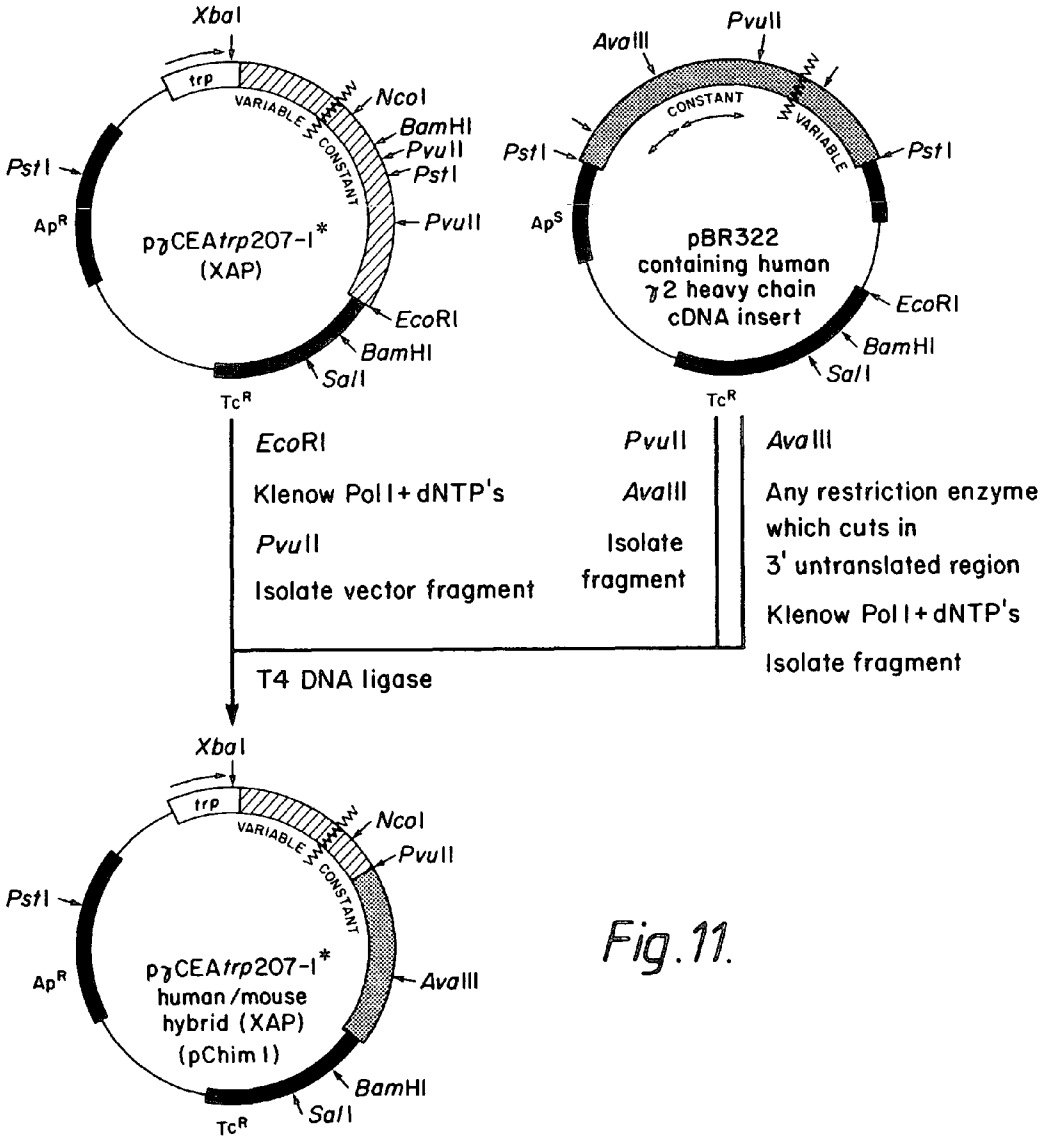

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
OD
1
10
100
1000
10000
Concentration (ng/ml)
STANDARD DATA
STANDARD CURVE

Fig. 10.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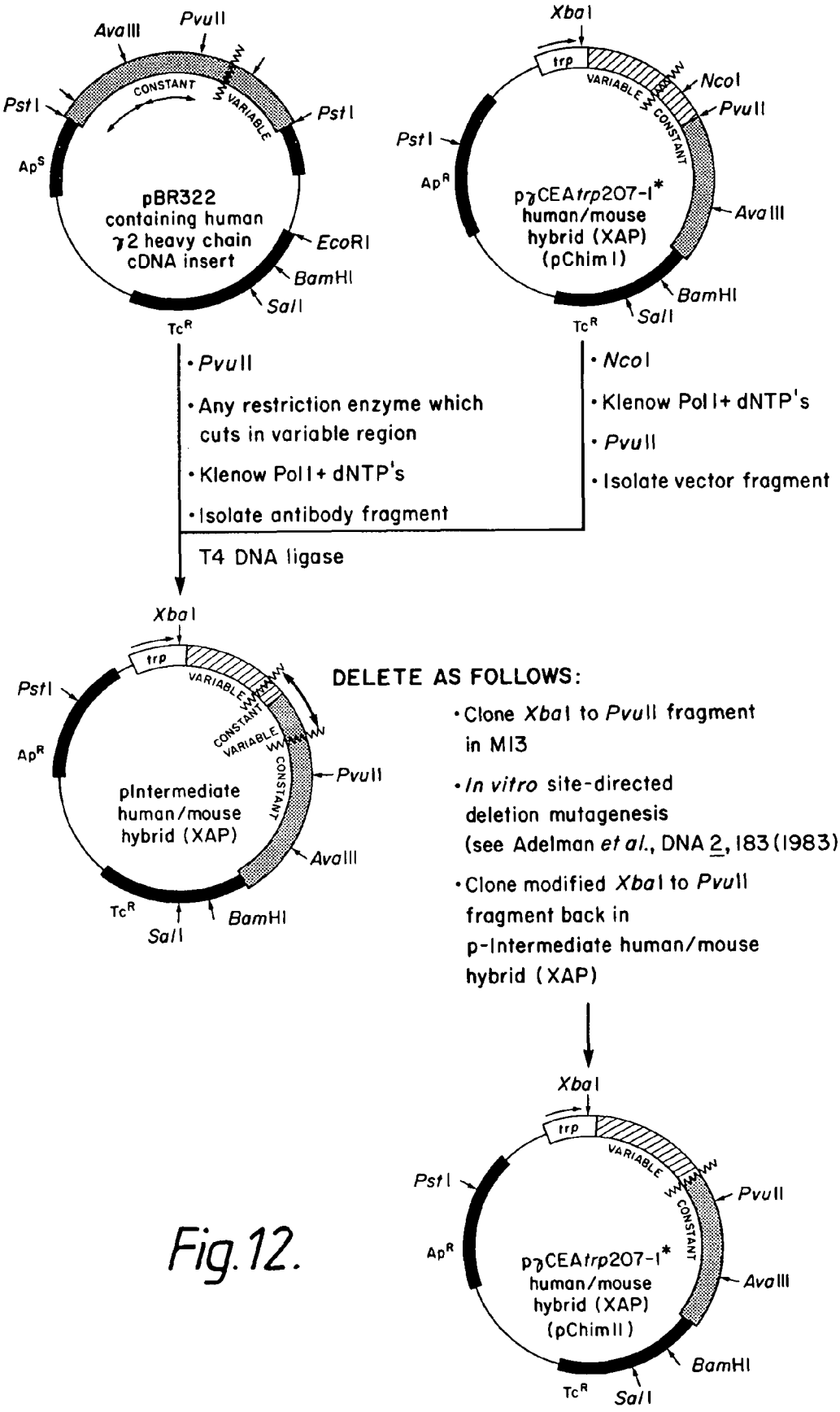
trp
VARIABLE
CONSTANT
NcoI
BamHI
PvuII
PstI
PvuII
EcoRI
BamHI
SalI
TcR
PstI
ApR
pγCEAtrp207-1*
(XAP)
AvaIII
PvuII
CONSTANT
VARIABLE
PstI
PstI
ApS
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
TcR
EcoRI
Klenow PolI+dNTP's
PvuII
Isolate vector fragment
PvuII
AvaIII
Isolate
fragment
AvaIII
Any restriction enzyme
which cuts in
3' untranslated region
Klenow PolI+dNTP's
Isolate fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
NcoI
PvuII
PstI
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim1)
AvaIII
BamHI
SalI
TcR

Fig. 11.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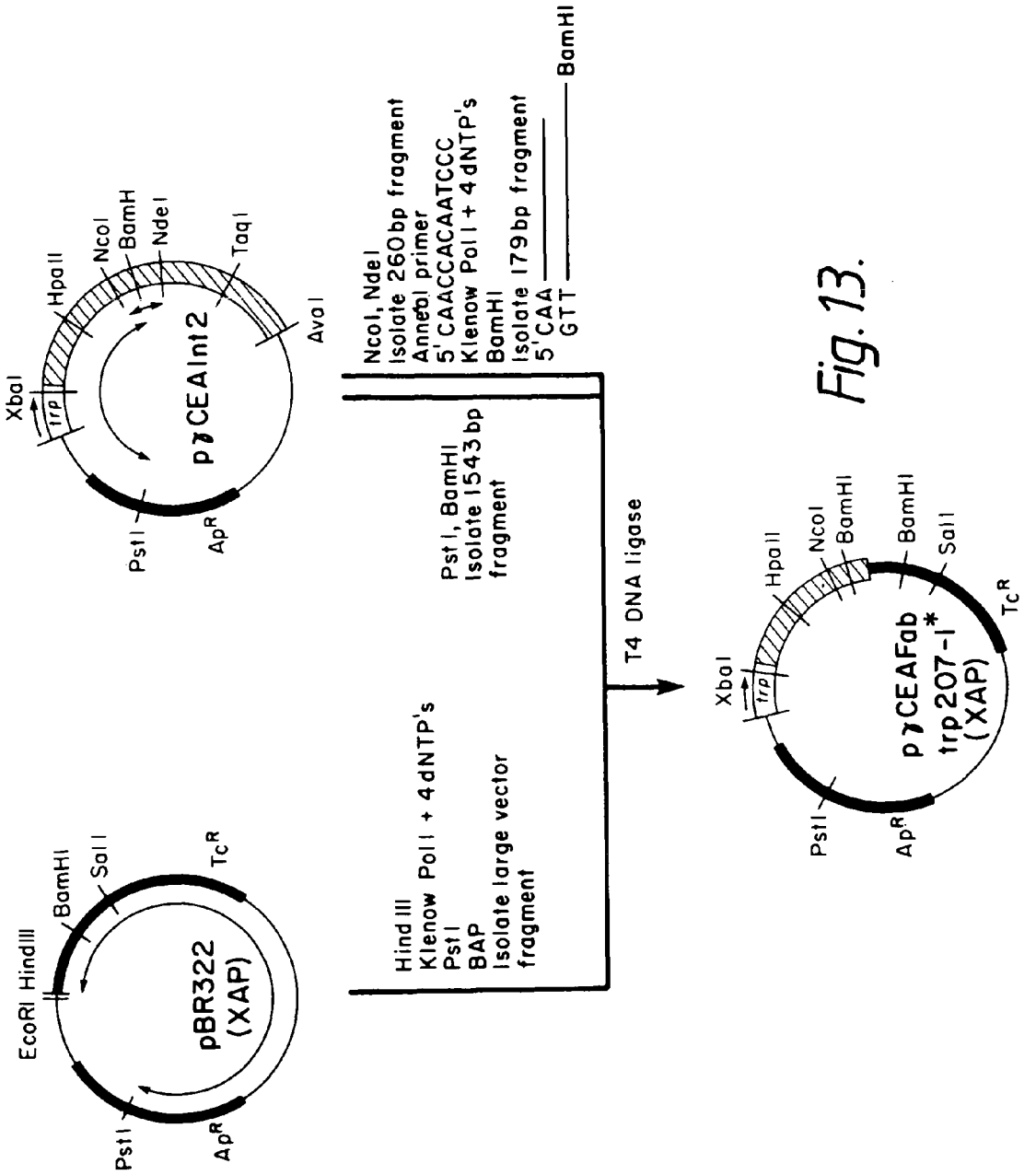
PvuII
PstI
CONSTANT
VARIABLE
PstI
Ap^S
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
Tc^R
XbaI
trp
VARIABLE
NcoI
PvuII
PstI
Ap^R
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim1)
AvaIII
BamHI
SalI
Tc^R
• PvuII
• Any restriction enzyme which cuts in variable region
• Klenow Pol I + dNTP's
• Isolate antibody fragment
• NcoI
• Klenow Pol I + dNTP's
• PvuII
• Isolate vector fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
VARIABLE
CONSTANT
PstI
Ap^R
pIntermediate
human/mouse
hybrid (XAP)
PvuII
AvaIII
Tc^R
SalI
BamHI
DELETE AS FOLLOWS:
• Clone XbaI to PvuII fragment in M13
• In vitro site-directed deletion mutagenesis (see Adelman et al., DNA 2, 183 (1983))
• Clone modified XbaI to PvuII fragment back in p-Intermediate human/mouse hybrid (XAP)
XbaI
trp
VARIABLE
CONSTANT
PstI
Ap^R
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim11)
PvuII
AvaIII
BamHI
Tc^R
SalI

Fig. 12.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EcoRI HindIII
BamHI
SalI
TcR
PstI
ApR
Hind III
Klenow Pol I + 4 dNTP's
PstI
BAP
Isolate large vector fragment
pγCEAIn12
XbaI
trp
HpaII
NcoI
BamH
NdeI
TaqI
AvaI
PstI
ApR
PstI, BamHI
Isolate 1543 bp
fragment
NcoI, NdeI
Isolate 260bp fragment
Anneal primer
5' CAACCACAATCCC
Klenow Pol I + 4 dNTP's
BamHI
Isolate 179bp fragment
5' CAA
GTT
BamHI
T4 DNA ligase
pγCEAFab trp207-1* (XAP)
XbaI
trp
HpaII
NcoI
BamHI
BamHI
SalI
TcR
PstI
ApR

Fig. 13.

METHODS OF MAKING ANTIBODY HEAVY AND LIGHT CHAINS HAVING SPECIFICITY FOR A DESIRED ANTIGEN

This is a continuation of application(s) Ser. No. 07/205,419 filed on 10 Jun. 1988, now U.S. Pat. No. 6,331,415, issued on 18 Dec. 2001, which is a continuation of Ser. No. 06/483,457 filed on 8 Apr. 1983, now U.S. Pat. No. 4,816,567, issued on 28 Mar. 1989, which applications are incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.

BACKGROUND OF THE INVENTION

This invention relates to the field of immunoglobulin production and to modification of naturally occurring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce both immunoglobulins which are analogous to those normally found in vertebrate systems and to take advantage of these gene modification techniques to construct chimeric or other modified forms.

A. Immunoglobulins and Antibodies

Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicited it.

Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

A.1 Source and Utility

Two major sources of vertebrate antibodies are presently utilized—generation in situ by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made in situ as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci,* 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The in situ immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B cells—hence in situ generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur. J. Immunol.,* 6: 511 (1976)). In this process, splenocytes or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed are segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci. (USA),* 77: 5429 (1980)); human-murine hybridomas (Schlom, J., et al. (ibid) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized in vitro by transformation with viral DNA.

Polyclonal, or, much more preferably, monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. These cells contain additional materials, notably nucleic acid fragments, but protein fragments as well, which are capable of enhancing, causing, or mediating carcinogic responses. Second, hybridoma lines producing monoclonal antibodies tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al., *Proc. Natl. Acad. Sci (USA)* 77: 2197 (1980); Morrison, S. L., *J. Immunol.* 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., *Biochemistry,* 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not

suffer from the foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

Even those immunoglobulins which lack the specificity of antibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in protein replacement therapy for globulin related anemia. In this context, an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of cancelling out specificity by manipulating the four chains of the tetramer separately.

A.2 General Structure Characteristics

The basic immunoglobin structural unit in vertebrate systems is now well understood (Edelman, G. M., *Ann. N.Y. Acad. Sci.,* 190: 5 (1971)). The units are composed of two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the Y and continuing through the divergent region as shown in FIG. **1**. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class" of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occurring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

The remaining portions of the chain are referred to as constant regions and within a particular class do not to vary with the specificity of the antibody (i.e., the antigen eliciting it).

As stated above, there are five known Major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding to γ, μ, α, δ, and ϵ heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E. A., *Structural Concepts in Immunology and Immunochemistry,* 2nd Ed., p. 413-436, Holt, Rinehart, Winston (1976)), and other cellular responses (Andrews, D. W., et al., *Clinical Immunobiology* pp 1-18, W. B. Sanders (1980); Kohl, S., et al., *Immunology,* 48: 187 (1983)); while the variable region determines the antigen with which it will react.

B. Recombinant DNA Technology

Recombinant DNA technology has reached sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformant. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment.

The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems.

SUMMARY OF THE INVENTION

The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. These antibodies and NSIs can be readily prepared in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras of variants which draw their homology from species which differ from each other. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell. Thus, there are a number of "types" of immunoglobulins encompassed by the invention.

First, immunoglobulins, particularly antibodies, are produced using recombinant techniques which mimic the amino acid sequence of naturally occurring antibodies produced by either mammalian B cells in situ, or by B cells fused with suitable immortalizing tumor lines, i.e., hybridomas. Second, the methods of this invention produce, and the invention is

directed to, immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. Such reassembly is particularly useful in producing "hybrid" antibodies capable of binding more than one antigen; and in producing "composite" immunoglobuins wherein heavy and light chains of different origins essentially damp out specificity. Third, by genetic manipulation, "chimeric" antibodies can be formed wherein, for example, the variable regions correspond to the amino acid sequence from one mammalian model system, whereas the constant region mimics the amino acid sequence of another. Again, the derivation of these two mimicked sequences may be from different species. Fourth, also by genetic manipulation, "altered" antibodies with improved specificity and other characteristics can be formed.

Two other types of immunoglobulin-like moieties may be produced: "univalent" antibodies, which are useful as homing carriers to target tissues, and "Fab proteins" which include only the "Fab" region of an immunoglobulin molecule i.e, the branches of the "Y". These univalent antibodies and Fab fragments may also be "mammalian" i.e., mimic mammalian amino acid sequences; novel assemblies of mammalian chains, or chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions thereof, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

In other aspects, the invention is directed to DNA which encodes the aforementioned NSIs, antibodies, and portions thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, the invention is directed to methods of producing these NSIs and antibodies, and the DNA sequences, plasmids, and transformed cells intermediate to them.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a representation of the general structure of immunoglobulins.

FIGS. **2**A-B show the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

FIG. **3** shows the coding sequence of the fragment shown in FIG. **2**, along with the corresponding amino acid sequence.

FIGS. **4**A-C show the combined detailed sequence of the cDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

FIGS. **5**A-B show the corresponding amino acid sequence encoded by the fragment in FIG. **4**.

FIGS. **6** and **7** outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively.

FIGS. **8**A, **8**B, and **8**C show the results of sizing gels run on extracts of *E. coli* expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

FIG. **9** shows the results of western blots of extracts of cells transformed as those in FIG. **8**.

FIG. **10** shows a standard curve for ELISA assay of anti CEA activity.

FIGS. **11** and **12** show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain.

FIG. **13** shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

DETAILED DESCRIPTION

A. Definitions

As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of FIG. **1**, with or without covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity—i.e., those which are not antibodies.

"Mammalian antibodies" refers to antibodies wherein the amino acid sequences of the chains are homologous with those sequences found in antibodies produced by mammalian systems, either in situ, or in hybridomas. These antibodies mimic antibodies which are otherwise capable of being generated, although in impure form, in these traditional systems.

"Hybrid antibodies" refers to antibodies wherein chains are separately homologous with referenced mammalian antibody chains and represent novel assemblies of them, so that two different antigens are precipitable by the tetramer. In hybrid antibodies, one pair of heavy and light chain is homologous to antibodies raised against one antigen, while the other pair of heavy and light chain is homologous to those raised against another antigen. This results in the property of "divalence" i.e., ability to bind two antigens simultaneously. Such hybrids may, of course, also be formed using chimeric chains, as set forth below.

"Composite" immunoglobulins means those wherein the heavy and light chains mimic those of different species origins or specificities, and the resultant is thus likely to be a non-specific immunoglobulin (NSI), i.e. —lacking in antibody character.

"Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals, while the constant portions are homologous to the sequences in antibodies derived from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non-human source.

However, the definition is not limited to this particular example. It includes any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, differing antigen responses, or differing species of origin and whether or not the fusion point is at the variable/constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation or to make other improvements in properties possessed by a particular constant region.

"Altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a mammalian or

other vertebrate antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the constant region. Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics. The antibody can also be engineered so as to aid the specific delivery of a toxic agent according to the "magic bullet" concept. Alterations, can be made by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques (Dalbadie-McFarland, et al *Proc. Natl. Acad. Sci.* (*USA*), 79:6409 (1982)).

"Univalent antibodies" refers to aggregations which comprise a heavy chain/light chain dimer bound to the Fc (or stem) region of a second heavy chain. Such antibodies are specific for antigen, but have the additional desirable property of targeting tissues with specific antigenic surfaces, without causing its antigenic effectiveness to be impaired—i.e., there is no antigenic modulation. This phenomenon and the property of univalent antibodies in this regard is set forth in Glennie, M. J., et al., *Nature,* 295: 712 (1982). Univalent antibodies have heretofore been formed by proteolysis.

"Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as $F(ab)_2$), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, as have univalent ones, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack the "effector" Fc portion they cannot effect, for example, lysis of the target cell by macrophages.

"Fab protein" has similar subsets according to the definition of the present invention as does the general term "antibodies" or "immunoglobulins". Thus, "mammalian" Fab protein, "hybrid" Fab protein "chimeric" Fab and "altered" Fab protein are defined analogously to the corresponding definitions set forth in the previous paragraphs for the various types of antibodies.

Individual heavy or light chains may of course be "mammalian", "chimeric" or "altered" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence—i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

B. Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537).
These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F^-, λ^-, prototrophic, ATTC No. 27325), bacilli such as *Bacillus subtilus*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al, *Nature,* 275: 615 (1978);

Itakura, et al, *Science,* 198: 1056 (1977); (Goeddel, et al *Nature* 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic Acids Res.,* 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, *Cell* 20: 269 (1980)).

In addition to prokaryates, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, (Stinchcomb, et al, *Nature,* 282: 39 (1979); Kingsman et al, *Gene,* 7: 141 (1979); Tschemper, et al, *Gene,* 10: 157 (1980)) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics,* 85: 12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.,* 255: 2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.,* 7: 149 (1968); Holland, et al, *Biochemistry,* 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (Holland, ibid.). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propogation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers, et al, *Nature,* 273: 113 (1978)) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those host cells, vectors and expression systems exemplified.

C. Methods Employed

C.1 Transformation

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology,* 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci.* (*USA*), 69: 2110 (1972).

C.2 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

Cleavage is performed by treating with restriction enzyme (or enyzmes) in suitable buffer. In general, about 1 μg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 μl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of *E. coli* DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, *Nucleic Acids Res.,* 8: 4057 (1980) incorporated herein by reference.

For ligation, approximately equimolar amounts of the desired components, suitably end tailored to provide correct

matching are treated with about 10 units T4 DNA ligase per 0.5 μg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

In the examples described below correct ligations for plasmid construction are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.,* 9:309 (1981) or by the method of Maxam, et al, *Methods in Enzymology,* 65:499 (1980).

D. Outline of Procedures

D.1 Mammalian Antibodies

The first type of antibody which forms a part of this invention, and is prepared by the methods thereof, is "mammalian antibody"-one wherein the heavy and light chains mimic the amino acid sequences of an antibody otherwise produced by a mature mammalian B lymphocyte either in situ or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA. The poly-A mRNA may, further, be fractionated to obtain sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli*, is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP^{32}. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. Pat. Nos. 307,473; 291,892; and 305,657, have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

In the present invention, the gene coding for the light chain and that coding for the heavy chain are recovered separately by the procedures outlined above. Thus they may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such conditions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circumstances, in yeast and bacterial strains, can be secreted into the medium (yeast and gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures. Tissue culture cells as hosts also appear, in general, to permit reasonably facile recovery of heterologous proteins.

When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished in vitro as described below, or might be possible in vivo in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

D.2 Chain Recombination Techniques

The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins, Fab regions, and univalent antibodies. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield native structure and activity (Freedman, R. B., et al. In *Enzymology*

of Post Translational Modification of Proteins, I: 157-212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by disulfide bonds are more difficult to reconstruct in vitro after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R. E., et al., In *Peptides: Proceedings of the Seventh Annual American Peptide Symposium* (Rich, D. H. and Gross, E., eds.) 721-728, Pierce Chemical Co., Rockford, Ill. (1981)).

Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by, 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the inter-chain disulfides (Edelman, G. M., et al., *Proc. Natl. Acad. Sci. (USA)* 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of ~50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., *Proc. Natl. Acad. Sci. (USA)* 52: 1099 (1964); Whitney, P. L., et al., *Proc. Natl. Acad. Sci. (USA)* 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding pathway (see discussion in Freedman, M. H., et al., *J. Biol. Chem.* 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (ibid).

A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolysis, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (supra)). Oxidative sulfitolysis is a mild disulfide cleavage reaction (Means, G. E., et al., *Chemical Modification of Proteins*, Holden-Day, San Francisco (1971)) which is sometimes more gentle than reduction (Wetzel, R., *Biochemistry*, submitted (1983)), and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur via thiol-disulfide interchange (Morehead, H., et al. *Biochemistry*, in press, (1983)). In the present invention the heavy and light chain S-sulfonates generated by oxidative sulfitolysis were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Pat. No. 452, 187, filed Dec. 22, 1982, incorporated herein by reference.

D.3 Variants Permitted by Recombinant Technology

Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of mammalian antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered mammalian immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, human-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

The present invention permits a more controlled assembly of desired chains, either by mixing the desired chains in vitro, or by transforming the same culture with the coding sequences for the desired chains.

D.4 Composite Immunoglobulins

The foregoing procedure, which describes in detail the recombinant production of mammalian antibodies is employed with some modifications to construct the remaining types of antibodies or NSIs encompassed by the present invention. To prepare the particular embodiment of composite non-specific immunoglobulin wherein the homology of the chains corresponds to the sequences of immunoglobulins of different specificities, it is of course, only necessary to prepare the heavy and light chains in separate cultures and reassemble them as desired.

For example, in order to make an anti-CEA light chain/anti-hepatitis heavy chain composite antibody, a suitable source for the mRNA used as a template for the light chain clone would comprise, for instance, the anti CEA producing cell line of paragraph E.1. The mRNA corresponding to heavy chain would be derived from B cells raised in response to hepatitis infection or from hybridoma in which the B cell was of this origin. It is clear that such composites can be assembled using the methods of the invention almost at will, and are limited only by available sources of mRNA suitable

for use as templates for the respective chains. All other features of the process are similar to those described above.

D.5 Hybrid Antibodies

Hybrid antibodies are particularly useful as they are capable of simultaneous reaction with more than one antigen. Pairs of heavy and light chains corresponding to chains of antibodies for different antigens, such as those set forth in paragraph D.4 are prepared in four separate cultures, thus preventing premature assembly of the tetramer. Subsequent mixing of the four separately prepared peptides then permits assembly into the desired tetramers. While random aggregation may lead to the formation of considerable undesired product, that portion of the product in which homologous light and heavy chains are bound to each other and mismatched to another pair gives the desired hybrid antibody.

D.6 Chimeric Antibodies

For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph D.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

For example, in a particularly preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and cloned from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.7 Altered Antibodies

Altered antibodies present, in essence, an extension of chimeric ones. Again, the techniques of D.1 and D.2 are applicable; however, rather than splicing portions of the chain(s), suitable amino acid alterations, deletions or additions are made using available techniques such as mutagenesis (supra). For example, genes which encode antibodies having diminished complement fixation properties, or which have enhanced metal binding capacities are prepared using such techniques. The latter type may, for example, take advantage of the known gene sequence encoding metalothionein II (Karin, M., et al., *Nature,* 299: 797 (1982)). The chelating properties of this molecular fragment are useful in carrying heavy metals to tumor sites as an aid in tumor imaging (Scheinberg, D. A., et al., *Science,* 215: 19 (1982).

D.8 Univalent Antibodies

In another preferred embodiment, antibodies are formed which comprise one heavy and light chain pair coupled with the Fc region of a third (heavy) chain. These antibodies have a particularly useful property. They can, like ordinary antibodies, be used to target antigenic surfaces of tissues, such as tumors, but, unlike ordinary antibodies, they do not cause the antigenic surfaces of the target tissue to retreat and become non-receptive. Ordinary antibody use results in aggregation and subsequent inactivation, for several hours, of such surface antigens.

The method of construction of univalent antibodies is a straightforward application of the invention. The gene for heavy chain of the desired Fc region is cleaved by restriction enzymes, and only that portion coding for the desired Fc region expressed. This portion is then bound using the technique of D.2 to separately produced heavy chain the desired pairs separated from heavy/heavy and Fc/Fc combinations, and separately produced light chain added. Pre-binding of the two heavy chain portions thus diminishes the probability of formation of ordinary antibody.

D.9 Fab Protein

Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that that portion of the heavy chain coding for the amino terminal 220 amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

The invention has been described above in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti CEA antibody components, i.e. for a "mammalian antibody". Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of mammalian, composite, hybrid and chimeric immunoglobulins, and Fab proteins and univalent antibodies. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

The examples set forth below are included for illustrative purposes and do not limit the scope of the invention.

E.1 Construction of Expression Vectors for Murine Anti-CEA Antibody Chains and Peptide Synthesis

Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., *J. Exp. Med.,* 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T. R., et al., *Cancer Res.* 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the $Ig\gamma_1$ class, CEA.66-E3, has been prepared as described by Wagener, C, et al., *J. Immunol.* (in press) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAse (Podell, D. N., et al., BBRC 81: 176 (1978)), and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C. water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a linear gradi-

ent from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H_2O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SOS gels by silver staining. The first two peaks were pure light chain (MW 25,000 daltons), the third peak showed a (7:3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J. E., *Methods in Enzymology,* 79: 31 (1981), with an NH_2-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

Total RNA from CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology,* 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM $MgCl_2$. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform:isoamyl alcohol 25:1 at 4° C. The aqueous phase was made 0.2 M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA was purified from total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l. Acad. Sci.* (*USA*), 69: 1408 (1972). 142 μg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of *E. coli* Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

5 μg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., *Nature* 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No. 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

The 14mer, 5' GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mouse MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5' GACCAGGCATCCCAG 3', complementary to a coding sequence located 72 basepairs 3' of the variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

Both probes were synthesized by the phosphotriester method described in German Offenlegungschrift 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 μl of 60 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 15 mM beta-mercaptoethanol, and 100 μCi (γ-^{32}P) ATP (Amersham, 5000 Ci/mMole). 5 units of T4 polynucleotide kinase were added and the reaction was allowed to proceed at 37° C. for 30 minutes and terminated by addition of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431-3, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. (1972))+5 μg/ml tetracycline and stored at −20° C. after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 μg/ml tetracycline. After ~10 hours growth at 37° C. the colony filters were transferred to agar plates containing LB+5 μg/ml tetracycline and 12.5 μg/ml chloramphenicol and reincubated overnight at 37° C. The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci.* (*USA*) 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5 N NaOH, 1.5 M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3 M NaCl, 0.5 M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2×SSC, and subsequently baked for 2 hours in an 80° C. vacuum oven. The filters were prehybridized for ~2 hours at room temperature in 0.9 M NaCl, 1×Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1 M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 μg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using ~40× 10^6 cpm of either the kinased kappa or gamma probe described above.

After extensive washing at 37° C. in 6×SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 16-24 hours at −80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith, *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). FIG. **2** shows the nucleotide sequence of the cDNA insert of pK17G4 and FIG. **3** shows the gene sequence with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain,

deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acid 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides 374-388 (FIG. **2**).

E.1.6 Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region. Selected plasmid DNAs were digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and NcoI. The C-terminal region of the cDNA insert of pγ11 was sequenced and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

FIG. **4** presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.*, 65: 560 (1980)) and FIG. **5** includes the translated sequence.

The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosolated protein (MW 52,258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 and pγ11 hybridized to nucleotides 528-542 (FIG. **4**).

E.1.7 Construction of a Plasmid for Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCE-Atrp207-1☆

FIG. **6** illustrates the construction of pKCEAtrp207-1☆

First, an intermediate plasmid pHGH207-1☆, having a single trp promoter, was prepared as follows:

The plasmid pHGH 207 (described in U.S. Pat. No. 307, 473, filed Oct. 1, 1981) has a double lac promoter followed by the trp promoter, flanked by EcoR I sites and was used to prepare pHGH207-1. pHGH207 was digested with BamH 1, followed by partial digestion with EcoR I. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoR I-BamH I fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet^R Amp^R colonies were isolated, and most of them contained pHGH207-1. pHGH207-1☆ which lacks the EcoR1 site between the amp^R gene and the trp promoter, was obtained by partial digestion of pHGH207-1 with EcoR I, filling in the ends with Klenow and dNTPs, and religation.

5 μg of pHGH207-1☆ was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 μl reaction containing 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C. for 1 hour, followed by extraction with phenol/$CHCl_3$ and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/$CHCl_3$ extraction and ethanol precipitation.

The DNA was resuspended in 50 μl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30' at 65° followed by phenol/$CHCl_3$ extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence was prepared as follows: 7 μg of pK17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolated by 6 percent gel electrophoresis, and electroelution. After phenol/$CHCl_3$ extraction, ethanol precipitation and resuspension in water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer was synthesized by the phosphotriester method G. O. 2,644,432 (supra) and has the following sequence:

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Met Asp Ile Val Met
5' ATG GAC ATT GTT ATG 3'
```

The 5' methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5' end with 10 units T4 DNA kinase in 20 μl reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20 μl of the phosphorylated primer, heated to 95° C. for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C. this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.

100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20 μl of 20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 2.5 mM ATP and 1 unit of 14 DNA ligase. After overnight ligation at 14° C. the reaction was transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequence analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1 (FIG. **6**).

The remainder of the coding sequence of the kappa light chain gene was prepared as follows:

The Pst I cDNA insert fragment from 7 μg of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686 basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isolated and purified after gel electrophoresis.

10 μg of pKCEAInt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment, and digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reaction and incubated overnight at 14°, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was designated pKCEAInt2.

Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared as described in U.S. Pat. No. 452,227, filed Dec. 22, 1982.)

The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis, and one plasmid DNA demonstrated the proper construction, and was designated pKCEAtrp207-I☆.

E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1☆

FIG. **7** illustrates the construction of pγCEAtrp207-1☆. This plasmid was constructed in two parts beginning with construction of the C-terminal region of the gamma 1 gene.

5 μg of plasmid pHGH207-1☆ was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol/$CHCl_3$, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution.

~5 μg of pγ11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified, digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment (fragment B) was isolated and purified by gel electrophoresis and electroelution.

9 μg of pγ298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).

Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20 μl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pγCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (FIG. **5**).

To obtain the N-terminal sequences, 30 μg of pγ298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer

```
      met glu val met leu
5' ATG GAA GTG ATG CTG 3'
```

was synthesized by the phosphotriester method (supra).

The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blunt-end to Hpa II DNA fragment (fragment D) was purified from the gel.

A second aliquot of pγ298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I and Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.

~5 μg of pγCEAIntI was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.

In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 μl reaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pγCEAInt2.

The expression plasmid, pγCEAtrp207-I☆ used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pγCEAInt2.

pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pγCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pγCEAInt2 with Pst

I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pγCEAInt2 with Ava I, blunt ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.

The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pγCEAtrp207-1☆.

E.1.9 Production of Immunoglobulin Chains by *E. coli*

E. coli strain W3110 (ATTC No. 27325) was transformed with pγCEAtrp207-1☆ or pKCEAtrp207-1☆ using standard techniques.

To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1☆, pKCEAtrp207-1☆Δ, which had been modified by cleaving a Pst I-Pvu I fragment from the amp^R gene and religating. Cells transformed with pKCEAtrp207-1☆Δ are thus sensitive to ampicillin but still resistant to tetracycline. Successful transformants were retransformed using pγCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1☆Δ and pγCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 μg/ml tetracycline, and induced with indoleacrylic acid (IAA) when the OD 550 reads 0.5. The induced cells were grown at 37° C. during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1 M β-mercaptoethanol and boiled for 5 minutes. A 10× volume of acetone was added and the cells kept at 22° C. for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell, P. H., *J. Biol. Chem.*, 250: 4007 (1975)); boiled 3 minutes, recentrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman, D. et al., *Science* 211: 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

Cells transformed with pγCEAtrp207-1☆ showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1☆ showed the proper molecular weight band for light chain as identified by Western blot; double transformed cells showed bands for both heavy and light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in FIGS. **8**A, **8**B, and **8**C.

FIG. **8**A shows results developed by silver stain from cells transformed with pγCEAtrp207-1☆. Lane 1 is monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b-5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a-5a are corresponding untransformed controls; Lanes 2c-5c are corresponding uninduced transformants.

FIG. **8**B shows results developed by Western blot from cells transformed with pKCEAtrp207-1☆. Lanes 1b-6b are extracts from induced cells immediately, 1 hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a-6a corresponding uninduced controls. Lane 7 is an extract from a pγCEAtrp207-1☆ control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

FIG. **8**C shows results developed by Western blot from four colonies of double transformed cells 24 hours after IAA addition (lanes 4-7). Lanes 1-3 are varying amounts of monoclonal gamma chain controls, lanes 8 and 9 are untransformed and pγCEAtrp207-1☆ transformed cell extracts, respectively.

In another quantitative assay, frozen, transformed *E. coli* cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS)/γ-mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using ^{125}I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in FIG. **9**. The figure shows that the *E. coli* products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in *E. coli*. Heavy chain from mammalian cells is expected to be slightly heavier than *E. coli* material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

	(Per gram of cells)
E. coli (W3110/pγCE Atrp207-1*)	5 mg γ
E. coli (W3110/pKCE Atrp207-1*)	1.5 mg K
E. coli (W3110/pKCE Atrp207-1*Δ, pγCEAInt2)	0.5 mg K, 1.0 mg γ

E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

In order to obtain heavy and light chain preparations for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

E. coli (W3110/pγCEAtrp207-1☆) were inoculated into 500 ml LB medium containing 5 μg/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transferred to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 μg/ml tetracycline. Additional glucose was added during growth and at OD 550=20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50 μg/ml. The cells were fed additional glucose to a final OD 550=40, achieved approximately 6 hours from the IAA addition.

E. coli (W3110) cells transformed with pKCEA trp 207-1☆ and double transformed (with pKCEAtrp207-1☆Δ and pγCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after IAA addition at harvest was 25-30.

The cells were then harvested by centrifugation, and frozen.

E.2 Assay Method for Reconstituted Antibody

Anti-CEA activity was determined by ELISA as a criterion for successful reconstitution. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubating 100 μl of 2-5 μg CEA/ml solution in 0.1M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 μl of 0.5 percent BSA in PBS for 2 hours at 37° C., followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in FIG. **10**), was run, which consisted of 50 μl samples of 10 μg, 5 μg, 1 μg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng anti-CEA/ml in 0.5 percent BSA in PBS, plus 50 μl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37° C.

The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 μl of an enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37° C. for 90 minutes. The plates were washed 4 times with PBS before adding the substrate, 100 μl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37° C. for color development.

The A_{450} of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A_{450} data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples' concentrations were calculated based on the A_{450} data.

E.3 Reconstitution of Recombinant Antibody and Assay

Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 3,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PMSF, and used immediately or stored frozen at −80° C.; frozen lysates were never thawed more than once.

The S-sulfonate of *E. coli* produced anti-CEA heavy chain (γ) was prepared as follows: Recombinant *E. coli* cells transformed with pγCEAtrp207-1☆ which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1 mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of γ-SSO_3.

650 μl of cell lysate from cells of various *E. coli* strains producing various IgG chains, was added to 500 mg urea. To this was added β-mercaptoethanol to 20 mM, Tris-HCl, pH 8.5 to 50 mM and EDTA to 1 mM, and in some experiments, γ-SSO_3 was added to 0.1 mg/ml. After standing at 25° for 30-90 mins., the reaction mixtures were dialyzed at 4° against a buffer composed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10 mM glycine ethyl ester, 5 mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N_2-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16-48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1 mM PMSF and dialysis continued another 16-24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in×ng/ml anti-CEA. Also shown are the reconstitution efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of γ and K chains in the reaction mixtures.

	ng/ml anti-CEA	Percent recombination
E. coli W3110 producing IFN-αA (control)	0	—
E. coli (W3110/pKCEAtrp207-1*)	108	—
E. coli (W3110/pKCEAtrp207-1*), plus γ-$SS0_3$	848	0.33
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEAInt2)	1580	0.76
Hybridoma anti-CEA K-$SS0_3$ and γ-$SS0_3$	540	0.40

E.4 Preparation of Chimeric Antibody

FIGS. **11** and **12** show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human γ-2 constant region.

A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA library obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACTCGACACAA 3' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., *Cell,* 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., *Proc. Natl. Acad. Sci.* (*USA*), 79: 1984 (1982) incorporated herein by reference).

As shown in FIG. **11**, two fragments are obtained from this cloned human gamma 2 plasmid (pγ2). The first fragment is formed by digestion with PvuII followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the pγ2 with any restriction enzyme which cleaves in the 3' untranslated region of γ2, as deduced from the nucleotide sequence, filling in with Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 6 percent PAGE. (The choice of a two step, two fragment composition to supply the PvuII-3' untranslated fragment provides a cleaner path to product due to the proximity of the AvaIII site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pγCEA207-1☆ is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

The location and DNA sequence surrounding the PvuII site in the mouse gamma-1 gene are identical to the location and DNA sequence surrounding the PvuII site in the human gamma-2 gene.

The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when transformed into *E. coli*, but one wherein the change from mouse to human does not take place at the variable to constant junction.

FIG. **12** shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human γ-2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described pγ2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and

isolating the short fragment which comprises the junction between variable and constant regions of this chain.

Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., *Nucleic Acids Res.* 9: 309 (1981), followed by in vitro site directed deletion mutagenesis as described by Adelman, et al., *DNA,* in press (1983) which is incorporated herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form pChim2. This plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable/human constant chimeric heavy chain.

In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than γ chain, the expression plasmid for chimeric light chain is prepared.

The foregoing two plasmids are then double transformed into *E. coli* W3110, the cells grown and the chains reconstituted as set forth in paragraph E.1-E.3 supra;

E.5 Preparation of Altered Murine Anti-CEA Antibody

E.5.1 Construction of Plasmid Vectors for Direct Expression of Altered Murine Anti-CEA Heavy Chain Gene

The cysteine residues, and the resultant disulfide bonds in the region of amino acids 216-230 in the constant region of murine anti-CEA heavy chain are suspected to be important for complement fixation (Klein, et al., *Proc. Natl. Acad. Sci.*, (*USA*), 78: 524 (1981)) but not for the antigen binding property of the resulting antibody. To decrease the probability of incorrect disulfide bond formation during reconstitution according to the process of the invention herein, the nucleotides encoding the amino acid residues 226-232 which includes codons for three cysteines, are deleted as follows:

A "deleter" deoxyoligonucleotide, 5' CTAACACCATGTCAGGGT is used to delete the relevant portions of the gene from pγCEAtrp207-1☆ by the procedure of Wallace, et al., *Science,* 209: 1396 (1980) or of Adelman, et al., DNA (in press) 1983. Briefly, the "deleter" deoxyoligonucleotide is annealed with denatured pγCEAtrp207-1☆ DNA, and primer repair synthesis carried out in vitro, followed by screening by hybridization of presumptive deletion clones with ρ^{32} labelled deleter sequence.

E.5.2 Production of Cysteine Deficient Altered Antibody

The plasmid prepared in E.5.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1☆ as described above. The cells are grown, extracted for recombinant antibody chains, and the altered antibody reconstituted as described in E.1.10.

E.6 Preparation of Fab

E.6.1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene pγCEAFabtrp207-1☆

FIG. **13** presents the construction of pγCEAFabtrp207-1☆. 5 μg of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment I, was recovered using 6 percent PAGE followed by electroelution.

5 μg of pγCEAtrp207-1☆ was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a termination codon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 μg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (FIG. **4**) which has the following sequence:

```
AspCysGlyStop
5' GGGATTGTGGTTG 3'
```

The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

E.6.2 Production of Fab Protein

The plasmid prepared in E.6.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1☆ as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

The invention claimed is:

1. A method for making an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, wherein the heavy chain or fragment thereof comprises a human constant region sequence and a variable region comprising non human mammalian variable region sequences, the method comprising culturing a recombinant host cell comprising DNA encoding the heavy chain or fragment thereof and the light chain or fragment thereof and recovering the heavy chain or fragment thereof and light chain or fragment thereof from the host cell culture.

2. The method of claim **1** wherein the light chain or fragment thereof comprises a human constant region sequence and a variable region comprising non human mammalian variable region sequences.

3. The method of claim **1** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or fragment thereof and DNA encoding the light chain or fragment thereof.

4. The method of claim **1** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or frag-

ment thereof and a further vector comprising DNA encoding the light chain or fragment thereof.

5. The method of claim **1** wherein the non human mammalian variable region sequences are murine.

6. The method of claim **1** wherein the host cell is a prokaryotic cell.

7. The method of claim **6** wherein the prokaryotic cell is an *E. coli* cell.

8. The method of claim **1** wherein the host cell is an eukaryotic cell.

9. The method of claim **8** wherein the eukaryotic cell is a mammalian cell.

10. The method of claim **9** wherein the mammalian cell is selected from the group consisting of a VERO, HeLa, Chinese Hamster Ovary (CHO), W138, BHK, COS-7 and MDCK cell.

11. The method of claim **10** wherein the mammalian cell is a CHO cell.

12. The method of claim **10** wherein the mammalian cell is a COS-7 cell.

13. The method of claim **8** wherein the eukaryotic cell is a yeast cell.

14. The method of claim **13** wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

15. A method for making an antibody or antibody fragment capable of specifically binding a desired antigen, wherein the antibody or antibody fragment comprises (a) an antibody heavy chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences and (b) an antibody light chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences, the method comprising coexpressing the heavy chain or fragment thereof and the light chain or fragment thereof in a recombinant host cell.

16. The method of claim **15** further comprising recovering the antibody or antibody fragment from a cell culture comprising the recombinant cell.

17. The method of claim **15** which results in the production of an antibody fragment.

18. The method of claim **17** wherein the antibody fragment is an $F(ab)_2$ fragment.

19. The method of claim **17** wherein the antibody fragment is a Fab fragment.

20. The method of claim **15** which results in the production of an antibody.

21. A method for making an antibody capable of specifically binding a desired antigen, the antibody comprising heavy and light immunoglobulin polypeptide chains each comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences, the method comprising the steps of (a) transforming a recombinant host cell with a replicable expression vector comprising DNA encoding the heavy immunoglobulin polypeptide chain and a replicable expression vector comprising DNA encoding the light immunoglobulin polypeptide chain, wherein each of the DNAs is operably linked to a promoter; and (b) culturing the host cell to produce a host cell culture that expresses said antibody.

22. A replicable expression vector comprising DNA encoding an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, the heavy chain or fragment thereof and the light chain or fragment thereof each comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences.

23. A recombinant host cell comprising the vector of claim **22**.

24. A recombinant host cell comprising (a) a vector comprising DNA encoding an antibody heavy chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences and (b) a vector comprising DNA encoding an antibody light chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences.

25. A method for making an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, wherein the heavy chain or fragment thereof comprises a variable region sequence and a human constant region sequence, the method comprising culturing a recombinant host cell comprising DNA encoding the heavy chain or fragment thereof and the light chain or fragment thereof and recovering the heavy chain or fragment thereof and light chain or fragment thereof from the host cell culture.

26. The method of claim **25** wherein the light chain or fragment thereof comprises a variable region sequence and a human constant region sequence.

27. The method of claim **25** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or fragment thereof and DNA encoding the light chain or fragment thereof.

28. The method of claim **25** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or fragment thereof and a further vector comprising DNA encoding the light chain or fragment thereof.

29. The method of claim **25** wherein the host cell is a prokaryotic cell.

30. The method of claim **29** wherein the prokaryotic cell is an *E. coli* cell.

31. The method of claim **25** wherein the host cell is a eukaryotic cell.

32. The method of claim **31** wherein the eukaryotic cell is a mammalian cell.

33. The method of claim **32** wherein the mammalian cell is selected from the group consisting of a VERO, HeLa, Chinese Hamster Ovary (CHO), W138, BHK, COS-7 and MDCK cell.

34. The method of claim **32** wherein the mammalian cell is a CHO cell.

35. The method of claim **32** wherein the mammalian cell is a COS-7 cell.

36. The method of claim **31** wherein the eukaryotic cell is a yeast cell.

37. The method of claim **36** wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

38. A method for making an antibody or antibody fragment capable of specifically binding a desired antigen, wherein the antibody or antibody fragment comprises (a) an antibody heavy chain or fragment thereof comprising a variable region sequence and a human constant region sequence and (b) an antibody light chain or fragment thereof comprising a variable region sequence and a human constant region sequence, the method comprising coexpressing the heavy chain or fragment thereof and the light chain or fragment thereof in a recombinant host cell.

39. The method of claim **38** further comprising recovering the antibody or antibody fragment from a cell culture comprising the recombinant cell.

40. The method of claim **38** which results in the production of an antibody fragment.

41. The method of claim **38** wherein the antibody fragment is an $F(ab)_2$ fragment.

42. The method of claim **40** wherein the antibody fragment is a Fab fragment.

43. The method of claim **38** which results in the production of an antibody.

44. A method for making an antibody capable of specifically binding a desired antigen, the antibody comprising heavy and light immunoglobulin polypeptide chains each comprising a variable region sequence and a human constant region sequence, the method comprising the steps of (a) transforming a recombinant host cell with a replicable expression vector comprising DNA encoding the heavy immunoglobulin polypeptide chain and a replicable expression vector comprising DNA encoding the light immunoglobulin polypeptide chain, wherein each of the DNAs is operably linked to a promoter; and (b) culturing the host cell to produce a host cell culture that expresses said antibody.

45. A replicable expression vector comprising DNA encoding an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, the heavy chain or fragment thereof and the light chain or fragment thereof each comprising a variable region sequence and a human constant region sequence.

46. A recombinant host cell comprising the vector of claim **45**.

47. A recombinant host cell comprising (a) a vector comprising DNA encoding an antibody heavy chain or fragment thereof comprising a variable region sequence and human constant region sequence and (b) a vector comprising DNA encoding an antibody light chain or fragment thereof comprising a variable region sequence and a human constant region sequence.

* * * * *

EXHIBIT C

US006407213B1

(12) **United States Patent**
Carter et al.

(10) **Patent No.: US 6,407,213 B1**
(45) **Date of Patent: Jun. 18, 2002**

(54) **METHOD FOR MAKING HUMANIZED ANTIBODIES**

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(57) **ABSTRACT**

Variant immunoglobulins, particularly humanized antibody polypeptides are provided, along with methods for their preparation and use. Consensus immunoglobulin sequences and structural models are also provided.

82 Claims, 9 Drawing Sheets

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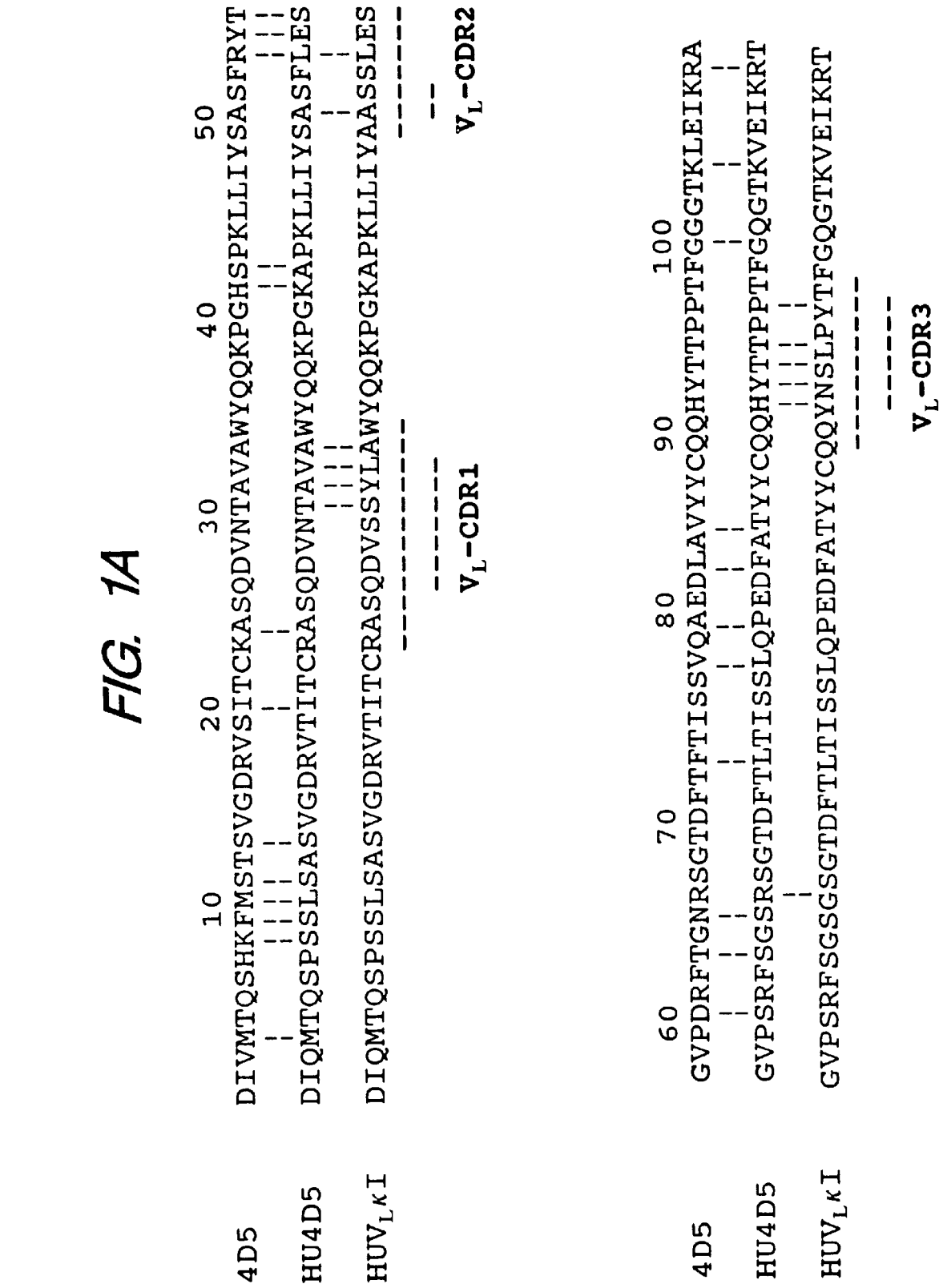
FIG. 1A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HU4D5 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLES
HUVLκI DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLES
VL-CDR1
VL-CDR2
60 70 80 90 100
4D5 GVPDRFTGNRSGTDFTFTISSVQAEDLAVYYCQQHYTTPPTFGGGTKLEIKRA
HU4D5 GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT
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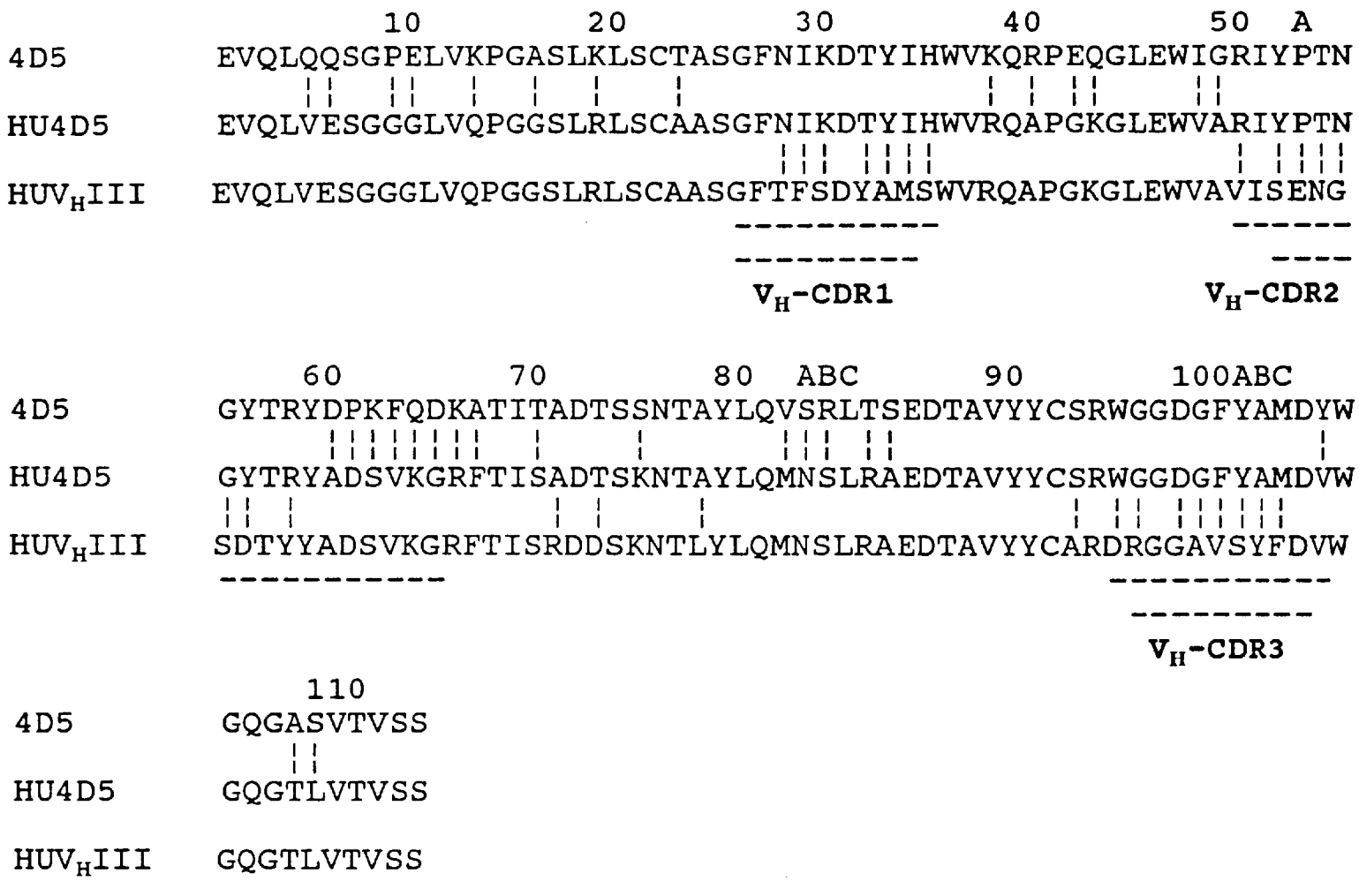
VL-CDR3

FIG. 1B



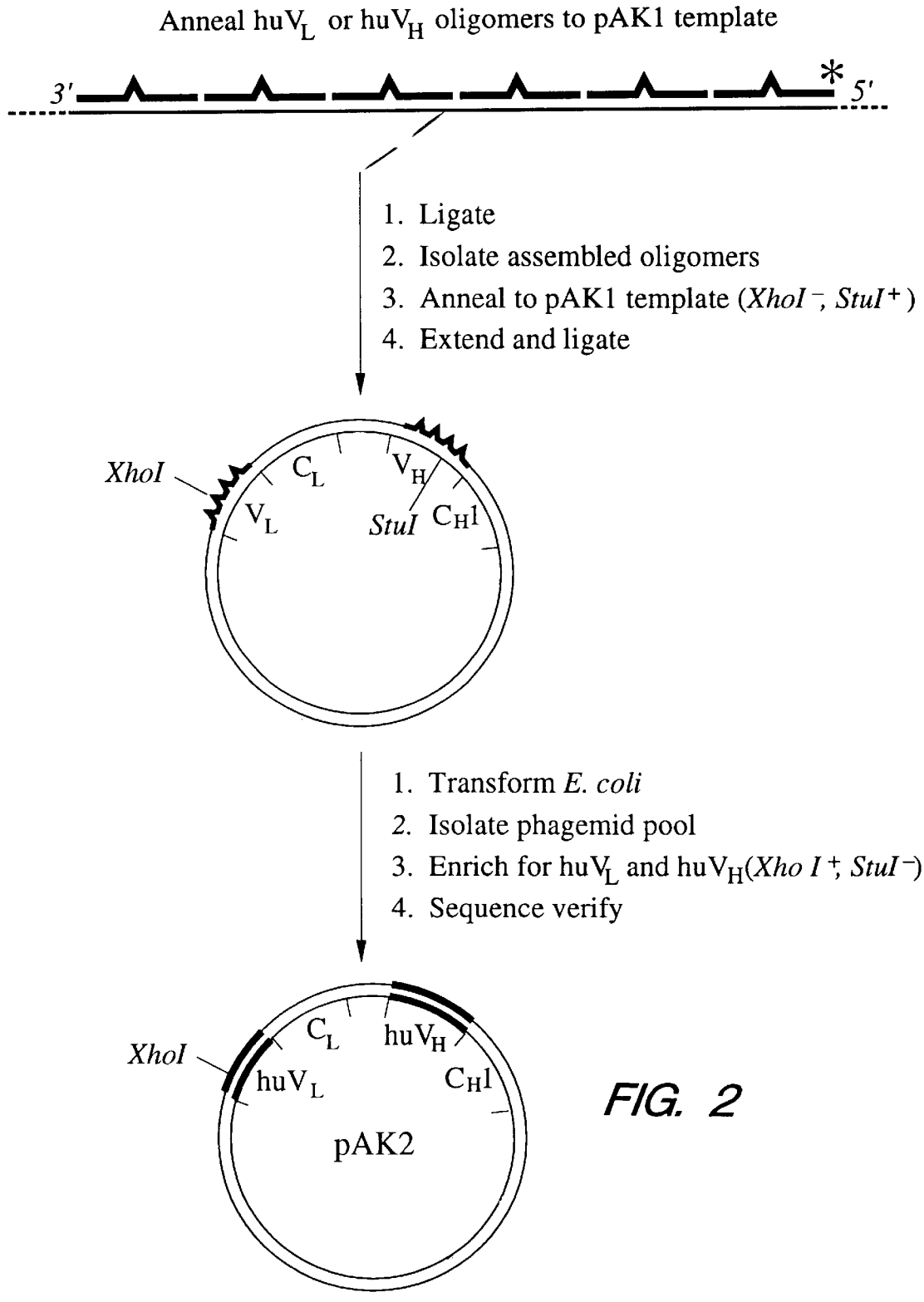

Anneal huV$_L$ or huV$_H$ oligomers to pAK1 template
3'
5'
*
1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (*XhoI*$^-$, *StuI*$^+$)
4. Extend and ligate
XhoI
C$_L$
V$_H$
V$_L$
StuI
C$_H$1
1. Transform *E. coli*
2. Isolate phagemid pool
3. Enrich for huV$_L$ and huV$_H$(*Xho I*$^+$, *StuI*$^-$)
4. Sequence verify
XhoI
C$_L$
huV$_H$
huV$_L$
C$_H$1
pAK2

FIG. 2

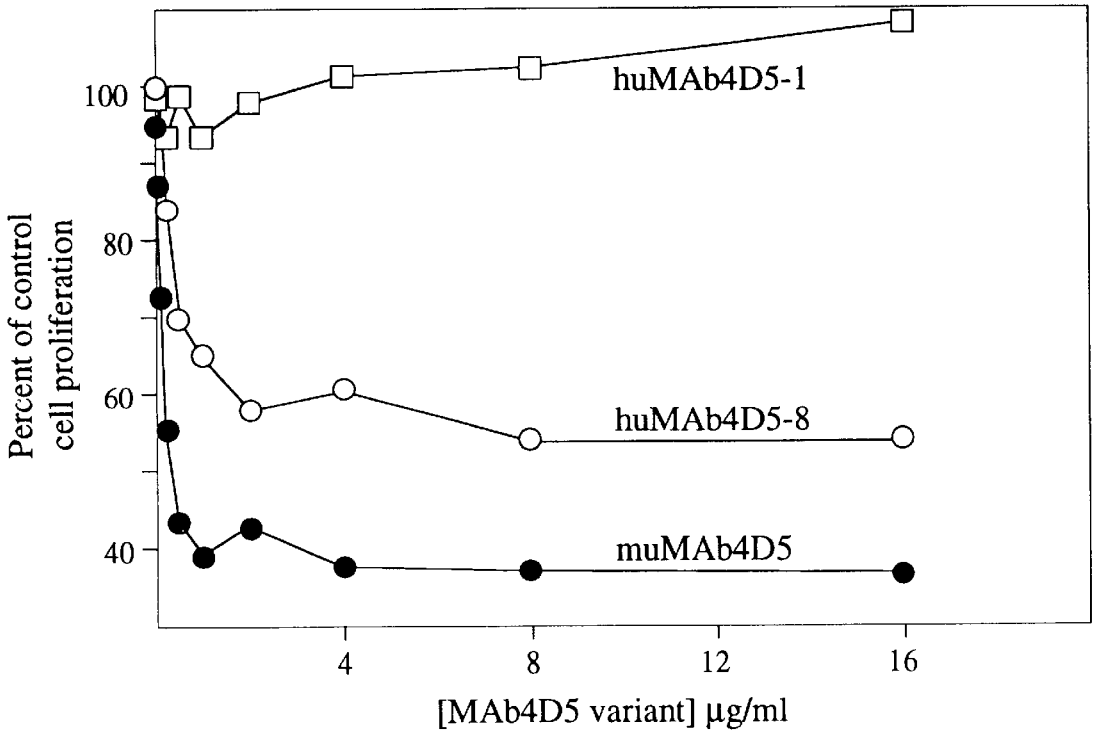

100
80
60
40
Percent of control cell proliferation
huMAb4D5-1
huMAb4D5-8
muMAb4D5
4
8
12
16
[MAb4D5 variant] µg/ml

FIG. 3

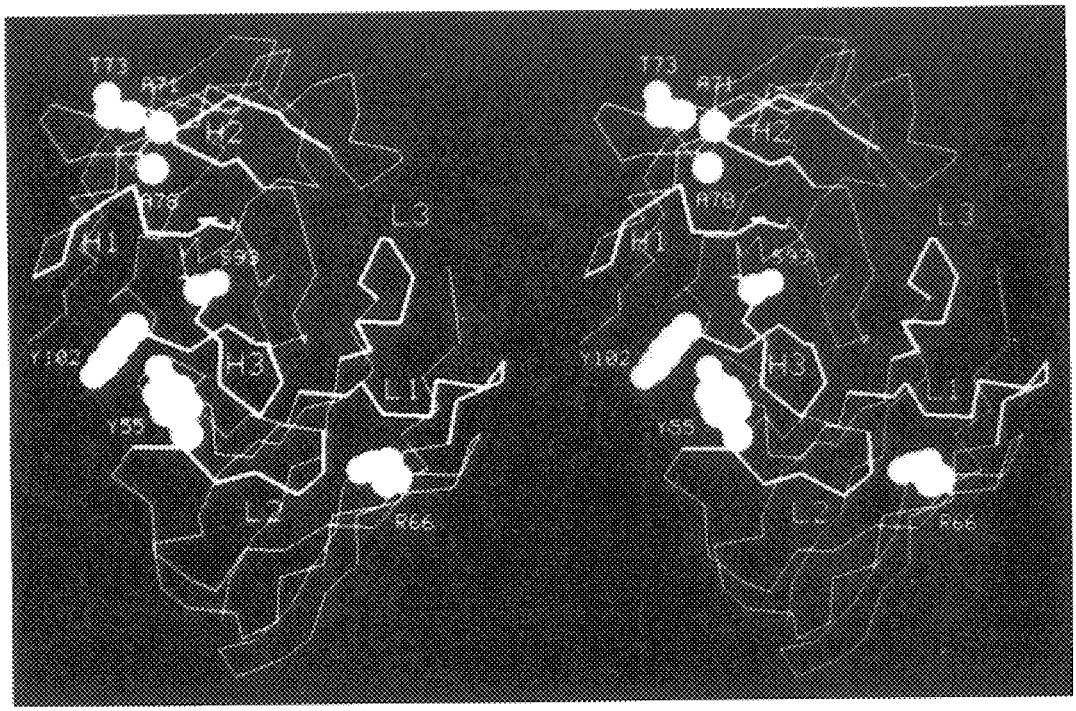


FIG. 4

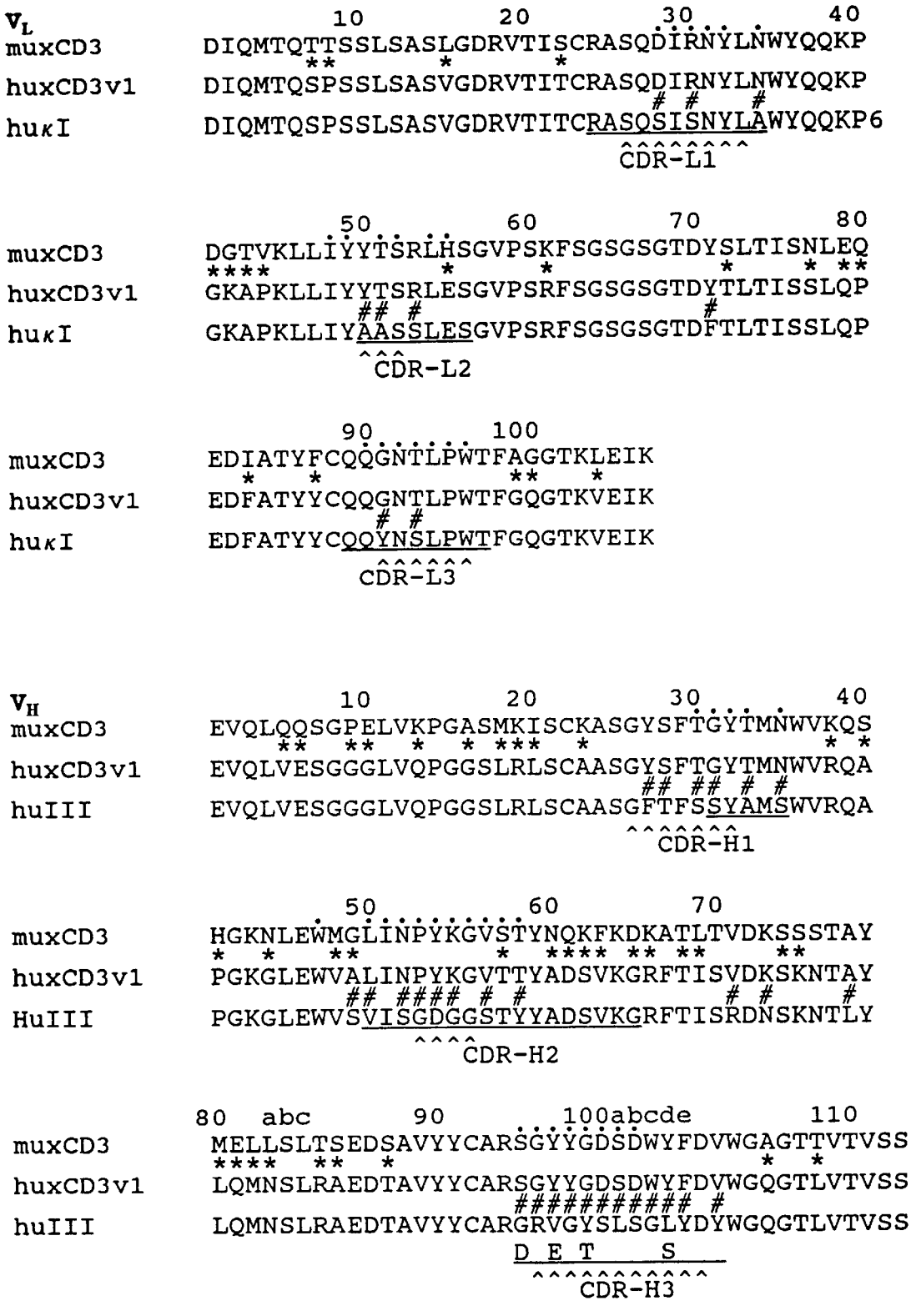

VL
muxCD3
huxCD3v1
huκI
CDR-L1
CDR-L2
CDR-L3
VH
muxCD3
huxCD3v1
huIII
CDR-H1
muxCD3
huxCD3v1
HuIII
CDR-H2
muxCD3
huxCD3v1
huIII
CDR-H3

FIG. 5

FIG. 6A-1

```
                                    10                  20                  30
H52H4-160                           QVQLQQSGPELVKPGASVKISCKTSGYTFTE
                                    .*** .**  **.**.*...** *********
pH52-8.0            MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE
                               10                  20                  30
                                    40                  50                  60                  70                  80
H52H4-160           YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM
                    *******.*. **.***..*.********.*********. *..**********
pH52-8.0            YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDRFTISVDKSTSTAYM
                                    60                  70                  80                  90                  100
                                    90                  100                 110                 120                 130
H52H4-160           ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS
                    .. ** .**....********************** ** **********
pH52-8.0            QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGTLVTVSSASTKGPS
                               110                 120                 130                 140                 150
                                    140                 150                 160                 170                 180
H52H4-160           VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
                    ****** *.*** .**********************************
pH52-8.0            VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
                                    160                 170                 180                 190                 200
                                    190                 200                 210                 220                 230
H52H4-160           QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
                    ************** **..***** ***.********** ** * *
pH52-8.0            QSSGLYSLSSVVTVTSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC---V
                               210                 220                 230                 240
                                    240                 250                 260                 270                 280
H52H4-160           TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
                    ********  .**********************************.
pH52-8.0            ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
                    250                 260                 270                 280                 290
```

FIG. 6A-2

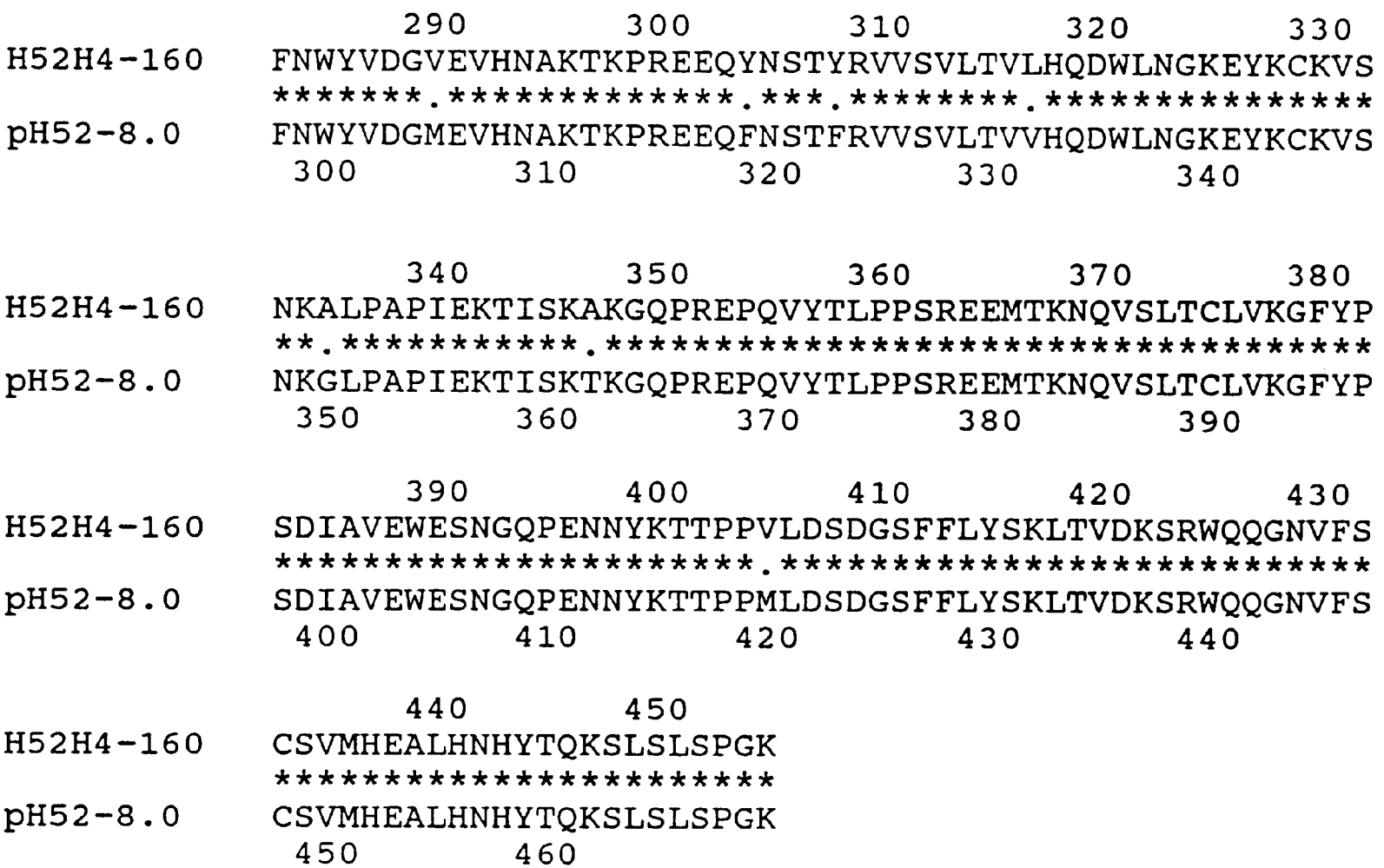


FIG. 6B

```
                                10        20        30
H52L6-158                       DVQMTQTTSSLSASLGDRVTINCRASQDINN
                                *.*****. *******.******* **********
pH52-9.0        MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN
                          10        20        30        40        50

                        40        50        60        70        80
H52L6-158       YLNWYQQKPNGTVKLLIYYTSTLHSGVPSRFSGSGSGTDYSLTISNLDQE
                ********** . ********************.*****.*. *
pH52-9.0        YLNWYQQKPGKAPKLLIYYTSTLHSGVPSRFSGSGSGTDYTLTISSLQPE
                          60        70        80        90       100

                        90       100       110       120       130
H52L6-158       DIATYFCQQGNTLPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
                *.****.************ ********************************
pH52-9.0        DFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
                          110       120       130       140       150

                       140       150       160       170       180
H52L6-158       VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
                **********************************************
pH52-9.0        VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
                          160       170       180       190       200

                       190       200       210
H52L6-158       SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
                **********************************
pH52-9.0        SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
                          210       220       230
```

METHOD FOR MAKING HUMANIZED ANTIBODIES

CROSS REFERENCES

This application is a continuation-in-part of U.S. application Ser. No. 07/715,272 filed Jun. 14, 1991 (abandoned) which application is incorporated herein by reference and to which application priority is claimed under 35 USC §120.

FIELD OF THE INVENTION

This invention relates to methods for the preparation and use of variant antibodies and finds application particularly in the fields of immunology and cancer diagnosis and therapy.

BACKGROUND OF THE INVENTION

Naturally occurring antibodies (immunoglobulins) comprise two heavy chains linked together by disulfide bonds and two light chains, one light chain being linked to each of the heavy chains by disulfide bonds. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain (V_L) at one end and a constant domain at its other end, the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains, see e.g. Chothia et al., *J. Mol. Biol.* 186:651–663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA* 82:4592–4596 (1985).

The constant domains are not involved directly in binding the antibody to an antigen, but are involved in various effector functions, such as participation of the antibody in antibody-dependent cellular cytotoxicity. The variable domains of each pair of light and heavy chains are involved directly in binding the antibody to the antigen. The domains of natural light and heavy chains have the same general structure, and each domain comprises four framework (FR) regions, whose sequences are somewhat conserved, connected by three hyper-variable or complementarity determining regions (CDRs) (see Kabat, E. A. et a., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda, Md., (1987)). The four framework regions largely adopt a β-sheet conformation and the CORs form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site.

Widespread use has been made of monoclonal antibodies, particularly those derived from rodents including mice, however they are frequently antigenic in human clinical use. For example, a major limitation in the clinical use of rodent monoclonal antibodies is an anti-globulin response during therapy (Miller, R. A. et al., *Blood* 62:988–995 (1983); Schroff, R. W. et al., *Cancer Res.* 45:879–885 (1985)).

The art has attempted to overcome this problem by constructing "chimeric" antibodies in which an animal antigen-binding variable domain is coupled to a human constant domain (Cabilly et al., U.S. Pat. No. 4,816,567; Morrison, S. L. et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984); Boulianne, G. L. et al., *Nature* 312:643–646 (1984); Neuberger, M. S. et al., *Nature* 314:268–270 (1985)). The term "chimeric" antibody is used herein to describe a polypeptide comprising at least the

antigen binding portion of an antibody molecule linked to at least part of another protein (typically an immunoglobulin constant domain).

The isotype of the human constant domain may be selected to tailor the chimeric antibody for participation in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (see e.g. Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988); Love et al., *Methods in Enzymology* 178:515–527 (1989); Bindon et al., *J. Exp. Med.* 168:127–142 (1988).

In the typical embodiment, such chimeric antibodies contain about one third rodent (or other non-human species) sequence and thus are capable of eliciting a significant anti-globulin response in humans. For example, in the case of the murine anti-CD3 antibody, OKT3, much of the resulting anti-globulin response is directed against the variable region rather than the constant region (Jaffers, G. J. et a., *Transplantation* 41:572–578 (1986)).

In a further effort to resolve the antigen binding functions of antibodies and to minimize the use of heterologous sequences in human antibodies, Winter and colleagues (Jones, P. T. et al., *Nature* 321:522–525 (1986); Riechmann, L. et al., *Nature* 332:323–327 (1988); Verhoeyen, M. et al., *Science* 239:1534–1536 (1988)) have substituted rodent CDRs or CDR sequences for the corresponding segments of a human antibody. As used herein, the term "humanized" antibody is an embodiment of chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The therapeutic promise of this approach is supported by the clinical efficacy of a humanized antibody specific for the CAMPATH-1 antigen with two non-Hodgkin lymphoma patients, one of whom had previously developed an anti-globulin response to the parental rat antibody (Riechmann, L. et al., *Nature* 332:323–327 (1988); Hale, G. et al., *Lancet i:*1394–1399 (1988)). A murine antibody to the interleukin 2 receptor has also recently been humanized (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)) as a potential immunosuppressive reagent. Additional references related to humanization of antibodies include Co et al., *Proc. Natl. Acad. Sci. USA* 88:2869–2873 (1991); Gorman et al., *Proc. Natl. Acad. Sci. USA* 88:4181–4185 (1991); Daugherty et al., *Nucleic Acids Research* 19(9):2471–2476 (1991); Brown et al., *Proc. Natl. Acad. Sci. USA* 88:2663–2667 (1991); Junghans et al., *Cancer Research* 50:1495–1502 (1990).

In some cases, substituting CDRs from rodent antibodies for the human CDRs in human frameworks is sufficient to transfer high antigen binding affinity (Jones, P. T. et al., *Nature* 321:522–525 (1986); Verhoeyen, M. et al., *Science* 239:1534–1536 (1988)), whereas in other cases it has been necessary to additionally replace one (Riechmann, L. et al., *Nature* 332:323–327 (1988)) or several (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)) framework region (FR) residues. See also Co et al., supra.

For a given antibody a small number of FR residues are anticipated to be important for antigen binding. Firstly for example, certain antibodies have been shown to contain a few FR residues which directly contact antigen in crystal structures of antibody-antigen complexes (e.g., reviewed in Davies, D. R. et al., *Ann. Rev. Biochem.* 59:439–473 (1990)).

Secondly, a number of FR residues have been proposed by Chothia, Lesk and colleagues (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987); Chothia, C. et al., *Nature* 342:877–883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) as critically affecting the conformation of particular CDRs and thus their contribution to antigen binding. See also Margolies et al., *Proc. Natl. Acad. Sci. USA* 72:2180–2184 (1975).

It is also known that, in a few instances, an antibody variable domain (either V_H or V_L) may contain glycosylation sites, and that this glycosylation may improve or abolish antigen binding, Pluckthun, *Biotechnology* 9:545–51 (1991); Spiegelberg et al., *Biochemistry* 9:4217–4223 (1970); Wallic et al., *J. Exp. Med.* 168:1099–1109 (1988); Sox et al., *Proc. Natl. Acad. Sci. USA* 66:975–982 (1970); Margni et al., *Ann. Rev. Immunol* 6:535–554 (1988). Ordinarily, however, glycosylation has no influence on the antigen-binding properties of an antibody, Pluckthun, supra, (1991).

The three-dimensional structure of immunoglobulin chains has been studied, and crystal structures for intact immunoglobulins, for a variety of immunoglobulin fragments, and for antibody-antigen complexes have been published (see e.g., Saul et al., *Journal of Biological Chemistry* 25:585–97 (1978); Sheriff et al., *Proc. Natl. Acad. Sci. USA* 84:8075–79 (1987); Segal et al., *Proc. Natl. Acad. Sci. USA* 71:4298–4302 (1974); Epp et al., *Biochemistry* 14(22) :4943–4952 (1975); Marquart et al., *J. Mol. Biol.* 141:369–391 (1980); Furey et al., *J. Mol. Biol.* 167:661–692 (1983); Snow and Amzel, *Protein: Structure, Function, and Genetics* 1:267–279, Alan R. Liss, Inc. pubs. (1986); Chothia and Lesk, *J. Mol. Bol.* 196:901–917 (1987); Chothia et al., *Nature* 342:877–883 (1989); Chothia et al., *Science* 233:755–58 (1986); Huber et al., *Nature* 264:415–420 (1976); Bruccoleri et al., *Nature* 335:564–568 (1988) and *Nature* 336:266 (1988); Sherman et al., *Journal of Biological Chemistry* 263:4064–4074 (1988); Amzel and Poljak, *Ann. Rev. Biochem.* 48:961–67 (1979); Silverton et al., *Proc. Natl. Acad. Sci. USA* 74:5140–5144 (1977); and Gregory et al., *Molecular Immunology* 24:821–829 (1987). It is known that the function of an antibody is dependent on its three dimensional structure, and that amino acid substitutions can change the three-dimensional structure of an antibody, Snow and Amzel, supra. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)).

Humanizing an antibody with retention of high affinity for antigen and other desired biological activities is at present difficult to achieve using currently available procedures. Methods are needed for rationalizing the selection of sites for substitution in preparing such antibodies and thereby increasing the efficiency of antibody humanization.

The proto-oncogene HER2 (human epidermal growth factor receptor 2) encodes a protein tyrosine kinase ($p185^{HER2}$) that is related to and somewhat homologous to the human epidermal growth factor receptor (see Coussens, L. et al., *Science* 230:1132–1139 (1985); Yamamoto, T. et al., *Nature* 319:230–234 (1986); King, C. R. et al., *Science* 229:974–976 (1985)). HER2 is also known in the field as c-erbB-2, and sometimes by the name of the rat homolog, neu. Amplification and/or overexpression of HER2 is associated with multiple human malignancies and appears to be integrally involved in progression of 25–30% of human breast and ovarian cancers (Slamon, D. J. et al., *Science* 235:177–182 (1987), Slamon, D. J. et al., *Science* 244:707–712 (1989)). Furthermore, the extent of amplification is inversely correlated with the observed median patient survival time (Slamon, supra, Science 1989).

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)), directed against the extracellular domain (ECD) of $p185^{HER2}$, specifically inhibits the growth of tumor cell lines overexpressing $p185^{HER2}$ in monolayer culture or in soft agar (Hudziak, R. M. et al., *Molec. Cell. Biol* 9:1165–1172 (1989); Lupu, R. et al., *Science* 249:1552–1555 (1990)). MuMAb4D5 also has the potential of enhancing tumor cell sensitivity to tumor necrosis factor, an important effector molecule in macrophage-mediated tumor cell cytotoxicity (Hudziak, supra, 1989; Shepard, H. M. and Lewis, G. D. *J. Clinical Immunology* 8:333–395 (1988)). Thus muMAb4D5 has potential for clinical intervention in and imaging of carcinomas in which $p185^{HER2}$ is overexpressed. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. However, this antibody may be immunogenic in humans.

It is therefore an object of this invention to provide methods for the preparation of antibodies which are less antigenic in humans than non-human antibodies but have desired antigen binding and other characteristics and activities.

It is a further object of this invention to provide methods for the efficient humanization is of antibodies, i.e. selecting non-human amino acid residues for importation into a human antibody background sequence in such a fashion as to retain or improve the affinity of the non-human donor antibody for a given antigen.

It is another object of this invention to provide humanized antibodies capable of binding $p185^{HER2}$.

Other objects, features, and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

SUMMARY OF THE INVENTION

The objects of this invention are accomplished by a method for making a humanized antibody comprising amino acid sequence of an import, non-human antibody and a human antibody, comprising the steps of:

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, the method of this invention comprises the additional steps of determining if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), retaining the consensus residue.

Additionally, in certain embodiments the method of this invention comprises the feature wherein the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In certain embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). If the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another embodiment of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

Certain alternate embodiments of the methods of this invention comprise obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b. (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 78H, 91H, 92H, 93H, and 103H.

In preferred embodiments, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody.

Optionally, this just-recited embodiment comprises the additional steps of following the method steps appearing at the beginning of this summary and determining whether a particular amino acid residue can reasonably be expected to have undesirable effects.

This invention also relates to a humanized antibody comprising the CDR sequence of an import, non-human antibody and the FR sequence of a human antibody, wherein an amino acid residue within the human FR sequence located at any one of the sites 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H has been substituted by another residue. In preferred embodiments, the residue substituted at the human FR site is the residue found at the corresponding location of the non-human antibody from which the non-human CDR was obtained. In other embodiments, no human FR residue other than those set forth in this group has been substituted.

This invention also encompasses specific humanized antibody variable domains, and isolated polypeptides having homology with the following sequences.

1. SEQ. ID NO. 1, which is the light chain variable domain of a humanized version of muMAb4D5: DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT

2. SEQ. ID NO. 2, which is the heavy chain variable domain of a humanized version of muMAb4D5): EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

In another aspect, this invention provides a consensus antibody variable domain amino acid sequence for use in the preparation of humanized antibodies, methods for obtaining, using, and storing a computer representation of such a consensus sequence, and computers comprising the sequence data of such a sequence. In one embodiment, the following consensus antibody variable domain amino acid sequences are provided:

SEQ. ID NO. 3 (light chain): DDIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT, and

SEQ. ID NO. 4 (heavy chain): EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAWYCSRWGGDGFYAMDVWGQGTLVTVSS

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1A** shows the comparison of the V_L domain amino acid residues of muMAb4D5, huMAb4D5, and a consensus sequence (FIG. **1A**, SEQ.ID NO. 5, SEQ. ID NO. 1 and SEQ. ID NO. 3, respectively). FIG. **1B** shows the comparison between the V_H domain amino acid residues of the muMAb4D5, huMAb4D5, and a consensus sequence (FIG. **1B**, SEQ. ID NO. 6, SEQ. ID NO. 2 and SEQ. ID NO. 4, respectively). Both FIGS. **1A** and **1B** use the generally accepted numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987)). In both FIG. **1A**

and FIG. **1**B, the CDR residues determined according to a standard sequence definition (as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are indicated by the first underlining beneath the sequences, and the CDR residues determined according to a structural definition (as in Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)) are indicated by the second, lower underlines. The mismatches between genes are shown by the vertical lines.

FIG. **2** shows a scheme for humanization of muMAb4D5 V_L and V_H by gene conversion mutagenesis.

FIG. **3** shows the inhibition of SK-BR-3 proliferation by MAb4D5 variants. Relative cell proliferation was determined as described (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)) and data (average of triplicate determinations) are presented as a percentage of results with untreated cultures for muMAb4D5 (●), huMAb4D5-8 (○) and huMAb4D5-1 (□).

FIG. **4** shows a stereo view of α-carbon tracing for a model of huMAb4D5-8 V_L and V_H. The CDR residues (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are shown in bold and side chains of V_H residues A71, T73, A78, S93, Y102 and V_L residues Y55 plus R66 (see Table 3) are shown.

FIG. **5** shows an amino acid sequence comparison of V_L (top panel) and V_H (lower panel) domains of the murine anti-CD3 monoclonal Ab UCHT1 (muxCD3, Shalaby et al., *J. Exp. Med.* 175, 217–225 (1992) with a humanized variant of this antibody (huxCD3v1). Also shown are consensus sequences (most commonly occurring residue or pair of residues) of the most abundant human subgroups, namely V_L κ 1 and V_H III upon which the humanized sequences are based (Kabat, E. A. et al., *Sequences of Proteins of immunological Interest,* 5^{th} edition, National Institutes of Health, Bethesda, Md., USA (1991)). The light chain sequences—muxCD3, huxCD3v1 and huKI—correspond to SEQ.ID. NOs 16, 17, and 18, respectively. The heavy chain sequences—muxCD3, huxCD3v1 and huxI—correspond to SEQ.ID.NOs 19, 26, and 21, respectively. Residues which differ between muxCD3 and huxCD3v1 are identified by an asterisk (*), whereas those which differ between humanized and consensus sequences are identified by a sharp sign (#). A bullet (•) denotes that a residue at this position has been found to contact antigen in one or more crystallographic structures of antibody/antigen complexes (Kabat et al., 1991; Mian, I. S. et al., *J. Mol. Biol* 217, 133–151 (1991)). The location of CDR residues according to a sequence definition (Kabat et al., 1991) and a structural definition (Chothia and Lesk, supra 1987) are shown by a line and carats (^) beneath the sequences, respectively.

FIG. **6**A compares murine and humanized amino acid sequences for the heavy chain of an anti-CD18 antibody. H52H4-160 (SEQ. ID. NO. 22) is the murine sequence, and pH52–8.0 (SEQ. ID. NO. 23) is the humanized heavy chain sequence. pH52–8.0 residue 143S is the final amino acid in the variable heavy chain domain V_H, and residue 144A is the first amino acid in the constant heavy chain domain C_{H1}.

FIG. **6**B compares murine and humanized amino acid sequences for the light chain of an anti-CD18 antibody. H52L6-158 (SEQ. ID. NO. 24) is the murine sequence, and pH52–9.0 (SEQ. ID. NO. 25) is the humanized light chain sequence. pH52–9.0 residue 128T is the final amino acid in the light chain variable domain V_L, and residue 129V is the first amino acid in the light chain constant domain C_L.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In general, the following words or phrases have the indicated definitions when used in the description, examples, and claims:

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) is directed against the extracellular domain (ECD) of $p185^{HER2}$. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. In this description and claims, the terms muMAb4D5, chMAb4D5 and huMAb4D5 represent murine, chimerized and humanized versions of the monoclonal antibody 4D5, respectively.

A humanized antibody for the purposes herein is an immunoglobulin amino acid sequence variant or fragment thereof which is capable of binding to a predetermined antigen and which comprises a FR region having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid sequence of a non-human immunoglobulin.

Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are referred to herein as "import" residues, which are typically taken from an "import" antibody domain, particularly a variable domain. An import residue, sequence, or antibody has a desired affinity and/or specificity, or other desirable antibody biological activity as discussed herein.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain.

The humanized antibody will be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and lgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

The FR and CDR regions of the humanized antibody need not correspond precisely to the parental sequences, e.g., the import CDR or the consensus FR may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or FR residue at that site does not correspond to either the consensus or the import antibody. Such mutations, however, will not be extensive. Usually, at least 75% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences, more often 90%, and most preferably greater than 95%.

In general, humanized antibodies prepared by the method of this invention are produced by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those

skilled in the art. Computer programs are available which illustrate and display probable three dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen.

Residues that influence antigen binding are defined to be residues that are substantially responsible for the antigen affinity or antigen specificity of a candidate immunoglobulin, in a positive or a negative sense. The invention is directed to the selection and combination of FR residues from the consensus and import sequence so that the desired immunoglobulin characteristic is achieved. Such desired characteristics include increases in affinity and greater specificity for the target antigen, although it is conceivable that in some circumstances the opposite effects might be desired. In general, the CDR residues are directly and most substantially involved in influencing antigen binding (although not all CDR residues are so involved and therefore need not be substituted into the consensus sequence). However, FR residues also have a significant effect and can exert their influence in at least three ways: They may noncovalently directly bind to antigen, they may interact with CDR residues and they may affect the interface between the heavy and light chains.

A residue that noncovalently directly binds to antigen is one that, by three dimensional analysis, is reasonably expected to noncovalently directly bind to antigen. Typically, it is necessary to impute the position of antigen from the spatial location of neighboring CDRs and the dimensions and structure of the target antigen. In general, only those humanized antibody residues that are capable of forming salt bridges, hydrogen bonds, or hydrophobic interactions are likely to be involved in non-covalent antigen binding, however residues which have atoms which are separated from antigen spatially by 3.2 Angstroms or less may also non-covalently interact with antigen. Such residues typically are the relatively larger amino acids having the side chains with the greatest bulk, such as tyrosine, arginine, and lysine. Antigen-binding FR residues also typically will have side chains that are oriented into an envelope surrounding the solvent oriented face of a CDR which extends about 7 Angstroms into the solvent from the CDR domain and about 7 Angstroms on either side of the CDR domain, again as visualized by three dimensional modeling.

A residue that interacts with a CDR generally is a residue that either affects the conformation of the CDR polypeptide backbone or forms a noncovalent bond with a CDR residue side chain. Conformation-affecting residues ordinarily are those that change the spatial position of any CDR backbone atom (N, Ca, C, O, Cβ) by more than about 0.2 Angstroms. Backbone atoms of CDR sequences are displaced for example by residues that interrupt or modify organized structures such as beta sheets, helices or loops. Residues that can exert a profound affect on the conformation of neighboring sequences include proline and glycine, both of which are capable of introducing bends into the backbone. Other residues that can displace backbone atoms are those that are capable of participating in salt bridges and hydrogen bonds.

A residue that interacts with a CDR side chain is one that is reasonably expected to form a noncovalent bond with a CDR side chain, generally either a salt bridge or hydrogen bond. Such residues are identified by three dimensional positioning of their side chains. A salt or ion bridge could be expected to form between two side chains positioned within about 2.5–3.2 Angstroms of one another that bear opposite charges, for example a lysinyl and a glutamyl pairing. A hydrogen bond could be expected to form between the side chains of residue pairs such as seryl or threonyl with aspartyl or glutamyl (or other hydrogen accepting residues). Such pairings are well known in the protein chemistry art and will be apparent to the artisan upon three dimensional modeling of the candidate immunoglobulin.

Immunoglobulin residues that affect the interface between heavy and light chain variable regions ("the V_L–V_H interface") are those that affect the proximity or orientation of the two chains with respect to one another. Certain residues involved in interchain interactions are already known and include V_L residues 34, 36, 38, 44, 46, 87, 89, 91, 96, and 98 and V_H residues 35, 37, 39, 45, 47, 91, 93, 95, 100, and 103 (utilizing the nomenclature setforth in Kabat et al., *Sequences of Proteins of immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)). Additional residues are newly identified by the inventors herein, and include 43L, 85L, 43H and 60H. While these residues are indicated for IgG only, they are applicable across species. In the practice of this invention, import antibody residues that are reasonably expected to be involved in interchain interactions are selected for substitution into the consensus sequence. It is believed that heretofore no humanized antibody has been prepared with an intrachain-affecting residue selected from an import antibody sequence.

Since it is not entirely possible to predict in advance what the exact impact of a given substitution will be it may be necessary to make the substitution and assay the candidate antibody for the desired characteristic. These steps, however, are per se routine and well within the ordinary skill of the art.

CDR and FR residues are determined according to a standard sequence definition (Kabat et al., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda Md. (1987), and a structural definition (as in Chothia and Lesk, *J. Mol. Biol.* 196:901–917 (1987). Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred, but the residues identified by the sequence definition method are considered important FR residues for determination of which framework residues to import into a consensus sequence.

Throughout this description, reference is made to the numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991). In these compendiums, Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. The Kabat numbering scheme is followed in this description.

For purposes of this invention, to assign residue numbers to a candidate antibody amino acid sequence which is not included in the Kabat compendium, one follows the following steps. Generally, the candidate sequence is aligned with any immunoglobulin sequence or any consensus sequence in Kabat. Alignment may be done by hand, or by computer using commonly accepted computer programs; an example of such a program is the Align 2 program discussed in this description. Alignment may be facilitated by using some amino acid residues which are common to most Fab

sequences. For example, the light and heavy chains each typically have two cysteines which have the same residue numbers; in V_L domain the two cysteines are typically at residue numbers 23 and 88, and in the V_H domain the two cysteine residues are typically numbered 22 and 92. Framework residues generally, but not always, have approximately the same number of residues, however the CDRs will vary in size. For example, in the case of a CDR from a candidate sequence which is longer than the CDR in the sequence in Kabat to which it is aligned, typically suffixes are added to the residue number to indicate the insertion of additional residues (see, e.g. residues 100abcde in FIG. **5**). For candidate sequences which, for example, align with a Kabat sequence for residues 34 and 36 but have no residue between them to align with residue 35, the number 35 is simply not assigned to a residue.

Thus, in humanization of an import variable sequence, where one cuts out an entire human or consensus CDR and replaces it with an import CDR sequence, (a) the exact number of residues may be swapped, leaving the numbering the same, (b) fewer import amino acid residues may be introduced than are cut, in which case there will be a gap in the residue numbers, or (c) a larger number of amino acid residues may be introduced then were cut, in which case the numbering will involve the use of suffixes such as 100abcde.

The terms "consensus sequence" and "consensus antibody" as used herein refers to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all immunoglobulins of any particular subclass or subunit structure. The consensus sequence may be based on immunoglobulins of a particular species or of many species. A "consensus" sequence, structure, or antibody is understood to encompass a consensus human sequence as described in certain embodiments of this invention, and to refer to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure. This invention provides consensus human structures and consensus structures which consider other species in addition to human.

The subunit structures of the live immunoglobulin classes in humans are as follows:

Class	Heavy Chain	Subclasses	Light Chain	Molecular Formula
IgG	γ	γ1, γ2, γ3, γ4	κ or λ	$(\gamma_2\kappa_2)$, $(\gamma_2\lambda_2)$
IgA	α	α1, α2	κ or λ	$(\alpha_2\kappa_2)_n$[8], $(\alpha_2\lambda_2)_n$[8]
IgM	μ	none	κ or λ	$(\mu_2\kappa_2)_5$, $(\mu_2\lambda_2)_5$
IgD	δ	none	κ or λ	$(\delta_2\kappa_2)$, $(\delta_2\lambda_2)$
IgE	ϵ	none	κ or λ	$(\epsilon_2\kappa_2)$, $(\epsilon_2\lambda_2)$

([8]n may equal 1, 2, or 3)

In preferred embodiments of an IgGγl human consensus sequence, the consensus variable domain sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda Md. (1987), namely V_L κ subgroup I and V_H group III. In such preferred embodiments, the V_L consensus domain has the amino acid sequence:

DIQMTQSPSSLSASVGDRVTITCRASQD-VSSYLAWYQQKPGKAPKLLIYAASSLES-GVPSRFSGSGSGTDFTLTISSLQPEDFA-TYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3);

the V_H consensus domain has the amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSW VRQAPGKGLEWVAVISENGGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 4).

These sequences include consensus CDRs as well as consensus FR residues (see for example in FIG. **1**).

While not wishing to be limited to any particular theories, it may be that these preferred embodiments are less likely to be immunogenic in an individual than less abundant subclasses. However, in other embodiments, the consensus sequence is derived from other subclasses of human immunoglobulin variable domains. In yet other embodiments, the consensus sequence is derived from human constant domains.

Identity or homology with respect to a specified amino acid sequence of this invention is defined herein as the percentage of amino acid residues in a candidate sequence that are identical with the specified residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into the specified sequence shall be construed as affecting homology. All sequence alignments called for in this invention are such maximal homology alignments. While such alignments may be done by hand using conventional methods, a suitable computer program is the "Align 2" program for which protection is being sought from the U.S. Register of Copyrights (Align 2, by Genentech, Inc., application filed Dec. 9, 1991).

"Non-homologous" import antibody residues are those residues which are not identical to the amino acid residue at the analogous or corresponding location in a consensus sequence, after the import and consensus sequences are aligned.

The term "computer representation" refers to information which is in a form that can be manipulated by a computer. The act of storing a computer representation refers to the act of placing the information in a form suitable for manipulation by a computer.

This invention is also directed to novel polypeptides, and in certain aspects, isolated novel humanized anti-$p185^{HER2}$ antibodies are provided. These novel anti-$p185^{HER2}$ antibodies are sometimes collectively referred to herein as huMAb4D5, and also sometimes as the light or heavy chain variable domains of huMAb4D5, and are defined herein to be any polypeptide sequence which possesses a biological property of a polypeptide comprising the following polypeptide sequence:

DIQMTQSPSSLSASVGDRVTITCRASODVNTAVAWY QQKPGKAPKLLIYSASFLESGVPSRFSGSRSGT DFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK VEIKRT (SEQ. ID NO. 1, which is the light chain variable domain of huMAb4D5); or

EVQLVESGGGLVOPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 2, which is the heavy chain variable domain of huMAb4D5).

"Biological property", as relates for example to anti-$p185^{HER2}$, for the purposes herein means an in vivo effector or antigen-binding function or activity that is directly or indirectly performed by huMAb4D5 (whether in its native or denatured conformation). Effector functions include $p185^{HER2}$ binding, any hormonal or hormonal antagonist activity, any mitogenic or agonist or antagonist activity, any

cytotoxic activity. An antigenic function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of huMAb4D5.

Biologically active huMAb4D5 is defined herein as a polypeptide that shares an effector function of huMAb4D5. A principal known effector function of huMAb4D5 is its ability to bind to $p185^{HER2}$.

Thus, the biologically active and antigenically active huMAb4D5 polypeptides that are the subject of certain embodiments of this invention include the sequence of the entire translated nucleotide sequence of huMAb4D5; mature huMAb4D5; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues comprising sequences from muMAb4D5 plus residues from the human FR of huMAb4D5; amino acid sequence variants of huMAb4D5 wherein an amino acid residue has been inserted N- or C-terminal to, or within, huMAb4D5 or its fragment as defined above; amino acid sequence variants of huMAb4D5 or its fragment as defined above wherein an amino acid residue of huMAb4D5 or its fragment as defined above has been substituted by another residue, including predetermined mutations by, e.g., site-directed or PCR mutagenesis; derivatives of huMAb4D5 or its fragments as defined above wherein huMAb4D5 or its fragments have been covalent modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety other than a naturally occurring amino acid; and glycosylation variants of huMAb4D5 (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such fragments and variants exclude any polypeptide heretofore identified, including muMAb4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S.C. 102 as well as polypeptides obvious thereover under 35 U.S.C. 103.

An "isolated" polypeptide means polypeptide which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, for example, a polypeptide product comprising huMAb4D5 will be purified from a cell culture or other synthetic environment (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a gas- or liquid-phase sequenator (such as a commercially available Applied Biosystems sequenator Model 470, 477, or 473), or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated huMAb4D5 includes huMAb4D5 in situ within recombinant cells since at least one component of the huMAb4D5 natural environment will not be present. Ordinarily, however, isolated huMAb4D5 will be prepared by at least one purification step.

In accordance with this invention, huMAb4D5 nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active huMAb4D5, is complementary to nucleic acid sequence encoding such huMAb4D5, or hybridizes to nucleic acid sequence encoding such huMAb4D5 and remains stably bound to it under stringent conditions, and comprises nucleic acid from a muMAb4D5 CDR and a human FR region.

Preferably, the huMAb4D5 nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with the huMAb4D5 amino acid sequence. Preferably, a nucleic acid molecule that hybridizes to the huMAb4D5 nucleic acid contains at least 20, more preferably 40, and most preferably 90 bases. Such hybridizing or complementary nucleic acid, however, is further defined as being novel under 35 U.S.C. 102 and unobvious under 35 U.S.C. 103 over any prior art nucleic acid.

Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% $NaDodSO_4$ at 50° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serumalbumin/0/1% Ficoll/0/1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5×Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42 C., with washes at 42 C. in 0.2×SSC and 0.1% SDS.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

An "exogenous" element is defined herein to mean nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032 published May

4, 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler et al., *Nucl. Acids Res.,* 14: 5399–5407 [1986]). They are then purified on polyacrylamide gels.

The technique of "polymerase chain reaction," or "PCR," as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued Jul. 28, 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.,* 51: 263 (1987); Erlich, ed., *PCR Technology,* (Stockton Press, N.Y., 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

Suitable Methods for Practicing the Invention

Some aspects of this invention include obtaining an import, non-human antibody variable domain, producing a desired humanized antibody sequence and for humanizing an antibody gene sequence are described below. A particularly preferred method of changing a gene sequence, such as gene conversion from a non-human or consensus sequence into a humanized nucleic acid sequence, is the cassette mutagenesis procedure described in Example 1. Additionally, methods are given for obtaining and producing antibodies generally, which apply equally to native non-human antibodies as well as to humanized antibodies.

Generally, the antibodies and antibody variable domains of this invention are conventionally prepared in recombinant cell culture, as described in more detail below. Recombinant synthesis is preferred for reasons of safety and economy, but it is known to prepare peptides by chemical synthesis and to purify them from natural sources; such preparations are included within the definition of antibodies herein.

Molecular Modeling

An integral step in our approach to antibody humanization is construction of computer graphics models of the import and humanized antibodies. These models are used to determine if the six complementarity-determining regions (CDRs) can be successfully transplanted from the import framework to a human one and to determine which framework residues from the import antibody, if any, need to be incorporated into the humanized antibody in order to maintain CDR conformation. In addition, analysis of the sequences of the import and humanized antibodies and reference to the models can help to discern which framework residues are unusual and thereby might be involved in antigen binding or maintenance of proper antibody structure.

All of the humanized antibody models of this invention are based on a single three-dimensional computer graphics structure hereafter referred to as the consensus structure. This consensus structure is a key distinction from the approach of previous workers in the field, who typically begin by selecting a human antibody structure which has an amino acid sequence which is similar to the sequence of their import antibody.

The consensus structure of one embodiment of this invention was built in five steps as described below.

Step 1

Seven Fab X-ray crystal structures from the Brookhaven Protein Data Bank were used (entries 2FB4, 2RHE, 3FAB, and 1 REI which are human structures, and 2MCP, 1 FBJ, and 2HFL which are murine structures). For each structure, protein mainchain geometry and hydrogen bonding patterns were used to assign each residue to one of three secondary structure types: alpha-helix, beta-strand or other (i.e. non-helix and non-strand). The immunoglobulin residues used in superpositioning and those included in the consensus structure are shown in Table 1.

TABLE I

	Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure							
Ig[a]	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
				$V_L\kappa$ domain				
								2–11
	18–24	18–24	19–25	18–24	19–25	19–25	19–25	16–27
	32–37	34–39	39–44	32–37	32–37	32–37	33–38	33–39
								41–49
	60–66	62–68	67–72	53–66	60–65	60–65	61–66	59–77
	69–74	71–76	76–81	69–74	69–74	69–74	70–75	
	84–88	86–90	91–95	84–88	84–88	84–88	85–89	82–91
								101–105
RMS[c]		0.40	0.60	0.53	0.54	0.48	0.50	
				V_H domain				
								3–8
	18–25		18–25	18–25	18–25	18–25		17–23
	34–39		34–39	34–39	34–39	34–39		33–41
	46–52		46–52	46–52	46–52	46–52		45–51
	57–61		59–63	56–60	57–61	57–61		57–61
	68–71		70–73	67–70	68–71	68–71		66–71
	78–84		80–86	77–83	78–84	78–84		75–82
	92–99		94–101	91–98	92–99	92–99		88–94
								102–108

TABLE I-continued

Ig[a]	Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure							
	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
RMS[c]			0.43	0.85	0.62	0.91		
RMS[d]	0.91		0.73	0.77	0.92			

[a]Four-letter code for Protein Data Bank file.
[b]Residue numbers for the crystal structures are taken from the Protein Data Bank files. Residue numbers for the consensus structure are according to Kabat et al.
[c]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2FB4.
[d]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2HFL.

Step 2

Having identified the alpha-helices and beta-strands in each of the seven structures, the structures were superimposed on one another using the INSIGHT computer program (Biosym Technologies, San Diego, Calif.) as follows: The 2FB4 structure was arbitrarily chosen as the template (or reference) structure. The 2FB4 was held fixed in space and the other six structures rotated and translated in space so that their common secondary structural elements (i.e. alpha-helices and beta-strands) were oriented such that these common elements were as close in position to one another as possible. (This superpositioning was performed using accepted mathematical formulae rather than actually physically moving the structures by hand.)

Step 3

With the seven structures thus superimposed, for each residue in the template (2FB4) Fab one calculates the distance from the template alpha-carbon atom (Cα) to the analogous Cα atom in each of the other six superimposed structures. This results in a table of Cα-Cα distances for each residue position in the sequence. Such a table is necessary in order to determine which residue positions will be included in the consensus model. Generally, is if all Cα-Cα distances for a given residue position were ≦1.0 Å, that position was included in the consensus structure. If for a given position only one Fab crystal structure was >1.0 Å, the position was included but the outlying crystal structure was not included in the next step (for this position only). In general, the seven β-strands were included in the consensus structure while some of the loops connecting the β-strands, e.g. complementarity-determining regions (CDRs), were not included in view of Cα divergence.

Step 4

For each residue which was included in the consensus structure after step 3, the average of the coordinates for individual mainchain N, Cα, C, O and Cβ atoms were calculated. Due to the averaging procedure, as well as variation in bond length, bond angle and dihedral angle among the crystal structures, this "average" structure contained some bond lengths and angles which deviated from standard geometry. For purposes of this invention, "standard geometry" is understood to include geometries commonly accepted as typical, such as the compilation of bond lengths and angles from small molecule structures in Weiner, S. J. et. al., *J. Amer. Chem. Soc.,* 106: 765–784 (1984).

Step 5

In order to correct these deviations, the final step was to subject the "average" structure to 50 cycles of energy minimization (DISCOVER program, Biosym Technologies) using the AMBER (Weiner, S. J. et. al., *J. Amer. Chem. Soc.,* 106: 765–784 (1984)) parameter set with only the Ca coordinates fixed (i.e. all other atoms are allowed to move) (energy minimization is described below). This allowed any deviant bond lengths and angles to assume a standard (chemically acceptable) geometry. See Table II.

TABLE II

Average Bond Lengths and Angles for "Average" (Before) and Energy-Minimized Consensus (After 50 Cycles) Structures

	V_LK before (Å)	V_LK after (Å)	V_H before (Å)	V_H after (Å)	Standard Geometry (Å)
N—Cα	1.459(0.012)	1.451(0.004)	1.451(0.023)	1.452(0.004)	1.449
Cα-C	1.515(0.012)	1.523(0.005)	1.507(0.033)	1.542(0.005)	1.522
O=C	1.208(0.062)	1.229(0.003)	1.160(0.177)	1.231(0.003)	1.229
C—N	1.288(0.049)	1.337(0.002)	1.282(0.065)	1.335(0.004)	1.335
Cα-Cβ	1.508(0.026)	1.530(0.002)	1.499(0.039)	1.530(0.002)	1.526
	(*)	(*)	(*)	(*)	(*)
C—N—Cα	123.5(4.2)	123.8(1.1)	125.3(4.6)	124.0(1.1)	121.9
N—Cα-C	110.0(4.0)	109.5(1.9)	110.3(2.8)	109.5(1.6)	110.1
Cα-C—N	116.6(4.0)	116.6(1.2)	117.6(5.2)	116.6(0.8)	116.6
O=C—N	123.1(4.1)	123.4(0.6)	122.2(4.9)	123.3(0.4)	122.9
N—Cα-Cβ	110.3(2.1)	109.8(0.7)	110.6(2.5)	109.8(0.6)	109.5
Cβ-Cα-C	111.4(2.4)	111.1(0.7)	111.2(2.2)	111.1(0.6)	111.1

Values in parentheses are standard deviations. Note that while some bond length and angle averages did not change appreciably after energy-minimization, the corresponding standard deviations are reduced due to deviant geometries assuming standard values after energy-minimization. Standard geometry values are from the AMBER forcefield as implemented in DISCOVER (Biosym Technologies).

The consensus structure might conceivably be dependent upon which crystal structure was chosen as the template on which the others were superimposed. As a test, the entire procedure was repeated using the crystal structure with the worst superposition versus 2FB4, i.e. the 2HFL Fab structure, as the new template (reference). The two consensus structures compare favorably (root-mean-squared deviation of 0.11 Å for all N, Cα and C atoms).

Note that the consensus structure only includes mainchain (N, Cα, C, O, Cβ atoms) coordinates for only those residues which are part of a conformation common to all seven X-ray crystal structures. For the Fab structures, these include the common β-strands (which comprise two β-sheets) and a few non-CDR loops which connect these β-strands. The consensus structure does not include CDRs or sidechains, both of which vary in their conformation among the seven structures. Also, note that the consensus structure includes only the V_L and V_H domains.

This consensus structure is used as the archetype. It is not particular to any species, and has only the basic shape without side chains. Starting with this consensus structure

the model of any import, human, or humanized Fab can be constructed as follows. Using the amino acid sequence of the particular antibody V_L and V_H domains of interest, a computer graphics program (such as INSIGHT, Biosym Technologies) is used to add sidechains and CDRs to the consensus structure. When a sidechain is added, its conformation is chosen on the basis of known Fab crystal structures (see the Background section for publications of such crystal structures) and rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193: 775–791 (1987)). The model also is constructed so that the atoms of the sidechain are positioned so as to not collide with other atoms in the Fab.

CDRs are added to the model (now having the backbone plus side chains) as follows. The size (i.e. number of amino acids) of each import CDR is compared to canonical CDR structures tabulated by Chothia et al., *Nature,* 342:877–883 (1989)) and which were derived from Fab crystals. Each CDR sequence is also reviewed for the presence or absence of certain specific amino acid residues which are identified by Chothia as structurally important: e.g. light chain residues 29 (CDR1) and 95 (CDR3), and heavy chain residues 26, 27, 29 (CDR1) and 55 (CDR2). For light chain CDR2, and heavy chain CDR3, only the size of the CDR is compared to the Chothia canonical structure. If the size and sequence (i.e. inclusion of the specific, structurally important residues as denoted by Chothia et al.) of the import CDR agrees in size and has the same structurally important residues as those of a canonical CDR, then the mainchain conformation of the import CDR in the model is taken to be the same as that of the canonical CDR. This means that the import sequence is assigned the structural configuration of the canonical CDR, which is then incorporated in the evolving model.

However, if no matching canonical CDR can be assigned for the import CDR, then one of two options can be exercised. First, using a program such as INSIGHT (Biosym Technologies), the Brookhaven Protein Data Bank can be searched for loops with a similar size to that of the import CDR and these loops can be evaluated as possible conformations for the import CDR in the model. Minimally, such loops must exhibit a conformation in which no loop atom overlaps with other protein atoms. Second, one can use available programs which calculate possible loop conformations, assuming a given loop size, using methods such as described by Bruccoleri et al., *Nature* 335: 564–568 (1988).

When all CDRs and sidechains have been added to the consensus structure to give the final model (import, human or humanized), the model is preferably subjected to energy minimization using programs which are available commercially (e.g. DISCOVER, Biosym Technologies). This technique uses complex mathematical formulae to refine the model by performing such tasks as checking that all atoms are within appropriate distances from one another and checking that bond lengths and angles are within chemically acceptable limits.

Models of a humanized, import or human antibody sequence are used in the practice of this invention to understand the impact of selected amino acid residues of the activity of the sequence being modeled. For example, such a model can show residues which may be important in antigen binding, or for maintaining the conformation of the antibody, as discussed in more detail below. Modeling can also be used to explore the potential impact of changing any amino acid residue in the antibody sequence.

Methods for Obtaining a Humanized Antibody Sequence

In the practice of this invention, the first step in humanizing an import antibody is deriving a consensus amino acid sequence into which to incorporate the import sequences. Next a model is generated for these sequences using the methods described above. In certain embodiments of this invention, the consensus human sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and have the sequences indicated in the definitions above.

While these steps may be taken in different order, typically a structure for the candidate humanized antibody is created by transferring the at least one CDR from the non-human, import sequence into the consensus human structure, after the entire corresponding human CDR has been removed. The humanized antibody may contain human replacements of the non-human import residues at positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) or as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)). For example, huMAb4D5 contains human replacements of the muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol Bol.* 196:901–917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S.

Differences between the non-human import and the human consensus framework residues are individually investigated to determine their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is desirably performed through modeling, by examination of the characteristics of the amino acids at particular locations, or determined experimentally through evaluating the effects of substitution or mutagenesis of particular amino acids.

In certain preferred embodiments of this invention, a humanized antibody is made comprising amino acid sequence of an import, non-human antibody and a human antibody, utilizing the steps of:

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus human variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least

one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, one determines if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), one may retain the consensus residue.

Additionally, in certain embodiments the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In preferred embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). if the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human sequence if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another preferred embodiment of the methods of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

In certain alternate embodiments, one need not utilize the modeling and evaluation steps described above, and may instead proceed with the steps of obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus human antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus human antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b. fin the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

Preferably, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody. If desired, one may utilize the other method steps described above for determining whether a particular amino acid residue can reasonably be expected to have undesirable effects, and remedying those effects.

If after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one preferably reexamines the potential effects of the amino acids at the specific locations recited above. Additionally, it is desirable to reinvestigate any buried residues which are reasonably expected to affect the V_L–V_H interface but may not directly affect CDR conformation. It is also desirable to reevaluate the humanized antibody utilizing the steps of the methods claimed herein.

In certain embodiments of this invention, amino acid residues in the consensus human sequence are substituted for by other amino acid residues. In preferred embodiments, residues from a particular non-human import sequence are substituted, however there are circumstances where it is desired to evaluate the effects of other amino acids. For example, if after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one may compare the sequences of other classes or subgroups of human antibodies, or classes or subgroups of antibodies from the particular non-human species, and determine which other amino acid side chains and amino acid residues are found at particular locations and substituting such other residues.

Antibodies

Certain aspects of this invention are directed to natural antibodies and to monoclonal antibodies, as illustrated in the Examples below and by antibody hybridomas deposited with the ATCC (as described below). Thus, the references throughout this description to the use of monoclonal antibodies are intended to include the use of natural or native antibodies as well as humanized and chimeric antibodies. As used herein, the term “antibody” includes the antibody variable domain and other separable antibody domains unless specifically excluded.

In accordance with certain aspects of this invention, antibodies to be humanized (import antibodies) are isolated from continuous hybrid cell lines formed by the fusion of antigen-primed immune lymphocytes with myeloma cells. in certain embodiments, the antibodies of this invention are obtained by routine screening. Polyclonal antibodies to an antigen generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the antigen and an adjuvant. It may be useful to conjugate the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic is anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

The route and schedule of the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodiesare prepared by recovering immune lymphoid cells—typically spleen cells or lymphocytes from lymph node tissue—from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, *Eur. J. Immunol.* 6:511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in vitro in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored. and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody. The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, Ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM as the case may be that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g. ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered, and optionally are conjugated to a detectable marker such as an enzyme or spin label for use in diagnostic assays of the antigen in test samples.

While routinely rodent monoclonal antibodies are used as the source of the import antibody, the invention is not limited to any species. Additionally, techniques developed for the production of chimeric antibodies (Morrison et al., *Proc. Natl. Acad. Sci.,* 81:6851 (1984); Neuberger et al., *Nature* 312:604 (1984); Takeda et al., *Nature* 314:452 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the scope of this invention.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as Fab fragments) which bypass the generation of monoclonal antibodies are encompassed within the practice of this invention. One extracts antibody-specific messenger ANA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the CDNA into a bacterial expressions system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system which contains a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional FAb fragments for those which bind the antigen. Such FAb fragments with specificity for the antigen are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

Amino Acid Sequence Variants

Amino acid sequence variants of the antibodies and polypeptides of this invention (referred to in herein as the target polypeptide) are prepared by introducing appropriate nucleotide changes into the DNA encoding the target polypeptide, or by in vitro synthesis of the desired target polypeptide. Such variants include, for example, humanized variants of non-human antibodies, as well as deletions from, or insertions or substitutions of, residues within particular amino acid sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the target polypeptide, such as changing the number or position of glycosylation sites, altering any membrane anchoring characteristics, and/or altering the intra-cellular location of the target polypeptide by inserting, deleting, or otherwise affecting any leader sequence of the native target polypeptide.

In designing amino acid sequence variants of target polypeptides, the location of the mutation site and the nature of the mutation will depend on the target polypeptide characteristics) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1–3. In certain embodiments, these choices are guided by the methods for creating humanized sequences set forth above.

A useful method for identification of certain residues or regions of the target polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesism" as described by Cunningham and Wells (*Science,* 244: 1081–1085 [1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the

expressed target polypeptide variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. In general, the location and nature of the mutation chosen will depend upon the target polypeptide characteristic to be modified.

Amino acid sequence deletions of antibodies are generally not preferred, as maintaining the generally configuration of an antibody is believed to be necessary for its activity. Any deletions will be selected so as to preserve the structure of the target antibody.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the target polypeptide sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions include the target polypeptide with an N-terminal methionyl residue, an artifact of the direct expression of target polypeptide in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the target polypeptide molecule to facilitate the secretion of the mature target polypeptide from recombinant host cells. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for *E. coli,* alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the target polypeptide include the fusion to the N- or C-terminus of the target polypeptide of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published Apr. 6, 1989.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the target polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of the target polypeptide, and sites where the amino acids found in the target polypeptide from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites for substitution are described infra, considering the effect of the substitution of the antigen binding, affinity and other characteristics of a particular target antibody.

Other sites of interest are those in which particular residues of the target polypeptides obtained from various species are identical. These positions may be important for the biological activity of the target polypeptide. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. If such substitutions result in a change in biological activity, then other changes are introduced and the products screened until the desired effect is obtained.

Substantial modifications in function or immunological identity of the target polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, lb) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gin, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of the target polypeptide that are homologous with other antibodies of the same class or subclass, or, more preferably, into the non-homologous regions of the molecule.

Any cysteine residues not involved in maintaining the proper conformation of target polypeptide also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DNA encoding amino acid sequence variants of the target polypeptide is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the target polypeptide. A particularly preferred method of gene conversion mutagenesis is described below in Example 1. These techniques may utilized target polypeptide nucleic acid (DNA or RNA), or nucleic acid complementary to the target polypeptide nucleic acid.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of target polypeptide DNA. This technique is well known in the art as described by Adelman et al., *DNA,* 2: 183 (1983). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the target polypeptide. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the target polypeptide DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci. USA,* 75: 5765 [1978]).

Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase 1, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for

synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the target polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the target polypeptide. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding target polypeptide variants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of target polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, supra, the chapter by R. Higuchi, p. 61–70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.), and 25 pmole of each oligonucleotide primer, to a final volume of 50μl. The reaction mixture is overlayed with 35 μl mineral oil. The reaction is denatured for 5 minutes at 100° C., placed briefly on ice, and then 1 μl *Thermus aquaticus* (Taq) DNA polymerase (5 units/μl, purchased from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows: 2 min. at 55° C., then 30 sec. at 72° C., then 19 cycles of the following: 30 sec. at 94° C., 30 sec. at 55° C., and 30 sec. at 72° C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50:vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene,* 34: 315 [1985]). The starting material is the plasmid (or other vector) comprising the target polypeptide DNA to be mutated. The codon(s) in the target polypeptide DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the target polypeptide DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated target polypeptide DNA sequence.

Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding the target polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(a) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector.

The target polypeptides of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. Included within the scope of this invention are target polypeptides with any native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin 11 leaders. For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

(b) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using Bacillus species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in Bacillus genomic DNA. Transfection of Bacillus with this vector results in homologous recombination with the genome and insertion of the target polypeptide DNA. However, the recovery of genomic DNA encoding the target polypeptide is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the target polypeptide DNA.

(c) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for Bacilli.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., *J. Molec. Appl. Genet.,* 1: 327 [1982]), mycophenolic acid (Mulligan et al., *Science=:* 1422 [1980]) or hygromycin (Sugden et al., *Mol. Cell. Biol.,* 5: 410–413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the target polypeptide nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants

under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the target polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the target polypeptide are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 [1980]. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the target polypeptide. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the target polypeptide, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., *Nature,* 22: 39 [1979]; Kingsman et al., *Gene,* 7: 141 [1979]; or Tschemper et al., *Gene,* 10: 157 [1980]). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics,* 5: 12 [1977]). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

(d) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the target polypeptide nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as that encoding the target polypeptide, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the target polypeptide by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native target polypeptide promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target polypeptide DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed target polypeptide as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang et al., *Nature,* 275: 615 [1978]; and Goeddel et al., *Nature,* 281: 544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.,* 8: 4057 [1980] and EP 36,776) and hybrid promoters such as the tao promoter (deBoer et al., *Proc. Natl. Acad. Sci, USA,* 80: 21–25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the target polypeptide (Siebenlist et al., *Cell,* 20: 269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the target polypeptide.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.,* 255: 2073 [1980]) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.,* 2: 149 [1968]; and Holland, *Biochemistry,* 17: 4900 [1978]), such asenolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also ate advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

Target polypeptide transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published Jul. 5, 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the target polypeptide sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that

also contains the SV40 viral origin of replication. Fiers et al., *Nature,* 273:113 (1978); Mulligan and Berg, *Science,* 209: 1422–1427 (1980); Pavlakis et al., *Proc. Natl. Acad. Sci. USA,* 78: 7398–7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., *Gene,* 18: 355–360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Gray et al., *Nature,* 29: 503–508 (1982) on expressing cDNA encoding immune interferon in monkey cells; , Reyes et al., *Nature,* 297: 598–601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, *Proc. Natl. Acad. Sci. USA,* 79: 5166–5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells, and Gorman et al., *Proc. Natl. Aced. Sci. USA,* 79: 6777–6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(e) Enhancer Element Component

Transcription of DNA encoding the target polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10–300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins et al., *Proc. Natl. Acad. Sci. USA,* 78: 993 [1981]) and 3' (Lusky et al., *Mol. Cell Bio.* 3: 1108 [1983]) to the transcription unit, within an intron (Banerji et al., *Cell,* 33: 729 [1983]) as well as within the coding sequence itself (Osborne et al., *Mol. Cell Bio.,* 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100–270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature,* 297: 17–18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the target polypeptide DNA, but is preferably located at a site 5' from the promoter.

(f) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the target polypeptide. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced is by the method of Messing et al., *Nucleic Acids Res.,* 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the target polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of the target polypeptide that have target polypeptide-like activity.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide in recombinant vertebrate cell culture are described in Gething et al., *Nature,* 293: 620–625 [1981]; Mantei et al., *Nature,* 281: 40–46 [1979]; Levinson et al.,; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the target polypeptide is pRK5 (EP pub. no. 307,247) or pSVI6B.

Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli* , Bacilli such as *B. subtilis*, Pseudomonas species such as *P. aeruginosa, Salmonella typhimurium,* or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, in vitro methods of cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for target polypeptide-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* [Beach and Nurse, *Nature,* 290: 140 (1981); EP 139,383 published May 2, 1985], Kluyveromyces hosts (U.S. Pat. No. 4,943,529) such as, e.g., *K. lactis* [Louvencourt et al., *J. Bacteriol.,* 737 (1983)], *K. fragilis, K. bulgaricus, K. thermotolerans,* and *K. marxianus,* yarrowia [EP 402,226], *Pichia pastoris* [EP 183,070; Sreekrishna et al., *J. Basic Microbiol.,* 28: 265–278 (1988)], Candida, *Trichoderma reesia* [EP 244,2341], *Neurospora crassa* [Case et al., *Proc. Natl. Acad. Sci. USA,* 76: 5259–5263 (1979)], and filamentous fungi such as, e.g, Neurospora, Penicillium, Tolypocladium [WO 91/00357 published Jan. 10, 1991], and Aspergillus hosts such as *A. nidulans* [Ballance et al., *Biochem. Biophys. Res. Commun.* 112: 284–289 (1983); Tilburn et al., *Gene,* 26: 205–221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA,* 81: 1470–1474 (1984)] and *A. niger* [Kelly and Hynes, *EMBO J.,* 4: 475–479 (1985)].

Suitable host cells for the expression of glycosylated target polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g., Luckow et al., *Bio/Technologvy* 6: 47–55 (1988); Miller et al., in *Genetic Engineering* Setlow, J. K. et a., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277–279; and Maeda et al., *Nature,* 315: 592–594 (1985). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cutures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens,* which has been previously manipulated to contain the target polypeptide DNA. During incubation of the plant cell culture with *A. tumefaciens,* the DNA encoding target polypeptide is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the target polypeptide DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al.,*J. Mol. Appl. Gen.,* 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published Jun. 21, 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.,* 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 [1980]); mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23: 243–251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HS 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.,* 383: 44–68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., supra, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene,* 23: 315 (1983) and WO 89/05859 published Jun. 29, 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30–16.37 of Sambrook et al., supra, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.,* 130: 946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA),* 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

Culturina the Host Cells

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce the target polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.,* 58: 44 (1979), Barnes and Sato, *Anal. Biochem.* 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

It is further envisioned that the target polypeptides of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the target polypeptide currently in use in the field.

For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired target polypeptide. The control element does not encode the target polypeptide of this invention, but the DNA is present in the host cell genome. One next screens for cells making the target polypeptide of this invention, or increased or decreased levels of expression, as desired.

Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA,* 77: 5201–5205 [1980]), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu et al., *Am. J. Clin. Path.,* 75: 734–738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native target polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

Purification of the Target Polypeptide

The target polypeptide preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When the target polypeptide is expressed in a recombinant cell other than one of human origin, the target polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the target polypeptide from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the target polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The target polypeptide may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the target polypeptide is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Target polypeptide variants in which residues have been deleted, inserted or substituted are recovered in the same fashion, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a target polypeptide fusion with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen (or containing antigen, where the target polypeptide is an antibody) can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-target polypeptide column can be employed to absorb the target polypeptide variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native target polypeptide may require modification to account for changes in the character of the target polypeptide or its variants upon expression in recombinant cell culture.

Covalent Modifications of Target Polypeptides

Covalent modifications of target polypeptides are included within the scope of this invention. One type of covalent modification included within the scope of this invention is a target polypeptide fragment. Target polypeptide fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length target polypeptide or variant target polypeptide. Other types of covalent modifications of the target polypeptide or fragments thereof are introduced into the molecule by reacting specific amino acid residues of the target polypeptide or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides,3-nitro-2-pyridyl disulfide, methyl2-pyridyldisulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5–7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal;

chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in so introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking target polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-target polypeptide antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis (diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modification include hydroxylation of proline and lysine, phophorylation of hydroxyl groups of seryl or threonyl resides, methylation of the α-amino groups of lysine, arginine, and histidine side chains, (T. E. Creighton, *Protein: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco, pp. 79–86 [1983]), acetylation of the N-terminal amine, and amidaatioon of any C-terminal carboxyl group.

Another type of covalent modification of the target polypeptide included within the scope of this invention comprises altering the native glucosylatuion pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native target polypeptide, and/or adding one or more glycosylation sites that are not present in the native target polypeptide.

Gylcosylation of polypeptides is typically either N-linked or O-linked refers to the attachment of the carbonhydrate moiety to the side chain of an asparagine reisdue. The tri-peptide sequences asparagine-X-resine and asparagine-X-threonine, where X is any aminoe acid except proline, are the recoginition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the target polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of hte above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or theonine resides to the native target polypeptide sequence (for O-linked glycosylation sites). For ease, the target polypeptide amino acid sequences is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that condons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of Target Polypeptide".

Another means of increasing the number of carbohydrate moieties on the target polypeptide is by chemical or enzymatic coupling glycosides to the polypeptides. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the couple mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston (*CRC Crit. Rev. Biochem., pp.* 259–306 [1981]).

Removal of carbohydrate moieties present on the native target polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al. (*Arch. Biochem. Biophys.,* 259:52 [1987]) and by Edge et al. (*Anal. Biochem.,* 118:131 [1981]). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. (*Meth. Enzymol.* 138:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al. (*J. Biol. Chem.,* 257:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of the target polypeptide comprises linking the target polypeptide to various nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the

manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The target polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate]microcapsules, respectively), in colloidal drug deliverysystems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Reminaton's Pharmaceutical Sciences,* 16th edition, Osol, A., Ed., (1980).

Target polypeptide preparations are also useful in generating antibodies, for screening for binding partners, as standards in assays for the target polypeptide (e.g. by labeling the target polypeptide for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant target polypeptide, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of the target polypeptide molecule, such as affinity for a given antigen or antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for the target polypeptide in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

Diagnostic and Related Uses of the Antibodies

The antibodies of this invention are useful in diagnostic assays for antigen expression in specific cells or tissues. The antibodies are detectably labeled and/or are immobilized on an insoluble matrix.

The antibodies of this invention find further use for the affinity purification of the antigen from recombinant cell culture or natural sources. Suitable diagnostic assays for the antigen and its antibodies depend on the particular antigen or antibody. Generally, such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of the antigen and for substances that bind the antigen, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for the antigen or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label antigen nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotintavidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalenily to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry) and U.S. Pat. No. 3,645,090 (enzymes); Hunter et al., *Nature,* 144: 945 (1962); David et al., *Biochemistry,* 13: 1014–1021 (1974); Pain et al., *J. Immunol. Methods,* 40: 219–230 (1981); and Nygren, *J. Histochem. and Cytochem.,* 30: 407–412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase.

The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan et al., "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in *Methods in in Enzymology*, ed. J. J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, N.Y., 1981), pp. 147–166. Such bonding methods are suitable for use with the antibodies and polypeptides of this invention.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Pat. No. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-

response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, the antigen or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with antibody so that binding of the antibody inhibits or potentiates the enzyme activity of the label. This method per se is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of antigen or antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-antigen monoclonal antibody as one antibody and a polyclonal anti-antigen antibody as the other is useful in testing samples for particular antigen activity.

The foregoing are merely exemplary diagnostic assays for the import and humanized antibodies of this invention. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

Immunotoxins

This invention is also directed to immunochemical derivatives of the antibodies of this invention such as immunotoxins (conjugates of the antibody and a cytotoxic moiety). Antibodies which carry the appropriate effector functions, such as with their constant domains, are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

For example, purified, sterile filtered antibodies are optionally conjugated to a cytotoxin such as ricin for use in AIDS therapy. U.S. patent application Ser. No. 07/350,895 illustrates methods for making and using immunotoxins for the treatment of HIV infection. The methods of this invention, for example, are suitable for obtaining humanized antibodies for use as immunotoxins for use in AIDS therapy.

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCI, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Immunotoxins can be made in a variety of ways, as discussed herein. Commonly known crosslinking reagents can be used to yield stable conjugates.

Advantageously, monoclonal antibodies specifically binding the domain of the antigen which is exposed on the infected cell surface, are conjugated to ricin A chain. Most advantageously the ricin A chain is deglycosylated and produced through recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta et al., *Science* 238:1098 (1987).

When used to kill infected human cells in vitro for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for in vitro use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques.

Cytotoxic radiopharmaceuticals for treating infected cells may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the antibodies. Advantageously alpha particle-emitting isotopes are used. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

In a preferred embodiment, ricin A chain is deglycosylated or produced without oligosaccharides, to decrease its clearance by irrelevant clearance mechanisms (e.g., the liver). In another embodiment, whole ricin (A chain plus B chain) is conjugated to antibody if the galactose binding property of B-chain can be blocked ("blocked ricin").

In a further embodiment toxin-conjugates are made with Fab or $F(ab')_2$ fragments. Because of their relatively small size these fragments can better penetrate tissue to reach infected cells.

In another embodiment, fusogenic liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding the particular antigen.

Antibody Dependent Cellular Cytotoxicity

Certain aspects of this invention involve antibodies which are (a) directed against a particular antigen and (b) belong to a subclass or isotype that is capable of mediating the lysis of cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with cell surface proteins, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.

Biological activity of antibodies is known to be determined, to a large extent, by the constant domains or Fc region of the antibody molecule (Uananue and Benacerraf,

Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect, as do antibodies from the same subclass but different species; according to the present invention, antibodies of those classes having the desired biological activity are prepared. Preparation of these antibodies involves the selection of antibody constant domains are their incorporation in the humanized antibody by known technique. For example, mouse immunoglobulins of the IgG3 and lgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen, and therefore humanized antibodies which incorporate IgG3 and lgG2a effector functions are desirable for certain therapeutic applications.

In general, mouse antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, IgG2a, and IgM subclasses bind and activate serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the target cell by complement activation and/or AOCC can be assayed. The cells of interest are grown and labeled in vitro; the antibody is added to the cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The antibody that is capable of activating complement or mediating ADCC in the in vitro test can then be used therapeutically in that particular patient.

This invention specifically encompasses consensus Fc antibody domains prepared and used according to the teachings of this invention.

Therapeutic and Other Uses of the Antibodies

When used in vivo for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that have desired therapeutic effect). They will normally be administered parenterally. The dose and dosage regimen will depend upon the degree of the infection, the characteristics of the particular antibody or immunotoxin used, e.g., its therapeutic index, the patient, and the patient's history. Advantageously the antibody or immunotoxin is administered continuously over a period of 1–2 weeks, intravenously to treat cells in the vasculature and subcutaneously and intraperitoneally to treat regional lymph nodes. Optionally, the administration is made during the course of adjunct therapy such as combined cycles of radiation, chemotherapeutic treatment, or administration of tumor necrosis factor, interferon or other cytoprotective or immunomodulatory agent.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate can also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

Use of IgM antibodies may be preferred for certain applications, however IgG molecules by being smaller may be more able than IgM molecules to localize to certain types of infected cells.

There is evidence that complement activation in vivo-leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uananue and Benecerraf, *Textbook of Immunology,* 2nd Edition, Williams & Wilkins, p. 218 (1984)). The increased vasodilation accompanying inflammation may increase the ability of various agents to localize in infected cells. Therefore, antigen-antibody combinations of the type specified by this invention can be used therapeutically in many ways. Additionally, purified antigens (Hakomori, *Ann. Rev. Immunol.* 2:103 (1984)) or anti-idiotypic antibodies (Nepom et al., *Proc. Natl. Acad. Sci.* 81:2864 (1985); Koprowski et al., *Proc. Natl. Acad. Sci.* 81:216 (1984)) relating to such antigens could be used to induce an active immune response in human patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause infected cell destruction.

Optionally, the antibodies of this invention are useful in passively immunizing patients, as exemplified by the administration of humanized anti-HIV antibodies.

The antibody compositions used in therapy are formulated and dosages established in a fashion consistent with good medical practice taking into account the disorder to be treated, the condition of the individual patient, the site of delivery of the composition, the method of administration and other factors known to practitioners. The antibody compositions are prepared for administration according to the description of preparation of polypeptides for administration, infra.

Deposit of Materials

As described above, cultures of the muMAb4D5 have been deposited with the American Type Culture Collection, 10801 University Blvd., Mauassas, Va., USA (ATCC).

This deposit was made under the provisions of the Budapest Treaty on the international Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of the deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures' availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed

when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the constructs deposited, since the deposited embodiments are intended to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

EXAMPLES

Example 1

Humanization of muMAb4D5

Here we report the chimerization of muMAb4D5 (chMAb4D5) and the rapid and simultaneous humanization of heavy (V_H) and light (V_L) chain variable region genes using a novel "gene conversion mutagenesis" strategy. Eight humanized variants (huMAb4D5) were constructed to probe the importance of several FR residues identified by our molecular modeling or previously proposed to be critical to the conformation of particular CDRs (see Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987); Chothia, C. et al., *Nature* 342:877–883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). Efficient transient expression of humanized variants in non-myeloma cells allowed us to rapidly investigate the relationship between binding affinity for $p185^{HER2}$ ECD and anti-proliferative activity against $p185^{HER2}$ overexpressing carcinoma cells.

Materials and Methods

Cloning of Variable Region Genes. The muMAb4D5 V_H and V_L genes were isolated by polymerase chain reaction (PCR) amplification of mRNA from the corresponding hybridoma (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) as described by Orlandi et al. (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989)). Amino terminal sequencing of muMAb4D5 V_L and V_H was used to design the sense strand PCR primers, whereas the anti-sense PCR primers were based upon consensus sequences of murine framework residues (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989); Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) incorporating restriction sites for directional cloning shown by underlining and listed after the sequences: V_L sense, 5'-TCC GATATCCAGCTGACCCAGTCTCCA-3' (SEQ. ID NO. 7), EcoRV; V_L anti-sense, 5'-GTTTGATCTCCAGCTT GGTACCHSCDCCGAA-3' (SEQ. ID NO. 8), Asp718; V_H sense, 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ. ID NO. 9), PstI and V_H anti-sense, 5'-TGAGGAGAC GGTGACCGTGGTCCCTTGGCCCCAG-3' (SEQ. ID. NO. 10), BstEII; where H=A or C or T, S=C or G, D=A or G or T, M=A or C, R=A or G and W=A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) and five clones for each variable domain sequenced by the dideoxy method (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)).

Molecular Modelling. Models for muMAb4D5 V_H and V_L domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1FB4, 2RHE, 2MCP, 3FAB, 1FBJ, 2HFL and 1REI). The Fab fragment KOL (Marquart, M. et al., *J. Mol. Biol.* 141:369–391 (1980)) was first chosen as a template for V_L and V_H domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Siosym Technologies). The distance from the template Cα to the analogous Cα in each of the superimposed structures was calculated for each residue position. If all (or nearly all) Cα—Cα distances for a given residue were ≦1 Å, then that position was included in the consensus structure. In most cases the β-sheet framework residues satisfied these criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, Cα, C, O and Cβ atoms were calculated and then corrected for resultant deviations from non-standard bond geometry by 50 cycles of energy minimization using the DISCOVER program (Biosym Technologies) with the AMBER forcefield (Weiner, S. J. et al., *J. Amer. Chem. Soc.* 106:765–784 (1984)) and Cα coordinates fixed. The side chains of highly conserved residues, such as the disulfide-bridged cysteine residues, were then incorporated into the resultant consensus structure. Next the sequences of muMAb4D5 V_L and V_H were incorporated starting with the CDR residues and using the tabulations of CDR conformations from Chothia et al. (Chothia, C. et al., *Nature* 342:877–883 (1989)) as a guide. Side-chain conformations were chosen on the basis of Fab crystal structures, rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193:775–791 (1987)) and packing considerations. Since V_H-COR3 could not be assigned a definite backbone conformation from these criteria, two models were created from a search of similar sized loops using the INSIGHT program. A third model was derived using packing and solvent exposure considerations. Each model was then subjected to 5000 cycles of energy minimization.

In humanizing muMAb4D5, consensus human sequences were first derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and a molecular model generated for these sequences using the methods described above. A structure for huMAb4D5 was created by transferring the CDRs from the muMAb4D5 model into the consensus human structure. All huMAb4D5 variants contain human replacements of muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but notas defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)):

V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S. Differences between muMAb4D5 and the human consensus framework residues (FIG. **1**) were individually modeled to investigate their possible influence on CDR conformation and/or binding to the $p185^{HER2}$ ECD.

Construction of Chimeric Genes. Genes encoding chMAb4D5 light and heavy chains were separately assembled in previously described phagemid vectors containing ihe human cytomegalovirus enhancer and promoter, a 5' intron and SV40 polyadenylation signal (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990)). Briefly, gene segments encoding muMAb4D5 V_L (FIG. **1**A) and REI human κ_1 light chain C_L (Palm, W. & Hilschmann, N., *Z. Physiol. Chem.* 356:167–191 (1975)) were precisely joined as were genes for muMAb4D5 V_H (FIG. **1**B) and human γ1 constant region (Capon, D. J. et al., *Nature* 337:525–531 (1989)) by simple subcloning (Boyle, A., in *Current Protocols in Molecular Biology,* Chapter 3 (F. A. Ausubel et al., eds., Greene Publishing & Wiley-Interscience, New York, 1990)) and site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The γ1 isotype was chosen as it has been found to be the preferred human isotype for supporting ADCC and complement dependent cytotoxicity using matched sets of chimeric (Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987)) or humanized antibodies (Riechmann, L. et al., *Nature* 332:323–327 (1988)). The PCR-generated V_L and V_H fragments (FIG. **1**) were subsequently mutagenized so that they faithfully represent the sequence of muMAb4D5 determined at the protein level: V_H Q1E, V_L V_{104}L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ1 constant regions are identical to those reported by Ellison et al. (Ellison, J. W. et al., *Nucleic Acids Res.* 13:4071–4079 (1982)) except for the mutations E359D and M361L (Eu numbering, as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) which we installed to convert the antibody from the naturally rare A allotype to the much more common non-A allotype (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). This was an attempt to reduce the risk of anti-allotype antibodies interfering with therapy.

Construction of Humanized Genes. Genes encoding chMAb4D5 light chain and heavy chain Fd fragment (V_H and C_H1 domains) were subcloned together into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) to create pAK1 and simultaneously humanized in a single step (FIG. **2**). Briefly, sets of 6 contiguous oligonucleotides were designed to humanize V_H and V_L (FIG. **1**). These oligonucleotides are 28 to 83 nucleotides in length, contain zero to 19 mismatches to the murine antibody template and are constrained to have 8 or 9 perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of V_H and V_L humanization oligonucleotides (5 pmol each) were phosphorylated with either ATP or γ-^{32}P-ATP (Carter, P. *Methods Enzymol.* 154: 382–403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μl 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ by cooling from 100° C. to room temperature over ~30 min. The annealed oligonucleotides were joined by incubation with T4 DNA ligase (12 units; New England Biolabs) in the presence of 2 μl 5 mM ATP and 2 μl 0.1 M DTT for 10 min at 14° C. After electrophoresis on a 6% acrylamide sequencing gel the assembled oligonucleotides were located by autoradiography and recovered by electroelution. The assembled oligonucleotides (~0.3 pmol each) were simultaneously annealed to 0.15 pmol single-stranded deoxyuridine-containing pAK1 prepared according to Kunkel et al. (Kunkel, T. A. et al., *Methods Enzymol.* 154:367–382 (1987)) in 10 μl 40 mM Tris-HCl (pH 7.5) and 16 mM $MgCl_2$ as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into *E. coli* BMH 71–18 mutL as previously described (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for huV_L by restriction purification using XhoI and then for huV_H by restriction selection using StuI as described in Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991); and in Wells, J. A. et al., *Phil. Trans. R. Soc. Lond., A* 317:415–423 (1986). Resultant clones containing both huV_L and huV_H genes were identified by nucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)) and designated pAK2. Additional humanized variants were generated by site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The muMAb4D5 V_L and V_H gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

Expression and Purification of MAb4D5 Variants. Appropriate MAb4D5 light and heavy chain cDNA expression vectors were co-transfected into an adenovirus transformed human embryonic kidney cell line, 293 (Graham, F. L. et al., *J. Gen. Virol.* 36:59–72 (1977)) using a high efficiency procedure (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990); Gorman, C., in *DNA Cloning,* vol II, pp 143–190 (D. M. Glover, ed., IRL Press, Oxford, UK 1985)). Media were harvested daily for up to 5 days and the cells re-fed with serum free media. Antibodies were recovered from the media and affinity purified on protein A sepharose CL-4B (Pharmacia) as described by the manufacturer. The eluted antibody was buffer-exchanged into phosphate-buffered saline by G25 gel filtration, concentrated by ultrafiltration (Centriprep-30 or Centricon-100, Amicon), sterile-filtered (Millex-GV, Millipore) and stored at 4° C. The concentration of antibody was determined by using both total immunoglobulin and antigen binding ELISAs. The standard used was huMAb4D5-5, whose concentration had been determined by amino acid composition analysis.

Cell Proliferation Assay. The effect of MAb4D5 variants upon proliferation of the human mammary adenocarcinoma cell line, SK-BR-3, was investigated as previously described (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) using saturating MAb4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAb4D5 variants was determined using a secreted form of the $p185^{HER2}$ ECD prepared as described in Fendly, B. M. et al., *J. Biol. Resp. Mod.* 9:449–455 (1990). Briefly, antibody and $p185^{HER2}$ ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized $p185^{HER2}$ ECD and used to calculate affinity (K_d) according to Friguet et al. (Friguet, B. et al., *J. Immunol. Methods* 77:305–319 (1985)).

Results

Humanization of muMAb4D5. The muMAb4D5 V_L and V_H gene segments were first cloned by PCR and sequenced (FIG. **1**). The variable genes were then simultaneously humanized by gene conversion mutagenesis using preassembled oligonucleotides (FIG. **2**). A 311-mer oligonucle-

otide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAb4D5 V_L. Humanization of muMAb4D5 V_H required 32 amino acid changes which were installed with a 361-mer containing 59 mismatches to the muMAb4D5 template. Two out of 8 clones sequenced precisely encode huMAb4D5-5, although one of these clones contained a single nucleotide imperfection. The 6 other clones were essentially humanized but contained a small number of errors: <3 nucleotide changes and <1 single nucleotide deletion per kilobase. Additional humanized variants (Table 3) were constructed by site-directed mutagenesis of huMAb4D5-5.

Expression levels of huMAb4D5 variants were in the range of 7 to 15 μg/ml as judged by ELISA using immobilized $p185^{HER2}$ ECD. Successive harvests of five 10 cm plates allowed 200 μg to 500 mg of each variant to be produced in a week. Antibodies affinity purified on protein A gave a single band on a Coomassie blue stained SDS polyacrylamide gel of mobility consistent with the expected M_r of ~150 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M_r of free heavy (48 kDa) and light (23 kDa) chains (not shown). Amino terminal sequence analysis (10-cycles) gave the mixed sequence expected (see FIG. **1**) from an equimolar combination of light and heavy chains (not shown).

huMAb4D5 Variants. In general, the FR residues were chosen from consensus human sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) and CDR residues from muMAb4D5. Additional variants were constructed by replacing selected human residues in huMAb4D5-1 with their muMAb4D5 counterparts. These are V_H residues 71, 73, 78, 93 plus 102 and V_L residues 55 plus 66 identified by our molecular modeling. V_H residue 71 has previously been proposed by others (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) to be critical to the conformation of V_H-CDR2. Amino acid sequence differences between huMAb4D5 variant molecules are shown in Table 3, together with their $p185^{HER2}$ ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K_d values were obtained for binding of MAb4D5 variants to either SK-BR-3 cells or to $p185^{HER2}$ ECD (Table 3). However, K_d estimates derived from binding of MAb4D5 variants to $p185^{HER2}$ ECD were more reproducible with smaller standard errors and consumed much smaller quantities of antibody than binding measurements with whole cells.

The most potent humanized variant designed by molecular modeling, huMAb4D5-8, contains 5 FR residues from muMAb4D5. This antibody binds the $p185^{HER2}$ ECD 3-fold more tightly than does muMAb4D5 itself (Table 3) and has comparable anti-proliferative activity with SK-BR-3 cells (FIG. **3**). In contrast, huMAb4D5-1 is the most humanized but least potent muMAb4D5 variant, created by simply installing the muMAb4D5 CDRs into the consensus human sequences. huMAb4D5-1 binds the $p185^{HER2}$ ECD 80-fold less tightly than does the murine antibody and has no detectable anti-proliferative activity at the highest antibody concentration investigated (16 μg/ml).

The anti-proliferative activity of huMAb4D5 variants against $p185^{HER2}$ overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the $p185^{HER2}$ ECD. For example, installation of three murine residues into the V_H domain of huMAb4D5-2 (D73T, L78A and A93S) to create huMAb4D5-3 does not change the antigen binding affinity but does confer significant anti-proliferative activity (Table 3).

The importance of V_H residue 71 (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) is supported by the observed 5-fold increase in affinity for $p185^{HER2}$ ECD on replacement of R71 in huMAb4D5-1 with the corresponding murine residue, alanine (huMAb4D5-2). In contrast, replacing V_H L78 in huMAb4D5-4 with the murine residue, alanine (huMAb4D5-5), does not significantly change the affinity for the $p185^{HER2}$ ECD or change anti-proliferative activity, suggesting that residue 78 is not of critical functional significance to huMAb4D5 and its ability to interact properly with the extracellular domain of $p185^{HER2}$.

V_L residue 66 is usually a glycine in human and murine κ chain sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but an arginine occupies this position in the muMAb4D5 κ light chain. The side chain of residue 66 is likely to affect the conformation of V_L-CDR1 and V_L-CDR2 and the hairpin turn at 68–69 (FIG. **4**). Consistent with the importance of this residue, the mutation V_L G66R (huMAb4D5-3→huMAb4D5-5) increases the affinity for the $p185^{HER2}$ ECD by 4-fold with a concomitant increase in anti-proliferative activity.

From molecular modeling it appears that the tyrosyl side chain of muMAb4D5 V_L residue 55 may either stabilize the conformation of V_H-CDR3 or provide an interaction at the V_L–V_H interface. The latter function may be dependent upon the presence of V_H Y102. In the context of huMAb4D5-5 the mutations V_L E55Y (huMAb4D5-6) and V_H V102Y (huMAb4D5-7) individually increase the affinity for $p185^{HER2}$ ECD by 5-fold and 2-fold respectively, whereas together (huMAb4D5-8) they increase the affinity by 11-fold. This is consistent with either proposed role of V_L Y55 and V_H Y102.

Secondary Immune Function of huMAb4D5-8. MuMAb4D5 inhibits the growth of human breast tumor cells which overexpress $p185^{HER2}$ (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)). The antibody, however, does not offer the possibility of direct tumor cytotoxic effects. This possibility does arise in huMAb4D5-8 as a result of its high affinity (Kd_d=0.1 μM) and its human IgG_1 subtype. Table 4 compares the ADCC mediated by huMAb4D5-8 with muMAb4D5 on a normal lung epithelial cell line, WI-38, which expresses a low level of $p185^{HER2}$ and on SK-BR-3, which expresses a high level of $p185^{HER2}$. The results demonstrate that: (1) huMAb4D5 has a greatly enhanced ability to carry out ADCC as compared with its murine parent; and (2) that this activity may be selective for cell types which overexpress $p185^{HER2}$.

Discussion

MuMAb4D5 is potentially useful for human therapy since it is cytostatic towards human breast and ovarian tumor lines overexpressing the HER2-encoded $p185^{HER2}$ receptor-like tyrosine kinase. Since both breast and ovarian carcinomas are chronic diseases it is anticipated that the optimal MAb4D5 variant molecule for therapy will have low immunogenicity and will be cytotoxic rather than solely cytostatic in effect. Humanization of muMAb4D5 should accomplish these goals. We have identified 5 different huMAb4D5 variants which bind tightly to $p185^{HER2}$ ECD ($K_d \leqq 1$ nM) and which have significant anti-proliferative activity (Table 3). Furthermore huMAb4D5-8 but not muMAb4D5 mediates ADCC against human tumor cell lines overexpressing $p185^{HER2}$ in the presence of human effector cells (Table 4) as anticipated for a human γ1 isotype (Brcüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988)).

Rapid humanization of huMAb4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler and more reliable than a variant protocol recently reported (Rostapshov, V. M. et al., *FEBS Lett.* 249: 379–382 (1989)). Transient expression of huMAb4D5 in human embryonic kidney 293 cells permitted the isolation of a few hundred micrograms of huMAb4D5 variants for rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, different combinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

The crucial role of molecular modeling in the humanization of muMAb4D5 is illustrated by the designed variant huMAb4D5-8 which binds the $p185^{HER2}$ ECD 250-fold more tightly than the simple CDR loop swap variant, huMAb4D5-1. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)). Here we have extended this earlier work by others with a designed humanized antibody which binds its antigen 3-fold more tightly than the parent rodent antibody. While this result is gratifying, assessment of the success of the molecular modeling must await the outcome of X-ray structure determination. From analysis of huMAb4D5 variants (Table 3) it is apparent that their anti-proliferative activity is not a simple function of their binding affinity for $p185^{HER2}$ ECD. For example the huMAb4D5–8 variant binds $p185^{HER2}$ 3-fold more tightly than muMAb4D5 but the humanized variant is slightly less potent in blocking the proliferation of SK-SR-3 cells. Additional huMAb4D5 variants are currently being constructed in an attempt to identify residues triggering the anti-proliferative activity and in an attempt to enhance this activity.

In addition to retaining tight receptor binding and the ability to inhibit cell growth, the huMAb4D5-8 also confers a secondary immune function (ADCC). This allows for direct cytotoxic activity of the humanized molecule in the presence of human effector cells. The apparent selectivity of the cytotoxic activity for cell types which overexpress $p185^{HER2}$ allows for the evolution of a straightforward clinic approach to those human cancers characterized by overexpression of the HER2 protooncogene.

TABLE 3

$p185^{HER2}$ ECD binding affinity and anti-proliferative activities of MAb4D5 variants

	V_H Residue*					V_L Residue*			
MAb4D5 cell Variant proliferation‡	71 FR3	73 FR3	78 FR3	93 FR3	102 CDR3	55 CDR2	56 FR3	K_d† nM	Relative
huMAb4D5-1	R	D	L	A	V	E	G	25	102
huMAb4D5-2	Ala	D	L	A	V	E	G	4.7	101
huMAb4D5-3	Ala	Thr	Ala	Ser	V	E	G	4.4	66
huMAb4D5-4	Ala	Thr	L	Ser	V	E	Arg	0.82	56
huMAb4D5-5	Ala	Thre	Ala	Ser	V	E	Arg	1.1	48
huMAb4D5-6	Ala	Thr	Ala	Ser	V	Tyr	Arg	0.22	51
huMAb4D5-7	Ala	Thr	Ala	Ser	Tyr	E	Arg	0.62	53
huMAb4D5-8	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.10	54
muMAb4D5	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.30	37

*Human and murine residues are shown in one letter and three letter amino acid code respectively.
†K_d values for the $p185^{HER2}$ ECD were determined using the method of Friguet et al. (43) and the standard error of each estimate is ≦ ± 10%.
‡Proliferation of SK-BR-3 cells incubated for 96 hr with MAb4D5 variants shown as a percentage of the untreated control as described (Hudziak, R. M. et al., Molec. Cell. Biol. 9: 1165–1172 (1989)). Data represent the maximal anti-proliferative effect for each variant (see FIG. 3A) calculated as the mean of triplicate determinations at a MAb4D5 concentration of 8 μg/ml. Data are all taken from the same experiment with an estimated standard error of ≦ ± 15%.

TABLE 4

Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8

Effector:Target ratio†	WI-38*		SK-BR-3	
	muMAb4D5	huMAb4D5-8	muMAb4D5	huMAb4D5-8
A.‡				
25:1	<1.0	9.3	7.5	40.6
12.5:1	<1.0	11.1	4.7	36.8
6.25:1	<1.0	8.9	0.9	35.2
3.13:1	<1.0	8.5	4.6	19.6
B.				
25:1	<1.0	3.1	6.1	33.4
12.5:1	<1.0	1.7	5.5	26.2
6.25:1	1.3	2.2	2.0	21.0
3.13:1	<1.0	0.8	2.4	13.4

*Sensitivity to ADCC of two human cell lines (WI-38, normal lung epithelium; and SK-BR-3, human breast tumor cell line) are compared. WI-38 expresses a low level of $p185^{HER2}$ (0.6 pg per μg cell protein) and SK-BR-3 expresses a high level of $p185^{HER2}$ (64 pg $p185^{HER2}$ per μg cell protein), as determined by ELISA (Fendly et al., J. Biol. Resp. Mod. 9:449–455 (1990)).
†ADCC assays were carried out as described in Brüggemann et al., J. Exp. Med. 166:1351–1361 (1987). Effector to target ratios were of IL-2 activated human peripheral blood lymphocytes to either WI-38 fibroblasts or SK-BR-3 tumor cells in 96-well microtiter plates for 4 hours at 37° C. Values given represent percent specific cell lysis as determined by ^{51}Cr release. Estimated standard error in these quadruplicate determinations was ≦±10%.
‡Monoclonal antibody concentrations used were 0.1 μg/ml (A) and 0.1 μg/ml (B).

Example 2
Schematic Method for Humanizing an Antibody Sequence

This example illustrates one stepwise elaboration of the methods for creating a humanized sequence described

above. It will be understood that not all of these steps are essential to the claimed invention, and that steps may be taken in different order.

1. ascertain a consensus human variable domain amino acid sequence and prepare from it a consensus structural model.
2. prepare model of import (the non-human domain to be humanized) variable domain sequences and note structural differences with respect to consensus human model.
3. identify CDR sequences in human and in import, both by using Kabat (supra, 1987) and crystal structure criteria. If there is any difference in CDR identity from the different criteria, use of crystal structure definition of the CDR, but retain the Kabat residues as important framework residues to import.
4. substitute import CDR sequences for human CDR sequences to obtain initial "humanized" sequence.
5. compare import non-CDR variable domain sequence to the humanized sequence and note divergences.
6. Proceed through the following analysis for each amino acid residue where the import diverges from the humanized.
   a. If the humanized residue represents a residue which is generally highly conserved across all species, use the residue in the humanized sequence. If the residue is not conserved across all species, proceed with the analysis described in 6b.
   b. If the residue is not generally conserved across all species, ask if the residue is generally conserved in humans.
      i. If the residue is generally conserved in humans but the import residue differs, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, leave the humanized residue unchanged.
      ii. If the residue is also not generally conserved in humans, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs be considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, proceed to the next step.
         a) Examine the structural models of the import and human sequences and determine if the residue is exposed on the surface of the domain or is buried within. If the residue is exposed, use the residue in the humanized sequence. If the residue is buried, proceed to the next step.
            (i) Examine the structural models of the import and human sequences and determine if the residue is likely to affect the V_L–V_H interface. Residues involved with the interface include: 34L, 36L, 38L, 43L, 33L, 36L, 85L, 87L, 89L, 91L, 96L, 98L, 35H, 37H, 39H, 43H, 45H, 47H, 60H, 91H, 93H, 95H, 100H, and 103H. If no effect is likely, use the residue in the humanized sequence. If some affect is likely, substitute the import residue.
7. Search the import sequence, the consensus sequence and the humanized sequence for glycosylation sites outside the CDRs, and determine if this glycosylation site is likely to have any affect on antigen binding and/or biological activity. If no effect is likely, use the human sequence at that site; if some affect is likely, eliminate the glycosylation site or use the import sequence at that site.
8. After completing the above analysis, determine the planned humanized sequence and prepare and test a sample. If the sample does not bind well to the target antigen, examine the particular residues listed below, regardless of the question of residue identity between the import and humanized residues.
   a. Examine particular peripheral (non-CDR) variable domain residues that may, due to their position, possibly interact directly with a macromolecular antigen, including the following residues (where the * indicates residues which have been found to interact with antigen based on crystal structures):
      i. Variable light domain: 36, 46, 49*, 63–70
      ii. Variable heavy domain: 2, 47*, 68, 70, 73–76.
   b. Examine particular variable domain residues which could interact with, or otherwise affect, the conformation of variable domain CDRs, including the following (not including CDR residues themselves, since it is assumed that, because the CDRs interact with one another, any residue in one CDR could potentially affect the conformation of another CDR residue) (L=LIGHT, H=HEAVY, residues appearing in bold are indicated to be structurally important according the Chothia et al., Nature 342:877 (1989), and residues appearing in italic were altered during humanization by Queen et al. (PDL), Proc. Natl. Acad. Sci. USA 86:10029 (1989) and Proc. Natl. Acad. Sci. USA 88:2869 (1991).):
      i. Variable light domain:
         a) CDR-1 (residues 24L–34L): 2L, 4L, 66L-69L, 71L
         b) CDR-2 (residues 50L–56L): 35L, 46L, 47L, 48L, 49L, 58L, 62L, 64L–66L, 71L, 73L
         c) CDR-3 (residues 89L–97L): 2L, 4L, 36L, 98L, 37H, 45H, 47H, 58H, 60H
      ii. Variable heavy domain:
         a) CDR-1 (residues 26H–35H): 2H, 4H, 24H, 36H, 71H, 73H, 76H, 78H, 92H, 94H
         b) CDR-2 (residues 50H–55H): 49H, 69H, 69H, 71H, 73H, 78H
         c) CDR-3 (residues 95H–102H): examine all residues as possible interaction partners with this loop, because this loop varies in size and conformation much more than the other CDRs.
9. If after step 8 the humanized variable domain still is lacking in desired binding, repeat step 8. In addition, re-investigate any buried residues which might affect the V_L–V_H interface (but which would not directly affect CDR conformation). Additionally, evaluate the accessibility of non-CDR residues to solvent.

Example 3

Engineering a Humanized Bisnecific F(ab')$_2$ Fragment

This example demonstrates the construction of a humanized bispecific antibody (BsF(ab')$_2$v1 by separate *E. coli* expression of each Fab' arm followed by directed chemical coupling in vitro. BsF(ab')$_2$v1 (anti-CD3/anti-p185^{HER2}) was demonstrated to retarget the cytotoxic activity of human

CD3+CTL in vitro against the human breast tumor cell line, SK-BR-3, which overexpresses the p185^{HER2} product of the protooncogene HER2. This example demonstrates the minimalistic humanization strategy of installing as few murine residues as possible into a human antibody in order to recruit antigen-binding affinity and biological properties comparable to that of the murine parent antibody. This strategy proved very successful for the anti-p185^{HER2}arm of BsF(ab')$_2$v1. In contrast BsF(ab')$_2$ v1 binds to T cells via its anti-CD3 arm much less efficiently than does the chimeric BsF(ab')$_2$ which contains the variable domains of the murine parent anti-CD3 antibody. Here we have constructed additional BsF(ab')$_2$ fragments containing variant anti-CD3 arms with selected murine residues restored in an attempt to improve antibody binding to T cells. One such variant, Ss F(ab')$_2$v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of BsF(ab')$_2$v1 with their counterparts from the murine parent anti-CD3 antibody. BsF(ab')$_2$v9 binds to T cells (Jurkat) much more efficiently than does BsF(ab')$_2$v1 and almost as efficiently as the chimeric BsF(ab')$_2$. This improvement in the efficiency of T cell binding of the humanized BsF(ab')$_2$ is an important step in its development as a potential therapeutic agent for the treatment of p185^{HER2}-overexpressing cancers.

Bispecific antibodies (BsAbs) with specificities for tumor-associated antigens and surface markers on immune effector cells have proved effective for retargeting effector cells to kill tumor targets both in vitro and in vivo (reviewed by Fanger, M. W. et al., *Immunol. Today* 10: 92–99 (1989); Fanger, M. W. et al., *Immunol. Today* 12: 51–54 (1991); and Nelson, H., *Cancer Cells* 3: 163–172 (1991)). BsF(ab')$_2$ fragments have often been used in preference to intact BsAbs in retargeted cellular cytotoxicity to avoid the risk of killing innocent bystander cells binding to the Fc region of the antibody. An additional advantage of BsF(ab')$_2$ over intact BsAbs is that they are generally much simpler to prepare free of contaminating monospecific molecules (reviewed by Songsivilai, S. and Lachmann, P. J., *Clin. Exp. Immunol.* 79: 315–321 (1990) and Nolan, O. and O'Kennedy, R., *Biochim. Biophys. Acta* 1040: 1–11 (1990)).

BsF(ab')$_2$ fragments are traditionally constructed by directed chemical coupling of Fab' fragments obtained by limited proteolysis plus mild reduction of the parent rodent monoclonal Ab (Brennan, M. et al., *Science* 229, 81–83 (1985) and Glennie, M. J. et al., *J. Immunol.* 139: 2367–2375 (1987)). One such BsF(ab')$_2$ fragment (anti-glioma associated antigen/anti-CD3) was found to have clinical efficacy in glioma patients (Nitta, T. et al., *Lancet* 335: 368–371 (1990) and another BsF(ab')$_2$ (anti-indium chelate/anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (Stickney, D. R. et al., *Antibody, Immunoconj. Radiopharm.* 2: 1–13 (1989)). Future SsF(ab')$_2$ destined for clinical applications are likely to be constructed from antibodies which are either human or at least "humanized" (Riechmann, L. et al., *Nature* 332: 323–327 (1988) to reduce their immunogenicity (Hale, G. et al., *Lancet i:* 1394–1399 (1988)).

Recently a facile route to a fully humanized BsF(ab')$_2$ fragment designed for tumor immunotherapy has been demonstrated (Shalaby, M. R. et al., *J. Exp. Med.* 175: 217–225 (1992)). This approach involves separate *E. coli* expression of each Fab' arm followed by traditional directed chemical coupling in vitro to form the BsF(ab')$_2$. One arm of the BsF(ab')$_2$ was a humanized version (Carter, P. et al., *Proc. Natl. Aced. Sci. USA* (1992a) and Carter, P., et al., *Bio/Technology* 10: 163–167 (1992b)) of the murine monoclonal Ab 4D5 which is directed against the p185^{HER2} product of the protooncogene HER2 (c-erbB-2) (Fendly, B. M. et al. *Cancer Res.* 50: 1550–1558 (1989)). The humanization of the antibody 4D5 is shown in Example 1 of this application. The second arm was a minimalistically humanized anti-CD3 antibody (Shalaby et al. supra) which was created by installing the CDR loops from the variable domains of the murine parent monoclonal Ab UCHT1 (Beverley, P. C. L. and Callard, R. E., *Eur. J. Immunol.* 11: 329–334 (1981)) into the humanized anti-p185^{HER2} antibody. The BsF(ab')$_2$ fragment containing the most potent humanized anti-CD3 variant (v1) was demonstrated by flow cytometry to bind specifically to a tumor target overexpressing p185^{HER2} and to human peripheral blood mononuclear cells carrying CD3. In addition, BsF(ab')$_2$v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-SR-3 tumor cells overexpressing p185^{HER2}. The example descries efforts to improve the antigen binding affinity of the humanized anti-CD3 arm by the judicious recruitment of a small number of additional murine residues into the minimalistically humanized anti-CD3 variable domains.

Materials and Methods

Construction of Mutations in the Anti-CD3 Variable Region Genes

The construction of genes encoding humanized anti-CD3 variant 1 (v1) variable light (V_1) and heavy (V_H) chain domains in phagemid pUC119 has been described (Shalaby et al. supra). Additional anti-CD3 variants were generated using an efficient site-directed mutagenesis method (Carter, P., *Mutagenesis: a practical approach,* (M. J. McPherson, Ed.), Chapter 1, IRL Press, Oxford, UK (1991)) using mismatched oligonucleotides which either install or remove unique restriction sites. Oligonucleotides used are listed below using lowercase to indicate the targeted mutations. Corresponding coding changes are denoted by the starting amino acid in one letter code followed by the residue numbered according to Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest,* 5th edition, National Institutes of Health, Bethesda, Md., USA (1991), then the replacement amino acid and finally the identity of the anti-CD3 variant:

HX11, 5' GTAGATAAATCCtctAACACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 11) V_HK75S, v6;

HX12, 5' GTAGATAAATCCAAAtctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 12) V_H N76S, v7;

HX13, 5' GTAGATAAATCCtcttctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 13) V_H K75S:N76S, v8;

X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcAgAaatTCAAGGatCGTTTCACgATAtcCGTAGATAAATCC 3' (SEO.ID.NO. 14) V_H T57S:A60N:D61Q:S62K:V63F:G65D, v9;

LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15) V_L E55H, v11.

Oliconucleotides HX11, HX12 and HX13 each remove a site for BspMI, whereas LX6 removes a site for XhoI and HX14 installs a site for EcoRV (bold). Anti-CD3 variant v10 was constructed from v9 by site-directed mutagenesis using oligonucleotide HX13. Mutants were verified by dideoxynucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad, Sci. USA* 74: 5463–5467 (1977)).

E. coli Expression of Fab' Fragments

The expression plasmid, pAK19, for the co-secretion of light chain and heavy chain Fd' fragment of the most preferred humanized anti-p185^{HER2} variant, HuMAb4D5-8, is described in Carter et al., 1992b, supre. Briefly, the Fab' expression unit is bicistronic with both chains under the

transcriptional control of the ohoA promoter. Genes encoding humanized V_L and V_H domains are precisely fused on their 5' side to a gene segment encoding the heat-stable enterotoxin II signal sequence and on their 3' side to human k_1 C_L and IgG1C_H1 constant domain genes, respectively. The C_H1gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ t_o transcriptional terminator. Fab' expression plasmids for chimeric and humanized anti-CD3 variants (v1 to v4, Shalaby et al.,supra; v6 to v12, this study) were created from pAK19 by precisely replacing anti-p185^{HER2} V_L and V_H gene segments with those encoding murine and corresponding humanized variants of the anti-CD3 antibody, respectively, by sub-cloning and site-directed mutagenesis. The Fab' expression plasmid for the most potent humanized anti-CD3 variant identified in this study (v9) is designated pAK22. The anti-p185^{HER2} Fab' fragment was secreted from *E. coli* K12 strain 25F2 containing plasmid pAK19 grown for 32 to 40 hr at 37° C. in an aerated 10 liter fermentor. The final cell density was 120–150 OD_{550} and the titer of soluble and functional anti-p185^{HER2} Fab' was 1–2 g/liter as judged by antigen binding ELISA (Carter et al., 1992b, suora). Anti-CD3 Fab' variants were secreted from *E. coli* containing corresponding expression plasmids using very similar fermentation protocols. The highest expression titers of chimeric and humanized anti-CD3 variants were 200 mgaliter and 700 mgaliter, respectively, as judged by total immunoglobulin ELISA.

Construction of BsF(ab')$_2$ Fragments

Fab' fragments were directly recovered from *E. coli* fermentation pastes in the free thiol form (Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b supra). Thioether linked BsF(ab')$_2$ fragments (anti-p185^{HER2}/anti-CD3) were constructed by the procedure of Glennie et al. supra with the following modifications. Anti-p185^{HER2} Fab'-SH in 100 mM Tris acetate, 5 mM EDTA (pH 5.0) was reacted with 0.1 vol of 40 mM N,N'-1,2-phenylenedimalemide (o-PDM) in dimethyl formamide for ~1.5 hr at 20° C. Excess o-PDM was removed by protein G purification of the Fab' maleimide derivative (Fab'-mal) followed by buffer exchange into 20 mM sodium acetate, 5 mM EDTA (pH 5.3) (coupling buffer) using centriprep-30 concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMAb4D5-8 Fab' $e^{0.1}$%=1.56, Carter et al., 1992b, supra). The free thiol content of Fab' preparations was estimated by reaction with 5,5' -dithiobis (2-nitrobenzoic acid) as described by Creighton, T. E., *Protein structure: a practical approach,* (T. E. Creighton, Ed.), Chapter 7, IRL Press, Oxford, UK (1990). Equimolar amounts of anti-p185^{HER2} Fab'-mal (assuming quantitative reaction of Fab'-SH with o-PDM) and each anti-CD3 Fab'-SH variant were coupled together at a combined concentration of 1 to 2.5 mg/ml in the coupling buffer for 14 to 48 hr at 4° C. The coupling reaction was adjusted to 4 mM cysteine at pH 7.0 and incubated for 15 min at 20 ° C. to reduce any unwanted disulfide-linked F(ab')$_2$ formed. These reduction conditions are sufficient to reduce inter-heavy chain disulfide bonds with virtually no reduction of the disulfide between light and heavy chains. Any free thiols generated were then blocked with 50 mM iodoacetamide. BsF(ab')$_2$ was isolated from the coupling reaction by S100-HR (Pharmacia) size exclusion chromatography (2.5 cm×100 cm) in the presence of PBS. The BsF(ab')$_2$ samples were passed through a 0.2 mm filter flash frozen in liquid nitrogen and stored at −70° C.

Flow Cytometric Analysis of F(ab')$_2$Binding to Jurkat Cells

The Jurkat human acute T cell leukemia cell line was purchased from the American Type Culture Collection (Manassas Va.) (ATCC TIB 152) and grown as. recommended by the ATCC. Aliquots of 10^6 Jurkat cells were incubated with appropriate concentrations of BsF(ab')$_2$ (anti-p185^{HER2}/anti-CD3 variant) or control mono-specific anti-p185^{HER2} F(ab')$_2$ in PBS plus 0.1% (w/v) bovine serum albumin and 10 mM sodium azide for 45 min at 4° C. The cells were washed and then incubated with fluorescein-conjugated goat anti-human F(ab')$_2$ (Organon Teknika, West Chester, Pa.) for 45 min at 4° C. Cells were washed and analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, Calif.). Cells (8×10^3) were acquired by list mode and gated by forward light scatter versus side light scatter excluding dead cells and debris.

Results

Design of Humanized anti-CD3 Variants

The most potent humanized anti-CD3 variant previously identified, v1, differs from the murine parent antibody, UCHT1 at 19 out of 107 amino acid residues within V_L and at 37 out of 122 positions within V_H (Shalaby et al.,supra) 1992). Here we recruited back additional murine residues into anti-CD3 v1 in an attempt to improve the binding affinity for CD3. The strategy chosen was a compromise between minimizing both the number of additional murine residues recruited and the number of anti-CD3 variants to be analyzed. We focused our attentions on a few CDR residues which were originally kept as human sequences in our minimalistic humanization regime. Thus human residues in V_H CDR2 of anti-CD3 v1 were replaced en bloc with their murine counterparts to give anti-CD3 v9: T57S:A60N:D61Q:S62K:V63F:G65D (SEQ ID NO:20). Similarly, the human residue E55 in V_L CDR2 of anti-CD3 v_1 was replaced with histidine from the murine anti-CD3 antibody to generate anti-CD3 v11. In addition, V_H framework region (FR) residues 75 and 76 in anti-CD3 v1 were also replaced with their murine counterparts to create anti-CD3 v8: K75S:N76S. V_H residues 75 and 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might influence antigen binding. Additional variants created by combining mutations at these three sites are described below.

Preparation of BsF(ab')$_2$ Fragments

Soluble and functional anti-p185^{HER2} and anti-CD3 Fab' fragments were recovered directly from corresponding *E. coli* fermentation pastes with the single hinge cysteine predominantly in the free thiol form (75–100% Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b, supra). Thioether-linked BsF(ab')$_2$ fragments were then constructed by directed coupling using o-PDM as described by Glennie et al., supra. One arm was always the most potent humanized anti-p185^{HER2} variant, HuMAb4D5-8 (Carter et al., 1992a, supra) and the other either a chimeric or humanized variant of the anti-CD3 antibody. Anti-p185^{HER2} Fab'-SH was reacted with o-PDM to form the maleimide derivative (Fab'-mal) and then coupled to the Fab'-SH for each anti-CD3 variant. F(ab')$_2$ was then purified away from unreacted Fab' by size exclusion chromatography as shown for a representative preparation (BsF(ab')$_2$ v8) in data not shown. The F(ab')$_2$ fragment represents ~54% of the total amount of antibody fragments (by mass) as judged by integration of the chromatograph peaks.

SDS-PAGE analysis of this BsF(ab')$_2$v8 preparation under non-reducing conditions gave one major band with the expected mobility (M, ~96 kD) as well as several very minor

bands (data not shown). Amino-terminal sequence analysis of the major band after electroblotting on to polyvinylidene difluoride 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might membrane Matsudaira, P., *J. Biol. Chem.* 262: 10035–10038 (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and heavy chains (V_L/V_H: D/E, I/V, Q/D, M/L, T/V, D/E, S/S) expected for BsF(ab')$_2$. The amino terminal region of both light chains are identical as are both heavy chains and correspond to consensus human FR sequences. We have previously demonstrated that F(ab')$_2$ constructed by directed chemical coupling carry both anti-p185^{HER2} and anti-CD3 antigen specificities (Shalaby et al., supra). The level of contamination of the BsF(ab')$_2$ with monospecific F(ab')$_2$ iS likely to be very low since mock coupling reactions with either anti-p185^{HER2} w Fab'-mal or anti-CD3 Fab'-SH alone did not yield detectable quantities of F(ab')$_2$. Furthermore the coupling reaction was subjected to a mild reduction step followed by alkylation to remove trace amounts of disulfide-linked F(ab')$_2$ that might be present. SDS-PAGE of the purified F(ab')$_2$ under reducing conditions gave two major bands with electrophoretic mobility and amino terminal sequence anticipated for free light chain and thioether-linked heavy chain dimers.

Scanning LASER densitometry of a o-PDM coupled F(ab')$_2$ preparation suggest that the minor species together represent ~10% of the protein. These minor contaminants were characterized by amino terminal sequence analysis and were tentatively identified on the basis of stoichiometry of light and heavy chain sequences and their electrophoretic mobility (data not shown). These data are consistent with the minor contaminants including imperfect F(ab')$_2$ in which the disulfide bond between light and heavy chains is missing in one or both arms, trace amounts of Fab' and heavy chain thioether-linked to light chain.

Binding of BsF(ab')$_2$ to Jurkat Cells

Binding of BsF(ab')$_2$ containing different anti-CD3 variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry (data not shown). BsF(ab')$_2$v9 binds much more efficiently to Jurkat cells than does our starting molecule, BsF(ab')$_2$vl, and almost as efficiently as the chimeric BsF(ab')$_2$. Installation of additional murine residues into anti-CD3 v9 to create v10 (V_HK75S:N76S) and v12 (V_HK75S:N76S plus V_L E55H) did not further improve binding of corresponding BsF(ab')$_2$to Jurkat cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding: V_HK75S (v6), V_HN76S (v7), V_HK75S:N76S (V8), V_LE55H (v11) (not shown). BsF(ab')$_2$v9 was chosen for future study since it is amongst the most efficient variants in binding to Jurkat cells and contains fewest murine residues in the humanized anti-CD3 arm. A monospecific anti-p185^{HEE2} F(ab')$_2$ did not show significant binding to Jurkat cells consistent with the interaction being mediated through the anti-CD3 arm.

Discussion

A minimalistic strategy was chosen to humanize the anti-p185^{HER2} (Carter et al., 1992a, supra) and anti-CD3 arms (Shalaby et al., supra) of the BsF(ab')$_2$ in this study in an attempt to minimize the potential immunogenicity of the resulting humanized antibody in the clinic. Thus we tried to install the minimum number of murine CDR and FR residues into the context of consensus human variable domain sequences as required to recruit antigen-binding affinity and biological properties comparable to the murine parent antibody. Molecular modeling was used firstly to predict the murine FR residues which might be important to antigen binding and secondly to predict the murine CDR residues that might not be required. A small number of humanized variants were then constructed to test these predictions.

Our humanization strategy was very successful for the anti-p185^{HER2} antibody where one out of eight humanized variants (HuMAb4D5-8, IgG1) was identified that bound the p185^{HER2} antigen ~3-fold more tightly than the parent murine antibody (Carter et al., 1992a, supra). HuMAb4D5-8 contains a total of five murine FR residues and nine murine CDR residues, including V_H CDR2 residues 60–65, were discarded in favor of human counterparts. In contrast, BsF(ab')$_2$v1 containing the most potent humanized anti-CD3 variant out of four originally constructed (Shalaby et al., supra) binds J6 cells with an affinity (K_d) of 140 nM which is ~70-fold weaker than that of the corresponding chimeric BsF(ab')$_2$.

Here we have restored T cell binding of the humanized anti-CD3 close to that of the chimeric variant by replacing six human residues in V_H CDR2 with their murine counterparts: T57S:A60N:D61Q:S62K:V63F:G65D (anti-CD3 v9, FIG. **5**). It appears more likely that these murine residues enhance antigen binding indirectly by influencing the conformation of residues in the N-terminal part of V_H CDR2 rather than by directly contacting antigen. Firstly, only N-terminal residues in V_H CDR2 (50–58) have been found to contact antigen in one or more of eight crystallographic structures of antibody/antigen complexes (Kabat et al., supra; and Mian, I. S. et al., *J. Mol. Biol* 217: 133–151 (1991), FIG. **5**). Secondly, molecular modeling suggests that residues in the C-terminal part of V_H CDR2 are at least partially buried (FIG. **5**). BsF(ab')$_2$v9 binds to SK-BR-3 breast tumor cells with equal efficiency to BsF(ab')$_2$v1 and chimeric BsF(ab')$_2$ as anticipated since the anti-p185^{HER2} arm is identical in all of these molecules (Shalaby et al., supra, not shown).

Our novel approach to the construction of BsF(ab')$_2$ fragments exploits an *E. coli* expression system which secretes humanized Fab' fragments at gram per liter titers and permits their direct recovery as Fab'-SH (Carter et al., 1992b, supra). Traditional directed chemical coupling of Fab'-SH fragments is then used to form BsF(ab')$_2$ in vitro (Brennan et al.,supra; and Glennie et al., supra). This route to Fab'-SH obviates problems which are inherent in their generation from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield as well as partial reduction that is not completely selective for the hinge disulfide bonds. The strategy of using *E. coli*-derived Fab'-SH containing a single hinge cysteine abolishes some sources of heterogeneity in BsF(ab')$_2$ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of F(ab')$_3$ fragments.

BsF(ab')2 fragments constructed here were thioether-linked as originally described by Glennie et al., supra with future in vivo testing of these molecules in mind. Thioether bonds, unlike disulfide bonds, are not susceptible to cleavage by trace amounts of thiol, which led to the proposal that thioether-linked F(ab')$_2$ may be more stable than disulfide-linked F(ab')$_2$ in vivo (Glennie et al., supra). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked BsF(ab')$_2$ v1 has a 3-fold longer plasma residence time than BsF(ab')$_2$ v1 linked by a single disulfide bond. Disulfide and thioether-linked chimeric BsF(ab')$_2$ were found to be indistinguishable in their efficiency of cell binding and in their retargeting of CTL cytotoxicity, which suggests that o-PDM directed coupling does not compromise binding of the

BsF(ab')$_2$ to either antigen (not shown). Nevertheless the nature of the linkage appears not to be critical since a disulfide-linked BsF(ab')$_2$ (murine anti-p185^{HER2}/murine anti-CD3) was recently shown by others (Nishimura et al., Int. *J. Cancer* 50: 800–804 (1992) to have potent anti-tumor activity in nude mice. Our previous study (Shalaby et al., supra) together with this one and that of Nishimura, T. et al., supra improve the potential for using BsF(ab')$_2$ in targeted immunotherapy of p185^{HER2}-overexpressing cancers in humans.

Example 4

Humanization of an anti-CD18 Antibody

A murine antibody directed against the leukocyte adhesion receptor β-chain (known as the H52 antibody) was humanized following the methods described above. FIGS. **6**A and **6**B provide amino acid sequence comparisons for the murine and humanized antibody light chains and heavy chains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                 1 05

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                65                  70                  75
```

-continued

```
Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                 95                 1 00                 1 05

Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                  120
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
                 20                  25                  30

Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

Tyr Asn Ser Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 1 00                 1 05

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                 20                  25                  30

Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Val Ile Ser Glu Asn Gly Ser Asp Thr Tyr Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Gly Ala Val Ser
                 95                 1 00                 1 05

Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                  120
```

-continued

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val
1 5 10 15

Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Asn
20 25 30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly His Ser Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Phe Arg Tyr Thr Gly Val Pro Asp
50 55 60

Arg Phe Thr Gly Asn Arg Ser Gly Thr Asp Phe Thr Phe Thr Ile
65 70 75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
80 85 90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
95 1 00 1 05

Ile Lys Arg Ala
109

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
20 25 30

Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
35 40 45

Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
50 55 60

Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
65 70 75

Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
95 1 00 1 05

Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCGATATCC AGCTGACCCA GTCTCCA 27

-continued

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGTSMARCT GCAGSAGTCW GG 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTAGATAAAT CCAAATCTAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG 36

-continued

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTATAAAGG TGTTTCCACC TATAACCAGA AATTCAAGGA TCGTTTCACG 50

ATATCCGTAG ATAAATCC 68

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTATACCTCC CGTCTGCATT CTGGAGTCCC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
1 5 10 15

Gly Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
35 40 45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser
50 55 60

Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
65 70 75

Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
80 85 90

Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys Leu Glu
95 1 00 1 05

Ile Lys
107

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Arg
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys

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                35                  40                  45

 Leu Leu Ile Tyr Tyr Thr Ser Arg Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
                65                  70                  75

 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

 Gly Asn Thr Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                1 05

 Ile Lys
     107


(2) INFORMATION FOR SEQ ID NO:18:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 107 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
   1               5                  10                  15

 Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
                20                  25                  30

 Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

 Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

 Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                1 05

 Ile Lys
     107


(2) INFORMATION FOR SEQ ID NO:19:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 122 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

 Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
   1               5                  10                  15

 Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

 Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
                35                  40                  45

 Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
                50                  55                  60

 Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser
                65                  70                  75

 Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp
                80                  85                  90
```

-continued

Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
95 1 00 1 05

Asp Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val
110 115 120

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
20 25 30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
50 55 60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
95 1 00 1 05

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
110 115 120

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
20 25 30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
50 55 60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
95 1 00 1 05

Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
110 115 120

-continued

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr
20 25 30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
35 40 45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His
50 55 60

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
65 70 75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp
80 85 90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95 1 00 1 05

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
110 115 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125 130 135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
140 145 150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155 160 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170 175 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185 190 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200 205 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215 220 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
230 235 240

Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
260 265 270

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
275 280 285

Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
305 310 315

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
320 325 330

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Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
                335                 340                 345

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
                350                 355                 360

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
                365                 370                 375

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
                380                 385                 390

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
                395                 400                 405

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
                410                 415                 420

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
                425                 430                 435

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
                440                 445                 450

Ser Pro Gly Lys
            454


(2) INFORMATION FOR SEQ ID NO:23:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 469 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
  1               5                  10                  15

Gly Val His Ser Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu
                20                  25                  30

Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly
                35                  40                  45

Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro
                50                  55                  60

Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly
                65                  70                  75

Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser
                80                  85                  90

Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu
                95                 1 00                1 05

Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly
                110                 115                 120

Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln
                125                 130                 135

Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
                140                 145                 150

Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
                155                 160                 165

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
                170                 175                 180

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr
                185                 190                 195

Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser
                200                 205                 210
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-continued

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Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr
                215                 220                 225

Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr
                230                 235                 240

Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
                245                 250                 255

Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
                260                 265                 270

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
                275                 280                 285

Val Asp Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
                290                 295                 300

Val Asp Gly Met Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
                305                 310                 315

Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu Thr Val
                320                 325                 330

Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
                335                 340                 345

Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
                350                 355                 360

Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
                365                 370                 375

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
                380                 385                 390

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
                395                 400                 405

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu
                410                 415                 420

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
                425                 430                 435

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
                440                 445                 450

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
                455                 460                 465

Ser Pro Gly Lys
            469


(2) INFORMATION FOR SEQ ID NO:24:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 214 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Asp Val Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Asn Cys Arg Ala Ser Gln Asp Ile Asn
                 20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asn Gly Thr Val Lys
                 35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
                 65                  70                  75
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Ser Asn Leu Asp Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
                 80                  85                  90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gly Gly Thr Lys Val Glu
                 95                 1 00                 1 05

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
            214
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

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Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
  1               5                  10                  15

Gly Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu
                 20                  25                  30

Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
                 35                  40                  45

Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly
                 50                  55                  60

Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser
                 65                  70                  75

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
                 80                  85                  90

Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
                 95                 1 00                 1 05

Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly
                110                 115                 120

Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe
                125                 130                 135

Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser
                140                 145                 150

Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val
                155                 160                 165

Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
                170                 175                 180

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                185                 190                 195
```

-continued

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Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
                200                 205                 210

Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr
                215                 220                 225

Lys Ser Phe Asn Arg Gly Glu Cys
                230          233


(2) INFORMATION FOR SEQ ID NO:26:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 122 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Thr Thr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                 1 00                1 05

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
               110                 115                 120

Ser Ser
    122
```

We claim:

1. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, and 92H, utilizing the numbering system set forth in Kabat.

2. The humanized variable domain of claim **1** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

3. The humanized variable domain of claim **1** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

4. The humanized variable domain of claim **1** wherein the human antibody variable domain is a consensus human variable domain.

5. The humanized variable domain of claim **1** wherein the residue at site 4L has been substituted.

6. The humanized variable domain of claim **1** wherein the residue at site 38L has been substituted.

7. The humanized variable domain of claim **1** wherein the residue at site 43L has been substituted.

8. The humanized variable domain of claim **1** wherein the residue at site 44L has been substituted.

9. The humanized variable domain of claim **1** wherein the residue at site 58L has been substituted.

10. The humanized variable domain of claim **1** wherein the residue at site 62L has been substituted.

11. The humanized variable domain of claim **1** wherein the residue at site 65L has been substituted.

12. The humanized variable domain of claim **1** wherein the residue at site 66L has been substituted.

13. The humanized variable domain of claim **1** wherein the residue at site 67L has been substituted.

14. The humanized variable domain of claim **1** wherein the residue at site 68L has been substituted.

15. The humanized variable domain of claim **1** wherein the residue at site 69L has been substituted.

16. The humanized variable domain of claim **1** wherein the residue at site 73L has been substituted.

17. The humanized variable domain of claim **1** wherein the residue at site 85L has been substituted.

18. The humanized variable domain of claim **1** wherein the residue at site 98L has been substituted.

19. The humanized variable domain of claim **1** wherein the residue at site 2H has been substituted.

20. The humanized variable domain of claim **1** wherein the residue at site 4H has been substituted.

21. The humanized variable domain of claim **1** wherein the residue at site 36H has been substituted.

22. The humanized variable domain of claim **1** wherein the residue at site 39H has been substituted.

23. The humanized variable domain of claim **1** wherein the residue at site 43H has been substituted.

24. The humanized variable domain of claim **1** wherein the residue at site 45H has been substituted.

25. The humanized variable domain of claim **1** wherein the residue at site 69H has been substituted.

26. The humanized variable domain of claim **1** wherein the residue at site 70H has been substituted.

27. The humanized variable domain of claim **1** wherein the residue at site 74H has been substituted.

28. The humanized variable domain of claim **1** wherein the residue at site 92H has been substituted.

29. An antibody comprising the humanized variable domain of claim **1**.

30. An antibody which binds $p185^{HER2}$ and comprises a humanized antibody variable domain, wherein the humanized antibody variable domain comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind $p185^{HER2}$ incorporated into a human antibody variable domain, and further comprises a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

31. The antibody of claim **30** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

32. The antibody of claim **30** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

33. The antibody of claim **30** wherein the human antibody variable domain is a consensus human variable domain.

34. The antibody of claim **30** wherein the residue at site 4L has been substituted.

35. The antibody of claim **30** wherein the residue at site 38L has been substituted.

36. The antibody of claim **30** wherein the residue at site 43L has been substituted.

37. The antibody of claim **30** wherein the residue at site 44L has been substituted.

38. The antibody of claim **30** wherein the residue at site 46L has been substituted.

39. The antibody of claim **30** wherein the residue at site 58L has been substituted.

40. The antibody of claim **30** wherein the residue at site 62L has been substituted.

41. The antibody of claim **30** wherein the residue at site 65L has been substituted.

42. The antibody of claim **30** wherein the residue at site 66L has been substituted.

43. The antibody of claim **30** wherein the residue at site 67L has been substituted.

44. The antibody of claim **30** wherein the residue at site 68L has been substituted.

45. The antibody of claim **30** wherein the residue at site 69L has been substituted.

46. The antibody of claim **30** wherein the residue at site 73L has been substituted.

47. The antibody of claim **30** wherein the residue at site 85L has been substituted.

48. The antibody of claim **30** wherein the residue at site 98L has been substituted.

49. The antibody of claim **30** wherein the residue at site 2H has been substituted.

50. The antibody of claim **30** wherein the residue at site 4H has been substituted.

51. The antibody of claim **30** wherein the residue at site 36H has been substituted.

52. The antibody of claim **30** wherein the residue at site 39H has been substituted.

53. The antibody of claim **30** wherein the residue at site 43H has been substituted.

54. The antibody of claim **30** wherein the residue at site 45H has been substituted.

55. The antibody of claim **30** wherein the residue at site 69H has been substituted.

56. The antibody of claim **30** wherein the residue at site 70H has been substituted.

57. The antibody of claim **30** wherein the residue at site 74H has been substituted.

58. The antibody of claim **30** wherein the residue at site 75H has been substituted.

59. The antibody of claim **30** wherein the residue at site 76H has been substituted.

60. The antibody of claim **30** wherein the residue at site 78H has been substituted.

61. The antibody of claim **30** wherein the residue at site 92H has been substituted.

62. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a consensus human variable domain, and further comprising an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

63. A humanized antibody which lacks immunogenicity compared to a non-human parent antibody upon repeated administration to a human patient in order to treat a chronic disease in that patient, wherein the humanized antibody comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprises an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

64. A humanized variant of a non-human parent antibody which binds an antigen and comprises a human variable domain comprising the most frequently occurring amino acid residues at each location in all human immunoglobulins of a human heavy chain immunoglobulin subgroup wherein amino acid residues forming Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprises a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) introduces a glycosylation site which affects the antigen binding or affinity of the antibody; or (d) participates in the V_L–V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another.

65. The humanized variant of claim **63** which binds the antigen up to 3-fold more in the binding affinity than the parent antibody binds antigen.

66. A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining

Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 73H, 76H, 78H, and 93H, utilizing the numbering system set forth in Kabat.

67. The humanized variable domain of claim **66** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

68. The humanized variable domain of claim **66** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

69. The humanized variable domain of claim **66** wherein the human antibody variable domain is a consensus human variable domain.

70. The humanized variable domain of claim **66** wherein the residue at site 24H has been substituted.

71. The humanized variable domain of claim **66** wherein the residue at site 73H has been substituted.

72. The humanized variable domain of claim **66** wherein the residue at site 76H has been substituted.

73. The humanized variable domain of claim **66** wherein the residue at site 78H has been substituted.

74. The humanized variable domain of claim **66** wherein the residue at site 93H has been substituted.

75. The humanized variable domain of claim **66** which further comprises an amino acid substitution at site 71H.

76. The humanized variable domain of claim **66** which further comprises amino acid substitutions at sites 71H and 73H.

77. The humanized variable domain of claim **66** which further comprises amino acid substitutions at sites 71H, 73H and 78H.

78. An antibody comprising the humanized variable domain of claim **66**.

79. A humanized variant of a non-human parent antibody which binds an antigen, wherein the humanized variant comprises Complementarity Determining Region (CDR) amino acid residues of the non-human parent antibody incorporated into a human antibody variable domain, and further comprises Framework Region (FR) substitutions at heavy chain positions 71H, 73H, 78H and 93H, utilizing the numbering system set forth in Kabat.

80. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution where the substituted FR residue:

(a) noncovalently binds antigen directly;

(b) interacts with a CDR; or

(c) participates in the V_L–V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another, and wherein the substituted FR residue is at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 24H, 36H, 39H, 43H, 45H, 69H, 70H, 73H, 74H, 76H, 78H, 92H and 93H, utilizing the numbering system set forth in Kabat.

81. The humanized variable domain of claim **80** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

82. The humanized variable domain of claim **80** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,407,213 B1
DATED : June 18, 2002
INVENTOR(S) : Carter et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 88,
Line 63, please delete "63" and insert therefor -- 79 --.

Signed and Sealed this

Third Day of December, 2002

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

EXHIBIT D

US007846441B1

(12) **United States Patent**
Hellmann

(10) **Patent No.: US 7,846,441 B1**
(45) **Date of Patent: Dec. 7, 2010**

(54) **TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventor: **Susan D. Hellmann**, San Carlos, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/208,649**

(22) Filed: **Dec. 10, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/069,346, filed on Dec. 12, 1997.

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.** **424/143.1**; 424/130.1; 424/133.1; 424/135.1; 424/136.1; 424/141.1; 424/142.1; 424/152.1; 424/155.1; 424/156.1; 424/172.1; 424/174.1

(58) **Field of Classification Search** 424/137.1, 424/138.1, 141.1, 227.1, 152.1, 130.1, 133.1, 424/135.1, 136.1, 142.1, 143.1, 155.1, 156.1, 424/172.1, 174.1
See application file for complete search history.

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(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

14 Claims, 2 Drawing Sheets

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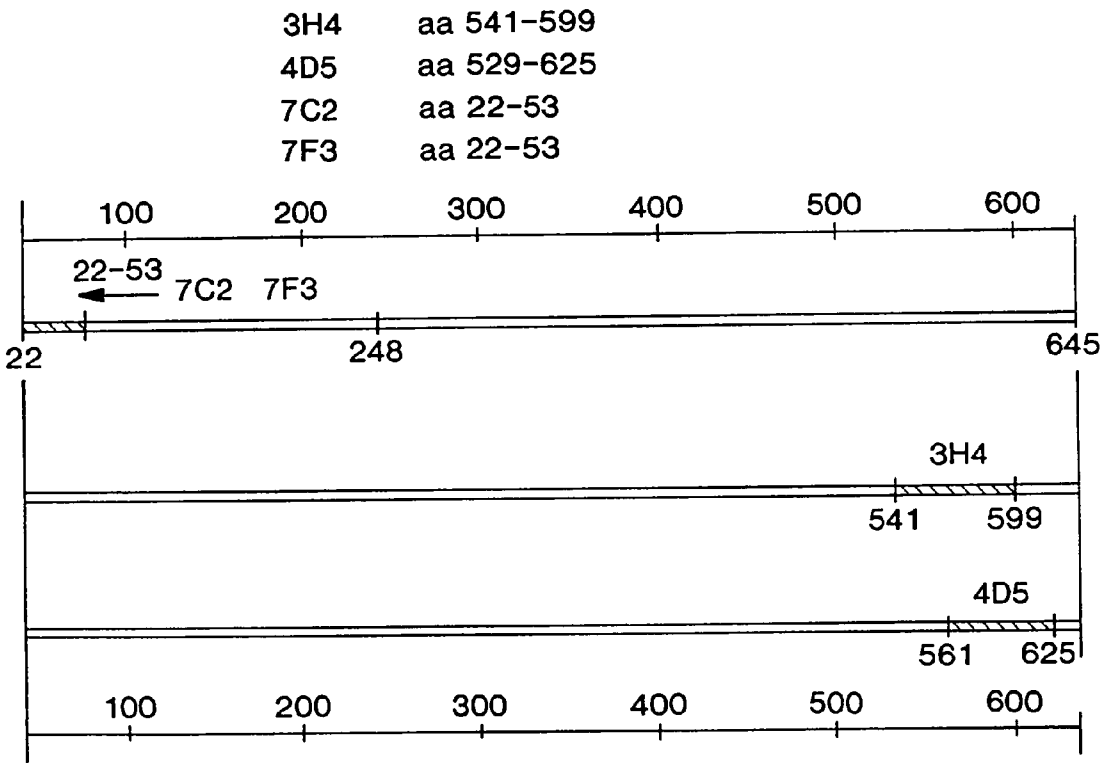
FIG. 1



3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR

541 599

4D5 epitope (SEQ ID NO:9) (64 residues)

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP

561 625

1 MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPA
38 SPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG. 2

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This is a non-provisional application claiming priority to provisional application No. 60/069,346, filed Dec. 12, 1997, the entire disclosure of which is hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237: 178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dysfunction that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and

electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO:1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein and "her2", "erbB2" and "c-erb-B2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al, *PNAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177\text{-}244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of

the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20): 14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a n-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the n-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. I, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the

techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al, *Proc. Natl. Acad. Sci. USA,* 81; 6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al, *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZEDT™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131}, I^{125}, Y^{90} and Re^{186}), chemo-

therapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (Taxotere®, Rhône-Poulenc Rorer, Antony, France), methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, caminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of Anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS* (*USA*) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc.*

Natl. Acad. Sci. USA, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity

chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5): 1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate

MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA,* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-de-

rivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatogra-

phy, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent, provided that the cytotoxic agent is other than an anthracycline derivative, e.g. doxorubicin, or epirubicin. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

The treatment of the present invention involved the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Va. 20110-2209 (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12226	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

Example

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci.* (*USA*) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for p185" (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of $p185^{HER2}$, induces antibody-dependent

cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

- Metastatic breast cancer
- Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].
- Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs.

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

- The ability to understand and willingness to sign a written informed consent form
- Women≧18 years
- Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

- Prior cytotoxic chemotherapy for metastatic breast cancer
- Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.
- Concomitant malignancy that has not been curatively treated
- A performance status of <60% on the Karnofsky scale
- Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator
- Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)
- Use of investigational or unlicensed agents within 30 days prior to study entry
- Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regiments for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68

-continued

	Enrolled	TTP(months)	RR(%)	AE(%)
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p <0.001 by log-rank test
**p <0.01 by X^2 test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade 3/4) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
                50                  55                  60

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
                65                  70                  75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
                80                  85                  90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
                95                 100                 105

Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
               110                 115                 120

Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
               125                 130                 135

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Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
35 40 45

Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
50 55 60

Gly Ala Cys Gln Pro
65

The invention claimed is:

1. A method for the treatment of a human patient with a malignant progressing tumor or cancer characterized by overexpression of ErbB2 receptor, comprising administering a combination of an intact antibody which binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

2. The method of claim **1** wherein said patient has a malignant tumor.

3. The method of claim **1** wherein said patient has cancer.

4. The method of claim **3** wherein said cancer is selected from the group consisting of breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

5. The method of claim **4** wherein said cancer is breast cancer.

6. The method of claim **5** wherein said cancer is metastatic breast carcinoma.

7. The method of claim **1** wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

8. The method of claim **1** wherein said taxoid is paclitaxel.

9. The method of claim **8** wherein the effective amount of said combination is lower than the sum of the effective amounts of said anti-ErbB2 antibody and said taxoid, when administered individually, as single agents.

10. The method of claim **1** wherein efficacy is further measured by determining the response rate.

11. A method for the treatment of a human patient with ErbB2 overexpressing progressing metastatic breast cancer, comprising administering a combination of a humanized 4D5 anti-ErbB2 antibody and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

12. The method of claim **11** wherein said taxoid is paclitaxel.

13. A method for the treatment of a human patient with a progressing malignant tumor or cancer characterized by over-expression of ErbB2 receptor, comprising administering a combination of a humanized 4D5 anti-ErbB2 antibody which comprises a human Fc region and that binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

14. A method for the treatment of a human patient with ErbB2 expressing progressing metastatic breast cancer, comprising administering a combination of an antibody which binds to epitope 4D5 within the extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

* * * * *

EXHIBIT E

US007892549B2

(12) **United States Patent**
Paton et al.

(10) **Patent No.: US 7,892,549 B2**
(45) **Date of Patent: *Feb. 22, 2011**

(54) **TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventors: **Virginia E. Paton**, Oakland, CA (US); **Steven Shak**, Burlingame, CA (US); **Susan D. Hellmann**, San Carlos, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1827 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/356,824**

(22) Filed: **Feb. 3, 2003**

(65) **Prior Publication Data**

US 2004/0037823 A9 Feb. 26, 2004

Related U.S. Application Data

(63) Continuation of application No. 09/208,649, filed on Dec. 10, 1998.

(60) Provisional application No. 60/069,346, filed on Dec. 12, 1997.

(51) **Int. Cl.**
A61K 39/395 (2006.01)
C07K 16/28 (2006.01)
C07K 16/30 (2006.01)

(52) **U.S. Cl.** **424/143.1**; 424/130.1; 424/133.1; 424/134.1; 424/135.1; 424/136.1; 424/138.1; 424/141.1; 424/152.1; 424/155.1; 424/156.1; 424/172.1; 424/174.1

(58) **Field of Classification Search** 424/130.1, 424/133.1, 138.1, 141.1, 143.1, 155.1, 174.1, 424/134.1, 135.1, 136.1, 152.1, 156.1, 172.1
See application file for complete search history.

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(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

17 Claims, 1 Drawing Sheet

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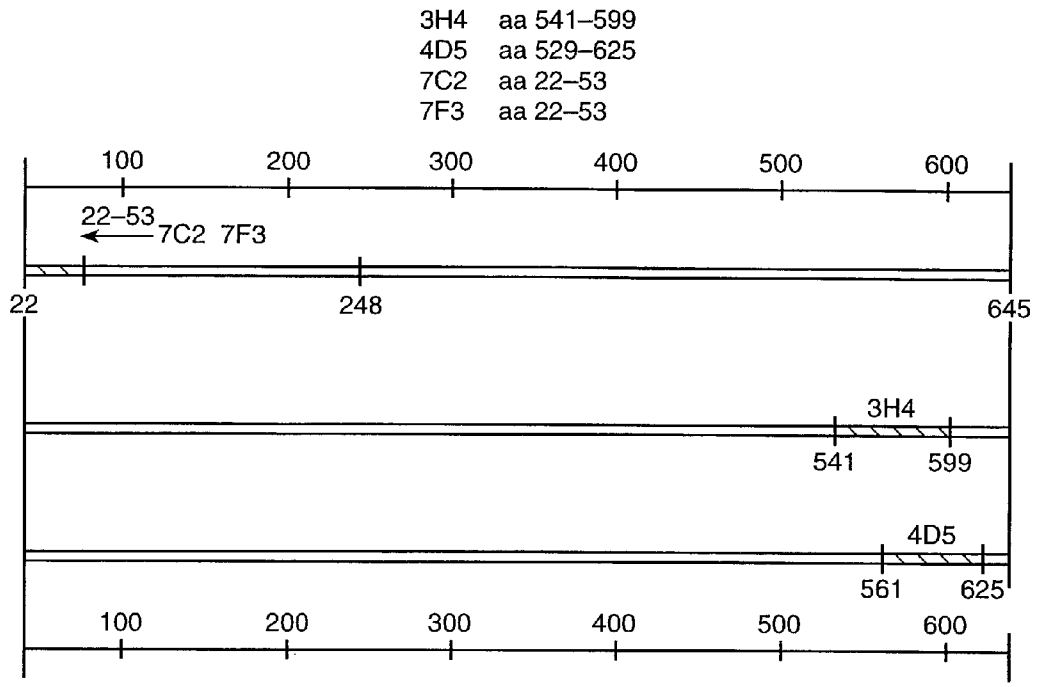

3H4 epitope (SEQ ID NO:8)

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
541 **599**

4D5 epitope (SEQ ID NO:9)

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP
561 **625**

FIG._1

1 MELAALCRWGLLLALLPPGAA**STQVCTGTDMKLRLPA**
38 **SPETHLDMLRHLYQGC**QVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG._2

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This is a continuation of non-provisional application Ser. No. 09/208,649, filed Dec. 10, 1998, which claims priority under 35 USC §119 to provisional application No. 60/069,346, filed Dec. 12, 1997, the entire disclosures of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712[1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci.* USA 84:7159-7163 [1987]; DiFiore et al., *Science* 237:178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B 104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al *PNAS* (USA) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects

of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al In Vitro 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Nail Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369[1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (USA) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of

this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dysfunction that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant

and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein and "her2", "erbB2" and "c-erb-B2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS* (USA) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce P1 uptake in the "P1 uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177-244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20): 14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. 1, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies"

may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci*. USA, 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al., *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci*. USA, 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemo-

therapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France), methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce GI arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest GI also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1a, IL-2, IL-3, IL-4, IL-5, L-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactamcontaining prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS* (USA) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R_1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Pluckthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554(1990). Clackson et al., *Nature,* 352:624-628(1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc.*

Natl Acad. Sci. USA, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525(1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "bestfit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci*. USA, 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci*. USA, 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity

chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli,* which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175:217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. *Natl. Acad. Sci.* USA, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147:60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate

MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp. Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MXDTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci.* USA, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci.* USA, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-de-

rivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO. 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328:457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312:604-608 [1984]).

(xi) Antibody-salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)(5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatogra-

phy, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immnol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent, provided that the cytotoxic agent is other than an anthracycline derivative, e.g. doxorubicin, or epirubicin. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

The treatment of the present invention involved the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin.

DEPOSIT OF MATERIALS

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

EXAMPLE

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 IgG_1K murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 5o:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci.* (USA) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse mycloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci.* USA 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that con-

tain high levels of $p185^{HER2}$, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligihility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

- Metastatic breast cancer
- Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].
- Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

- The ability to understand and willingness to sign a written informed consent form
- Women≧18 years
- Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

- Prior cytotoxic chemotherapy for metastatic breast cancer
- Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.
- Concomitant malignancy that has not been curatively treated
- A performance status of <60% on the Karnofsky scale
- Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator
- Bilateral breast cancer (either both primary tumors must have 2+ to 3+HER2 overexpression, or the metastatic site must have 2+ to 3+HER2 overexpression)
- Use of investigational or unlicensed agents within 30 days prior to study entry
- Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regiments for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed.2), New York, N.Y., Wiley, 1981, pp 13-17).

RESULTS

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test
**p < 0.001 by X^2 test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL®).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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                35                  40                  45

Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
                50                  55                  60

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
                65                  70                  75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
                80                  85                  90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
                95                 100                 105

Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
               110                 115                 120

Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
               125                 130                 135

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys
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Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
35 40 45

Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
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Gly Ala Cys Gln Pro
65

The invention claimed is:

1. A method for the treatment of a human patient with breast cancer that overexpresses ErbB2 receptor, comprising administering a combination of an antibody that binds ErbB2, a taxoid, and a further growth inhibitory agent to the human patient in an amount effective to extend the time to disease progression in the human patient, wherein the antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

2. The method of claim **1** wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

3. The method of claim **1** wherein the antibody cross-blocks binding of 4D5 to the ErbB2 extracellular domain sequence.

4. The method of claim **1** wherein the antibody binds to amino acid residues in the region from about residue 529 to about residue 625 of the ErbB2 extracellular domain sequence.

5. A method for the treatment of a human patient with breast cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of a combination of an anti-ErbB2 antibody which binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid, and a further therapeutic agent, to the human patient.

6. The method of claim **5** wherein the breast cancer is metastatic breast carcinoma.

7. The method of claim **5** wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

8. The method of claim **7** wherein the antibody is administered as a 4 mg/kg dose and then weekly administration of 2 mg/kg.

9. The method of claim **5** wherein the taxoid is paclitaxel.

10. The method of claim **5** wherein efficacy is measured by determining the time to disease progression or the response rate.

11. The method of claim **5**, wherein the further therapeutic agent is selected from the group consisting of: another ErbB2 antibody, EGFR antibody, ErbB3 antibody, ErbB4 antibody, vascular endothelial growth factor (VEGF) antibody, cytokine, and growth inhibitory agent.

12. The method of claim **5** wherein the further therapeutic agent is another ErbB2 antibody.

13. The method of claim **5** wherein the further therapeutic agent is a vascular endothelial growth factor (VEGF) antibody.

14. The method of claim **5** wherein the further therapeutic agent is a growth inhibitory agent.

15. The method of claim **14** wherein the growth inhibitory agent is a DNA alkylating agent.

16. A method for the treatment of a human patient with ErbB2 overexpressing breast cancer, comprising administering a combination of an antibody that binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in the human patient.

17. The method of claim **16** wherein the breast cancer is metastatic breast carcinoma.

* * * * *

EXHIBIT F

US008425908B2

(12) **United States Patent**
Hellmann

(10) **Patent No.: US 8,425,908 B2**
(45) **Date of Patent: Apr. 23, 2013**

(54) **TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventor: **Susan D. Hellmann**, San Carlos, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/185,329**

(22) Filed: **Jul. 18, 2011**

(65) **Prior Publication Data**

US 2012/0034213 A1 Feb. 9, 2012

Related U.S. Application Data

(60) Continuation of application No. 11/780,640, filed on Jul. 20, 2007, now Pat. No. 8,075,892, which is a division of application No. 10/909,998, filed on Aug. 2, 2004, which is a continuation of application No. 09/209,023, filed on Dec. 10, 1998, now abandoned.

(60) Provisional application No. 60/069,346, filed on Dec. 12, 1997.

(51) **Int. Cl.**

A61K 39/395	(2006.01)
C07K 14/75	(2006.01)
C07K 16/28	(2006.01)

(52) **U.S. Cl.**

USPC **424/143.1**; 424/130.1; 424/133.1; 424/134.1; 424/135.1; 424/136.1; 424/138.1; 424/141.1; 424/142.1; 424/155.1; 424/174.1

(58) **Field of Classification Search** 424/130.1, 424/133.1, 134.1, 135.1, 136.1, 138.1, 141.1, 424/142.1, 143.1, 155.1, 174.1

See application file for complete search history.

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Primary Examiner — Misook Yu
Assistant Examiner — Anne Holleran
(74) *Attorney, Agent, or Firm* — Diane Marschang; Ginger R. Dreger; Arnold & Porter LLP

(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The invention further provides a method of treating cancer in a human patient comprising administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to the patient.

7 Claims, 1 Drawing Sheet

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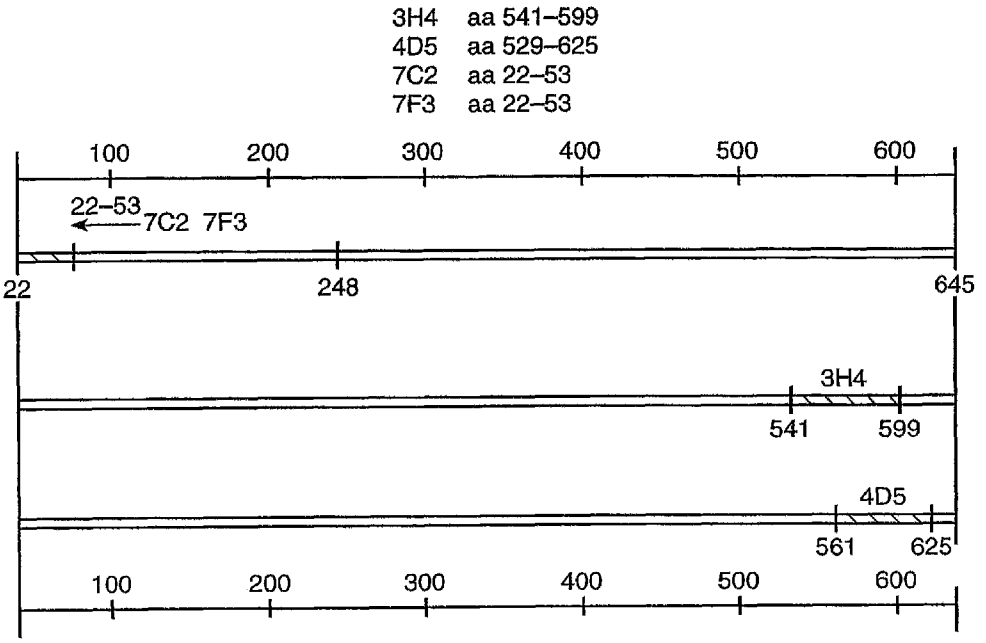

3H4 epitope (SEQ ID NO:8)

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
541 **599**

4D5 epitope (SEQ ID NO:9)

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP
561 **625**

FIG._1

1 MELAALCRWGLLLALLPPGAA**STQVCTGTDMKLRLPA**
38 **SPETHLDMLRHLYQGC**QVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG._2

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This application is a continuation of U.S. application Ser. No. 11/780,640, filed Jul. 20, 2007, now U.S. Pat. No. 8,075,892 which application is a divisional of U.S. application Ser. No. 10/909,998, filed Aug. 2, 2004, which is a continuation of U.S. application Ser. No. 09/209,023, filed Dec. 10, 1998 (now abandoned) which claims priority under 35 U.S.C. Section 119(e) and the benefit of U.S. Provisional Application Ser. No. 60/069,346, filed Dec. 12, 1997, the entire disclosures of which are hereby incorporated by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Sep. 30, 2011, is named GNE0329C.txt and is 5,761 bytes in size.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The invention further provides a method of treating cancer in a human patient comprising administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to the patient.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. In Vitro 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of

murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dysfunction that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

In a further embodiment, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to the patient. The cancer is preferably characterized by overexpression of ErbB2. In one embodiment, the method further comprises administering an anthracyline antibiotic to the patient. In another embodiment, an anthracycline antibiotic is not administered to the patient

with the anti-ErbB2 antibody or cardioprotectant. One or more additional chemotherapeutic agents may also be administered to the patient.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a cardioprotectant to a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2). FIG. **2** discloses the full length sequence as SEQ ID NO: 10.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2", "c-Erb-B2" and "HER2" when used herein refer to the human protein and "her2", "erbB2" and "c-erb-B2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells.

Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most

preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177-244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20): 14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. I, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al., *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

An "ErbB2-expressing cancer" is one comprising cells which have ErbB2 protein present at their cell surface, such that an anti-ErbB2 antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; antimetabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; carminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as antiestrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and antiandrogens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione. Other anthracycline antibiotics include

epirubicin, daunorubicin, carminomycin, detorubicin, esorubicin, marcellomycin, quelamycin, rodorubicin, idarubicin, as well as pharmaceutically active salts, acids or derivatives of any of these.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions,* 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

A "cardioprotectant" is a compound or composition which prevents or reduces myocardial dysfunction (i.e. cardiomyopathy and/or congestive heart failure) associated with administration of a drug, such as an anthracycline antibiotic and/or an anti-ErbB2 antibody, to a patient. The cardioprotectant may, for example, block or reduce a free-radical-mediated cardiotoxic effect and/or prevent or reduce oxidative-stress injury. Examples of cardioprotectants encompassed by the present definition include the iron-chelating agent dexrazoxane (ICRF-187) (Seifert et al. *The Annals of Pharmacotherapy* 28:1063-1072 [1994]); a lipid-lowering agent and/or anti-oxidant such as probucol (Singal et al. *J. Mol. Cell Cardiol.* 27:1055-1063 [1995]); amifostine (aminothiol 2-[(3-aminopropyl)amino]ethanethiol-dihydrogen phosphate ester, also called WR-2721, and the dephosphorylated cellular uptake form thereof called WR-1065) and S-3-(3-methylaminopropylamino)propylphosphorothioic acid (WR-151327), see Green et al. *Cancer Research* 54:738-741 (1994); digoxin (Bristow, M. R. In: Bristow M R, ed. *Drug-Induced Heart Disease*. New York: Elsevier 191-215 [1980]); beta-blockers such as metoprolol (Hjalmarson et al. *Drugs* 47:Suppl 4:31-9 [1994]; and Shaddy et al. *Am. Heart J.* 129:197-9 [1995]); vitamin E; ascorbic acid (vitamin C); free radical scavengers such as oleanolic acid, ursolic acid and N-acetylcysteine (NAC); spin trapping compounds such as alpha-phenyl-tert-butyl nitrone (PBN); (Paracchini et al., *Anticancer Res.* 13:1607-1612 [1993]); selenoorganic compounds such as P251 (Elbesen); and the like.

II. Production of Anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS* (*USA*) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuc-

cinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130: 151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348: 552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to

have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in W096/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow

cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODEFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl Acad. Sci. USA,* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A che-

motherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)(5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse

isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and cardioprotectants of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The present invention contemplates admin-

istration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

The present invention also provides a method of treating ErbB2 expressing cancer, especially cancer characterized by overexpression of ErbB2, by administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to a patient susceptible to, or diagnosed with, cancer.

In one embodiment, the method further comprises administering one or more chemotherapeutic agents to the patient. For example, an anthracycline antibiotic may be administered to the patient along with the cardioprotectant and anti-ErbB2 antibody (as well as one or more other optional chemotherapeutic agents).

In an alternative embodiment, an anthracycline antibiotic is not administered to the patient with the anti-ErbB2 antibody or cardioprotectant. Thus, while the patient may have been exposed to an anthracycline antibiotic in a previous treatment regimen, in this embodiment of the invention, the therapeutic regimen does not entail the administration of an antracycline antibiotic (i.e. the method is performed in the absence of an anthracycline antibiotic). This embodiment of the invention contemplates however the optional administration of one or more other chemotherapeutic agents (other than anthracycline antibiotics) to the patient.

Combined administration of the anti-ErbB2 antibody and cardioprotectant includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Moreover, administration of the cardioprotectant may be delayed until the patient has been given a lifetime dose of anti-ErbB2 antibody and/or anthracycline antibiotic which is considered to result in a high risk of cardiac side effects (e.g. a lifetime doxorubicin dose of 350 mg/m^2). In one embodiment, the cardioprotectant is administered to the patient both prior to administration of the anti-ErbB2 antibody and concurrently therewith.

The effective amount of the cardioprotectant is that which reduces or prevents myocardial dysfunction (e.g. cardiomyopathy and/or congestive heart failure) resulting from administration of the anti-ErbB2 antibody (and optionally an anthracycline antibiotic or other chemotherapeutic agent) to the patient. Myocardial dysfunction can be assessed by a variety of different methods including physical examination, electrocardiography, echocardiography, angiocardiography, and endomyocardial biopsy (reviewed in Singal et al. *NEJM* 339:900-905 (1998), expressly incorporated herein by reference). The preferred method for determining myocardial dysfunction comprises measuring the ejection fraction by echocardiography or angiocardiography. Suitable dosages for the cardioprotectants dexrazoxane and probucol have been described, for example, in Seifert et al. *The Annals of Pharmacotherapy* 28:1063-1072 (1994) and Singal et al. *J. Mol. Cell Cardiol.* 27:1055-1063 (1995), respectively. The effective amount of the cardioprotectant may be determined based on the amount of anti-ErbB2 antibody and/or anthracycline antibiotic agent administered to the patient. Generally, the amount of cardioprotectant will exceed the amount of chemotherapeutic agent or anti-ErbB2 antibody causing the cardiotoxicity. For example, the ratio of cardioprotectant: antibody or chemotherapeutic agent may be from about 1:1 to about 100:1.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in

the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including for example a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin, or instructing the user of the composition to administer the anti-ErbB2 antibody composition and a cardioprotectant to a patient.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

Example

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci.* (*USA*) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody) (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of $p185^{HER2}$, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

- Metastatic breast cancer
- Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].
- Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

- The ability to understand and willingness to sign a written informed consent form
- Women≧18 years
- Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

- Prior cytotoxic chemotherapy for metastatic breast cancer Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.
- Concomitant malignancy that has not been curatively treated
- A performance status of <60% on the Karnofsky scale
- Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator
- Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)
- Use of investigational or unlicensed agents within 30 days prior to study entry
- Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regiments for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP (months)	RR (%)	AE (%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*$p < 0.001$ by log-rank test
**$p < 0.01$ by X^2 test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade 3/4) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL®).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

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Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu Ile Ala His Asn
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Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg Ile Val Arg Gly Thr
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Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala Val Leu Asp Asn Gly Asp
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Pro Leu Asn Asn Thr Thr Pro Val Thr Gly Ala Ser Pro Gly Gly Leu
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Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
65                  70                  75                  80

Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
                85                  90                  95

Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
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Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
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Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
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Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145                 150                 155                 160

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175

Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala
            180                 185                 190
```

What is claimed is:

1. A method for the treatment of a human patient with gastric cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of a combination of an anti-ErbB2 antibody, or an antigen-binding fragment thereof, and a chemotherapeutic agent, to the human patient, wherein the chemotherapeutic agent is capecitabine or 5-fluorouracil, wherein the attending physician is provided with instructions including a warning that the treatment is not to be performed in combination with an anthracycline derivative and wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

2. The method of claim **1** wherein the chemotherapeutic agent is capecitabine.

3. The method of claim **2** wherein said antibody is a humanized 4D5 anti-ErbB2 antibody, or an antigen-binding fragment thereof.

4. The method of claim **3** wherein the antibody is rhuMAb HER2.

5. The method of claim **1** further comprising the administration of a further chemotherapeutic agent.

6. The method of claim **5** wherein the further chemotherapeutic agent is cisplatin.

7. The method of claim **1** wherein efficacy is defined in terms of time to disease progression or response rate.

* * * * *

EXHIBIT G

US006627196B1

(12) **United States Patent**
Baughman et al.

(10) **Patent No.: US 6,627,196 B1**
(45) **Date of Patent: Sep. 30, 2003**

(54) **DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventors: **Sharon A. Baughman**, Ventura, CA (US); **Steven Shak**, Burlingame, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/648,067**

(22) Filed: **Aug. 25, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/213,822, filed on Jun. 23, 2000, and provisional application No. 60/151,018, filed on Aug. 27, 1999.

(51) **Int. Cl.**[7] **A61K 39/395**

(52) **U.S. Cl.** **424/138.1**; 424/131.1; 424/133.1; 424/134.1; 424/135.1; 424/136.1; 424/137.1; 424/139.1; 424/141.1; 424/142.1; 424/143.1; 424/144.1; 424/145.1; 424/146.1; 424/147.1; 424/150.1; 424/151.1; 424/152.1; 424/153.1; 424/154.1; 424/155.1; 424/156.1; 424/158.1; 424/172.1; 424/174.1

(58) **Field of Search** 424/130.1, 138.1, 424/141.1, 142.1, 152.1, 155.1, 131.1, 133.1, 134.1, 135.1, 136.1, 137.1, 139.1, 143.1, 144.1, 145.1, 146.1, 147.1, 150.1, 151.1, 153.1, 154.1, 156.1, 158.1, 172.1, 174.1

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(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with anti-ErbB2 antibody.

33 Claims, 5 Drawing Sheets

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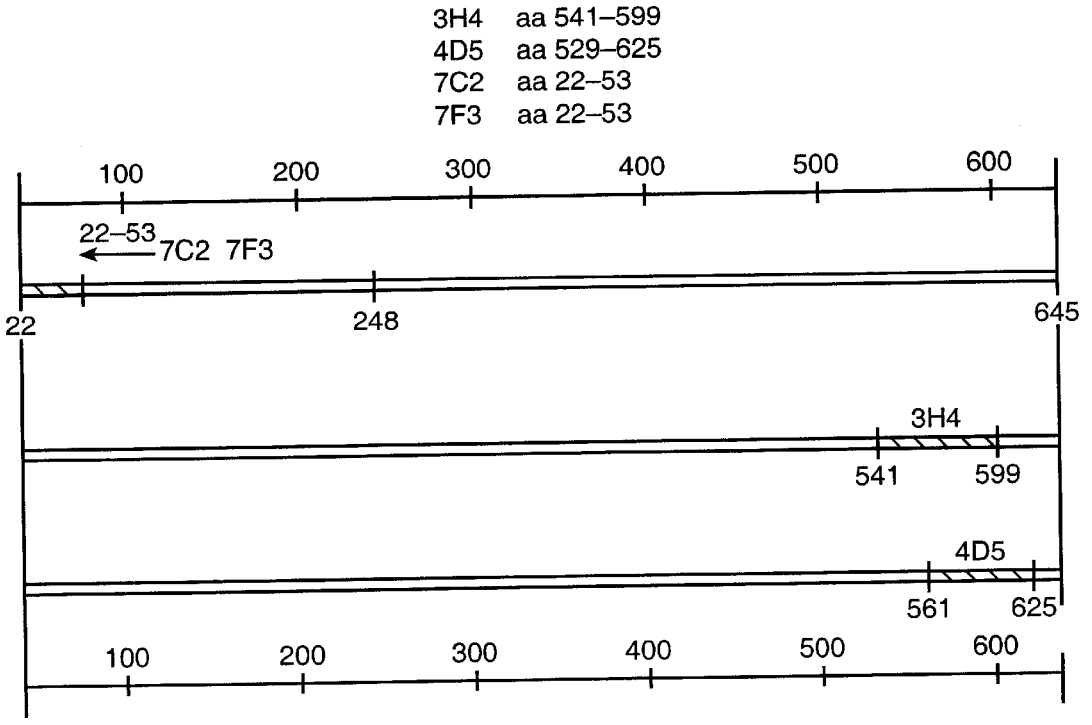

3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR

541 **599**

4D5 epitope (SEQ ID NO:9) 64 residues

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP

561 **625**

FIG._1

1 MELAALCRWGLLLALLPPGAA**STQVCTGTDMKLRLPA**

38 **SPETHLDMLRHLYQGC**QVVQGNLELTYLPTNASLSFL

75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN

112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI

149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID

186 TNRSRA

FIG._2

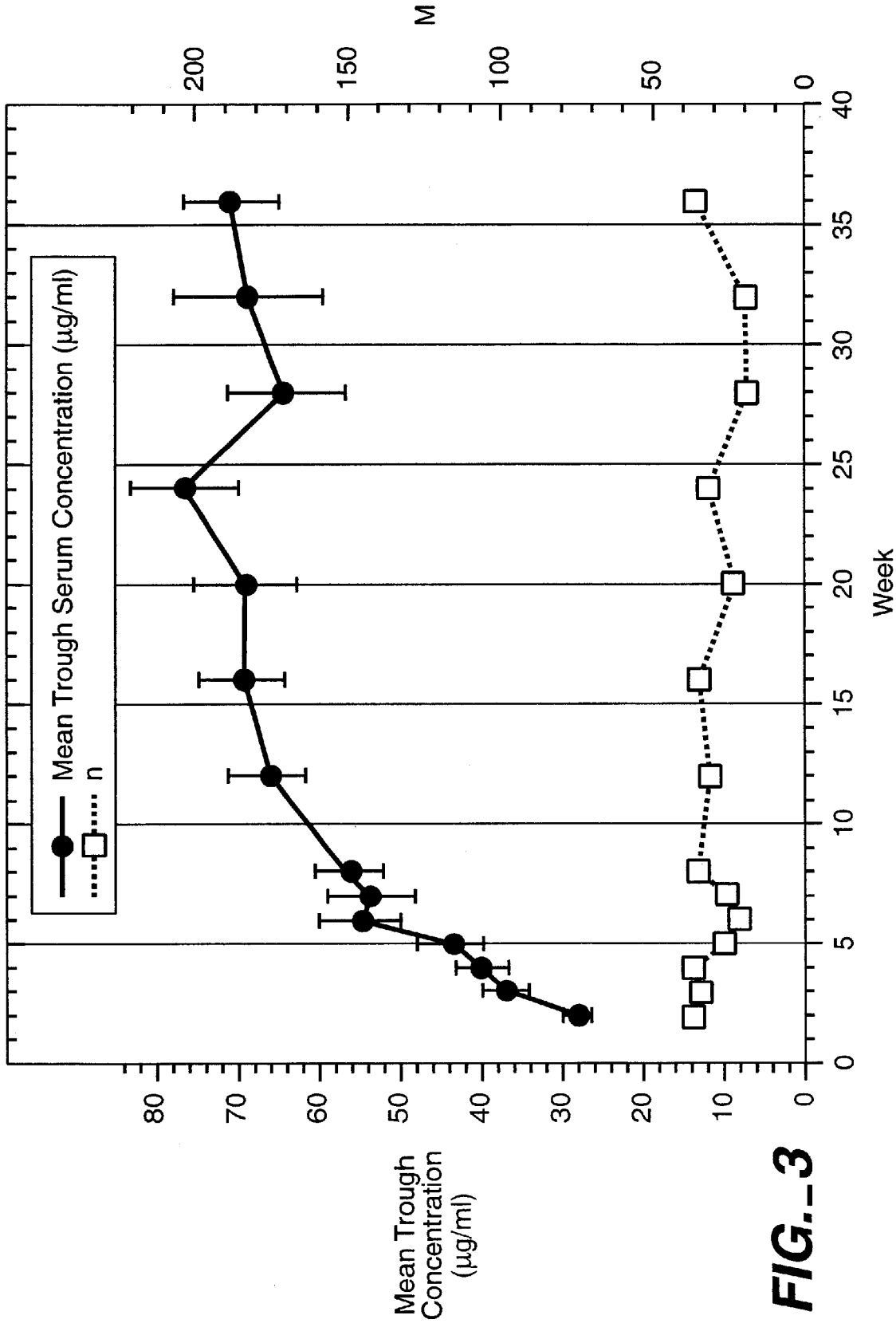
Mean Trough Serum Concentration (µg/ml)
n
Mean Trough Concentration (µg/ml)
M
Week
0
10
20
30
40
50
60
70
80
0
50
100
150
200
0
5
10
15
20
25
30
35
40

FIG._3

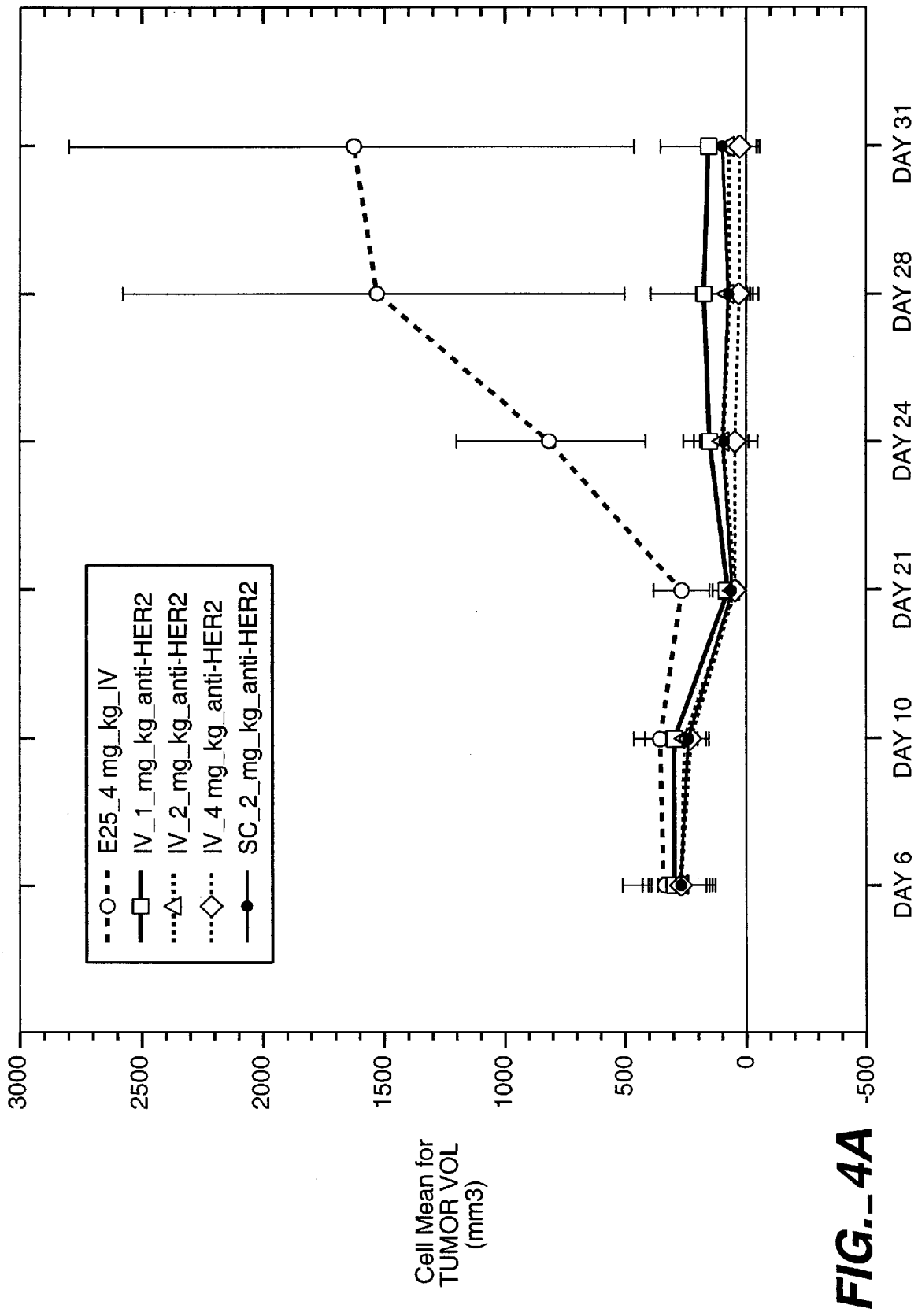
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
3000
2500
2000
1500
1000
500
0
-500
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4A

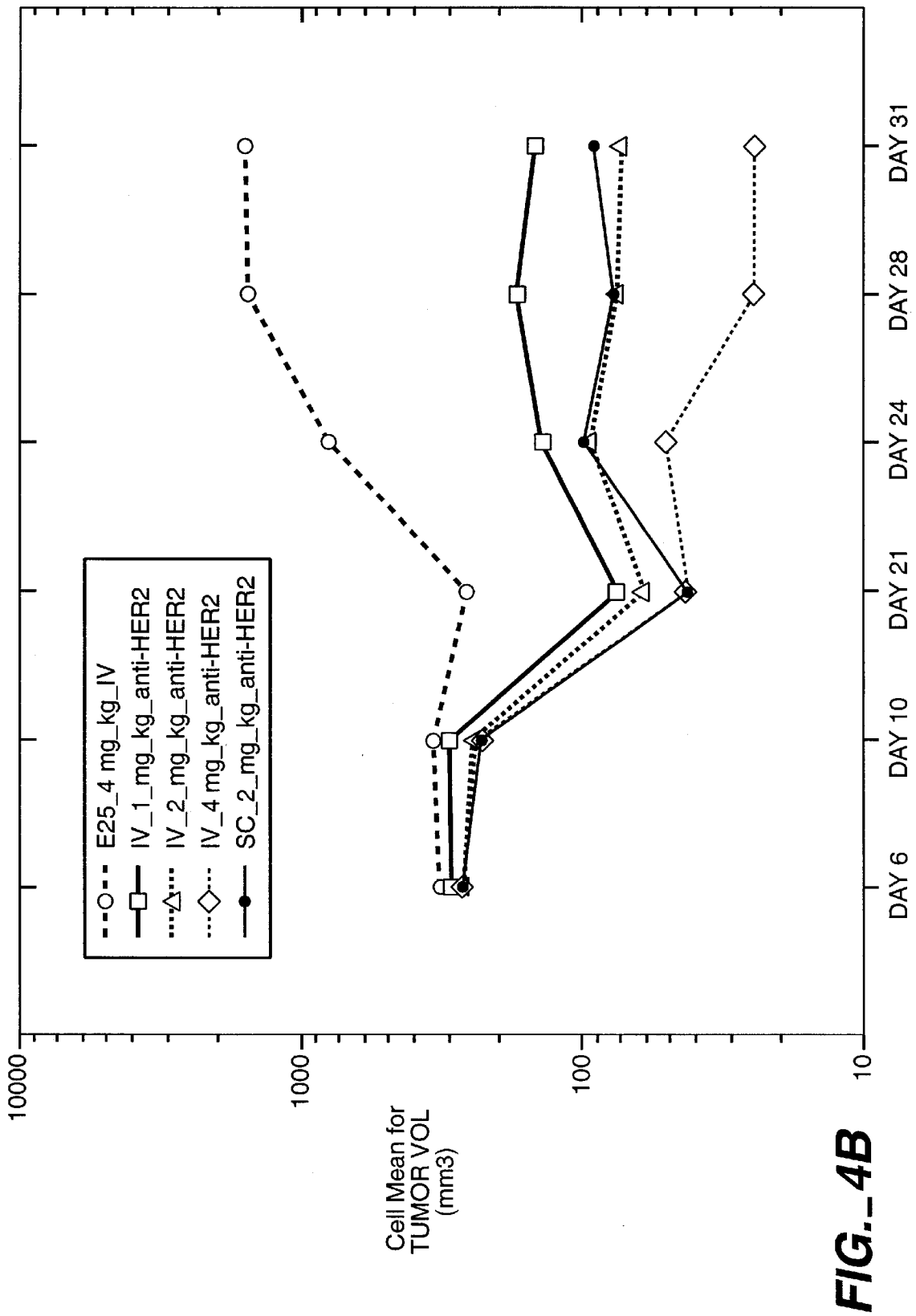
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
10000
1000
100
10
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4B

VARIABLE LIGHT

```
          1       10        20          30              40
2C4       DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA----] WYQQRP
           **    **** *      *                        *
574       DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA----] WYQQKP
                                   *   *  ****
hum kI    DIQMTQSPSSLSASVGDRVTITC [RASQSVSTSSYSYMH] WYQQKP

                    50          60        70        80
2C4       GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
           **                   *  *          *    * *
574       GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                     *  ****
hum kI    GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                   90          100
2C4       EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:10)
            * *                  *   *
574       EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:12)
                      ***
hum kI    EDFATYYC [QQYNSLPYT] FGQGTKVEIK (SEQ ID NO:14)
```

FIG._5A

VARIABLE HEAVY

```
          1       10        20          30          40
2C4       EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
              **  **  *  * ***  **                   *
574       EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                     ** * *
humIII    EVQLVESGGGSVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                    50        60          70        80
2C4       HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                     *** *    **** *
574       PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                  *  ******  ** ****       * * *
humIII    PGKGLEWVS [VISGDGGSTYYADSVKG] RFTISRDDSKNTLYL

                     90         100          110
2C4       ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLVTSS (SEQ ID NO:11)
          ***  **                              *
574       QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:13)
                            ** ***
humIII    QMNSLRAEDTAVYYCAR [GRGGGS--DY] WGQGTLVTVSS (SEQ ID NO:15)
```

FIG._5B

DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES

RELATED APPLICATIONS

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application No. 60/151,018, filed Aug. 27, 1999 and No. 60/213,822, filed Jun. 23, 2000, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2 or disorders expressing epidermal growth factor receptor (EGFR), comprising administering to a human or animal presenting the disorders a therapeutically effective amount of an antibody that binds ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 or expressing EGFR, where the treatment is with an anti-ErbB2 antibody administered by front loading the dose of antibody during treatment by intravenous and/or subcutaneous administration. The invention optionally includes treatment of cancer in a human patient with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, a taxoid. The taxoid may be, but is not limited to paclitaxel or docetaxel. The invention further includes treatment of cancer in a human patient with a combination of anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, an anthracycline derivative. Optionally, treatment with a combination of anti-ErbB2 and an anthracycline derivative includes treatment with an effective amount of a cardioprotectant. The present invention further concerns infrequent dosing of anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177–182 [1987]; Slamon et al., *Science* 244:707–712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159–7163 [1987]; DiFiore et al., *Science* 237:78–182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578–10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695–706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) a inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129–9133 (1986), the 7.16.4 antibody was shown to

inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387–394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273–277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277–290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165–1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel,including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p 185 -overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550–1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72–82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117–127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979–986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255–263 (1993); Pietras et al. *Oncogene* 9:1829–1838 (1994); Vitetta et al. *Cancer Research* 54:5301–5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20): 14661–14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300–5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.*91:7202–7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933–937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543–548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361–5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350–362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691–8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580–2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth, whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress-ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et a. *Int. J. Cancer* 53:401–408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771–2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575–4580 (1991); Shawver et al. *Cancer Res.* 54:1367–1373 (1994); Arteaga et al. *Cancer Res.* 54:3758–3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160–15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2, HERCEPTIN®, or HERCEPTIN® anti-ErbB2 antibody) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin Oncol.* 14:737–744 [1996]). The recommended initial loading dose for HERCEPTIN®O is 4 mg/kg administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg and can be administered as a 30-minute infusion if the initial loading dose is well tolerated.

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], *supra*; Ravdin and Chamness, *Gene* 159:19–27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165–184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11 (3 Suppl 1):43–48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the discovery that an early attainment of an efficacious target trough serum concentration by providing an initial dose or doses of anti-ErbB2 antibodies followed by subsequent doses of equal or smaller amounts of antibody (greater front loading) is more efficacious than conventional treatments. The efficacious target trough serum concentration is reached in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The target serum concentration is thereafter maintained by the administration of maintenance doses of equal or smaller amounts for the remainder of the treatment regimen or until suppression of disease symptoms is achieved.

The invention further concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of an anti-ErbB2 antibody subcutaneously. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance to the anti-ErbB2 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable.

According to the invention, the method of treatment involves administration of an initial dose of anti-ErbB2 antibody of more than approximately 4 mg/kg, preferably more than approximately 5 mg/kg. The maximum initial dose or a subsequent dose does not exceed 50 mg/kg, preferably does not exceed 40 mg/kg, and more preferably does not exceed 30 mg/kg. Administration is by intravenous or subcutaneous administration, preferably intravenous infusion or bolus injection, or more preferably subcutaneous bolus injection. The initial dose may be one or more administrations of drug sufficient to reach the target trough serum concentration in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less.

According to the invention, the initial dose or doses is/are followed by subsequent doses of equal or smaller amounts of antibody at intervals sufficiently close to maintain the trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or subsequent dose does not exceed 50 mg/kg, and each subsequent dose is at least 0.01 mg/kg. Preferably the amount of drug

administered is sufficient to maintain the target trough serum concentration such that the interval between administration cycles is at least one week. Preferably the trough serum concentration does not exceed 2500 μg/ml and does not fall below 0.01 μg/ml during treatment. The front loading drug treatment method of the invention has the advantage of increased efficacy by reaching a target serum drug concentration early in treatment. The subcutaneous delivery of maintenance doses according to the invention has the advantage of being convenient for the patient and health care professionals, reducing time and costs for drug treatment. Preferably, the initial dose (or the last dose within an initial dose series) is separated in time from the first subsequent dose by 4 weeks or less, preferably 3 weeks or less, more preferably 3 weeks or less, most preferably 1 week or less.

In an embodiment of the invention, the initial dose of anti-ErbB2 is 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous administration, such as intravenous infusion or subcutaneous bolus injection. The subsequent maintenance doses are 2 mg/kg delivered once per week by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated, the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In another embodiment, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In still another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

In yet another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

In another embodiment, the invention includes initial doses of at least 1 mg/kg, preferably 4 mg/kg, anti-ErbB2 antibody on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In another embodiment, the invention includes an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

In still another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is 2–3 times per week for 3 weeks. In one embodiment of the invention, each dose is approximately 25 mg/kg or less for a human patient, preferably approximately 10 mg/kg or less. This 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

In another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The method of the invention may further comprise administration of a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind o the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form. Other preferred ErbB2-binding antibodies include, but are not limited to, antibodies 7C2, 7F3, and 2C4, preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

The present application also provides a method of therapy involving infrequent dosing of an anti-ErbB2 antibody. In particular, the invention provides a method for the treatment of cancer (e.g. cancer characterized by overexpression of the ErbB2 receptor) in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). For instance, the antibody may be administered about every three weeks, about two to about 20 times, e.g. about six times. The first dose and subsequent dose may each be from about 2 mg/kg to about 16 mg/kg; e.g. from about 4 mg/kg to about 12 mg/kg; and optionally from about 6 mg/kg to about 12 mg/kg. Generally, two or more subsequent doses (e.g. from about two to about ten subsequent doses) of the antibody are administered to the patient, and those subsequent doses are preferably separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). The two or more subsequent doses may each be from about 2 mg/kg to about 16 mg/kg; or from about 4 mg/kg to about 12 mg/kg; or from about 6 mg/kg to about 12 mg/kg. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, and a package insert containing instructions to administer the antibody according to such methods.

The presently described dosing protocols may be applied to other anti-ErbB antibodies such as anti-epidermal growth factor receptor (EGFR), anti-ErbB3 and anti-ErbB4 antibodies. Thus, the invention provides a method for the treatment of cancer in a human patient, comprising administering an effective amount of an anti-ErbB antibody to the human patient, the method comprising administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose. Alternatively, or additionally, the invention pertains to a method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two

weeks. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB antibody, and a package insert containing instructions to administer the antibody according to such methods.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition. According to the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody at an initial dose of 5 mg/kg followed by the same or smaller subsequent dose or doses. In another embodiment of the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose, most preferably for all doses.

In a further aspect, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering to the patient effective amounts of an anti-ErbB2 antibody and a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is a taxoid including, but not limited to, paclitaxel and docetaxel. In another embodiment, the chemotherapeutic agent is an anthracyline derivative including, but not limited to, doxorubicin or epirubicin. In still another embodiment of the invention, treatment with an anti-ErbB2 antibody and an anthracycline derivative further includes administration of a cardioprotectant to the patient. In still another embodiment, an anthracycline derivative is not administered to the patient with the anti-ErbB2 antibody. One or more additional chemotherapeutic agents may also be administered to the patient. The cancer is preferably characterized by overexpression of ErbB2.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a chemotherapeutic agent to a patient. In another embodiment, the chemotherapeutic agent is other than an anthracycline, and is preferably a taxoid, such as TAXOL®. In still another embodiment, the chemotherapeutic agent is an anthracycline, including but not limited to, doxorubicin or epirubicin. In yet another embodiment, the chemotherapeutic agent is an anthracycline and the package insert further instructs the user to administer a cardioprotectant.

The methods and compositions of the invention comprise an anti-ErbB2 antibody and include a humanized anti-ErbB2 antibody. Thus, the invention further pertains to a composition comprising an antibody that binds ErbB2 and the use of the antibody for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. The invention also pertains to the use of the antibody for treating EGFR expressing cancer. Preferably the antibody is a monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5–8 (HERCEPTIN® anti-ErbB2 antibody); or monoclonal antibody 2C4, e.g., humanized 2C4. The antibody may be an intact antibody (e.g., an intact IgG, antibody) or an antibody fragment (e.g., a Fab, $F(ab')_2$, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-ErbB2 antibody 2C4 are shown in FIGS. **5**A and **5**B.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67 (10):6179–6191 [October 1993]; Renz et *J. Cell Biol.* 125(6):1395–1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 b bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2–4 hours at 4° C. The complexes were precipitated, applied to a 10–20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

FIG. **3** is a graph of anti-ErbB2 antibody (HERCEPTIN®) trough serum concentration (μg/ml, mean ±SE, dark circles) by week from week 2 through week 36 for ErbB2 overexpressing patients treated with HERCEPTIN® anti-ErbB2 antibody at 4 mg/kg initial dose, followed by 2 mg/kg weekly. The number of patients at each time point is represented by "n" (white squares).

FIG. **4**A is a linear plot of tumor volume changes over time in mice treated with HERCEPTIN® anti-ErbB2 antibody. FIG. **4**B is a semi-logarithmic plot of the same data as in FIG. **4**A such that the variation in tumor volume for the treated animals is observed more readily.

FIGS. **5**A and **5**B depict alignments of the amino acid sequences of the variable light (V_L)(FIG. **5**A) and variable heavy (V_H) (FIG. **5**B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 10 and 11, respectively); V_L and V_H domains of humanized Fab version 574 (SEQ ID Nos. 12 and 13, respectively), and human V_L and V_H consensus frameworks (hum κl, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 14 and 15, respectively). Asterisks identify differences between humanized Fab version 574 and murine monoclonal antibody 2C4 or between humanized Fab version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g. substituted as follows-IleL2Thr; ArgL54Leu; TyrL55Glu; ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured in order to further improve or refine its affinity and/or other biological activities.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

An "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3 and ErbB4 receptors as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881–914 (1987), including variants thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207–4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL RB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.).

"ErbB3" and"HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193–9197 (1989), including variants thereof. Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof.

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA*, 90:1746–1750 (1993); and Plowman et al., *Nature*, 366:473–475 (1993), including variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein, and "erbB2," "c-erb-B2," and "her2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS (USA)* 82:6497–650 (1985)and Yamamoto et al. *Nature* 319:230–234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1–4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N-terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1–11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed-in the example herein; and nuclear/chromatin condensation-along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the

ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0–10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545–548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science*, 256:1205–1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.*, 14(3): 1909–1919 (1994); *Nature*, 362:312–318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177\text{-}244}$)

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661–14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91–3242, Vol. I, pages 647–669 [1991]). The constant domains involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H–V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is use d in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal

antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.* 8(10): 1057–1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991), for example

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851–6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human inmmunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522–525 (1986); Reichmann et al., *Nature*, 332:323–329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593–596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269–315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H–V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444–6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see Example 1, below). Therapeutically effective amount also refers to a target serum concentration, such as a trough serum concentration, that has been shown to be effective in suppressing disease symptoms when maintained for a period of time.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY 117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W B Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,

3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375–382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247–267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g.,an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum HERCEPTIN® anti-ErbB2 antibody concentration" refers to the concentration of a drug, such as HERCEPTIN® anti-ErbB2 antibody, in the blood serum of an animal or human patient being treated with the drug. Serum concentration of HERCEPTIN® anti-ErbB2 antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is an ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient,

preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example,- by pinching or drawing the skin up and away from underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration. According to the present invention, front loading is achieved by an initial dose or doses delivered over three weeks or less that causes the animal's or patient's serum concentration to reach a target serum trough concentration. Preferably, the initial front loading dose or series of doses is administered in two weeks or less, more preferably in 1 week or less, including 1 day or less. Most preferably, where the initial dose is a single dose and is not followed by a subsequent maintenance dose for at least 1 week, the initial dose is administered in 1 day or less. Where the initial dose is a series of doses, each dose is separated by at least 3 hours, but not more than 3 weeks or less, preferably 2 weeks or less, more preferably 1 week or less, most preferably 1 day or less. To avoid adverse immune reaction to an antibody drug such as an anti-ErbB2 antibody (e.g., HERCEPTIN® anti-ErbB2 antibody) in an animal or patient who has not previously been treated with the antibody, it may be preferable to deliver initial doses of the antibody by intravenous infusion. The present invention includes front loading drug delivery of initial and maintenance doses by infusion or bolus administration, intravenously or subcutaneously.

Published information related to anti-ErbB2 antibodies includes the following issued patents and published applications: PCT/US89/00051,published Jan. 5,1989; PCT/US90/02697, published May 18,1990; EU 0474727 issued Jul. 23, 1997; DE 69031120.6, issued Jul. 23, 1997; PCT/US97/18385, published Oct. 9 1997; SA 97/9185, issued Oct. 14, 1997; U.S. Pat. No. 5,677,171, issued Oct. 14, 1997; U.S. Pat. No. 5,720,937, issued Feb. 24, 1998; U.S. Pat. No. 5,720,954, issued Feb. 24, 1998; U.S. Pat. No. 5,725,856, issued Mar. 10, 1998; U.S. Pat. No. 5,770,195, issued Jun. 23, 1998; U.S. Pat. No. 5,772,997, issued Jun. 30, 1998; PCT/US98/2626, published Dec. 10, 1998; and PCT/US99/06673, published Mar. 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., *PNAS (USA)* 88:8691–8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC- 11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51–63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells-is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-M EM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional imnmunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256–262 (1993) and Plückthun, *Immunol. Revs.*, 130:151–188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990). Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779–783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265–2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA*, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import"variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522–525 (1986); Riechmann et al., *Nature*, 332:323–327 (1988); Verhoeyen et al., *Science* 239:1534–1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the

functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581–597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al. , *Journal of Biochemical and Biophysical Methods* 24:107–117(1992) and Brennan et al., *Science*, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10: 163–167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537–539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target

immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547–1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444–6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies With the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM) :Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50–100%, the SKBR3 assay described in WO 89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish

(2 mls/35 mm dish) 2.5μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50–100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191–1195 (1992) and Shopes, B. *J. Immunol.* 148:2918–2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560–2565 (1993). Alternatively, an antibody can be engineered which has dual Fe regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219–230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as inununoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). S Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., *J. Biol. Chem.* 257: 286–288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al., *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active

portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312: 604–608 [1984]).

(xi) Antibody-salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H, region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163–167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1–13 [1983]). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5–4.5, preferably performed at low salt concentrations (e.g. from about 0–0.25M salt).

III. Determination of anti-ErbB2 Antibody Concentration in Serum

The following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific rhuMAb HER2 (humanized anti-$p185^{HER2}$ monoclonal antibody, including HERCEPTIN® anti-ErbB2 antibody) in a body fluid of a mammal including, but not limited to, serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids, and ocular vitreous gel.

Plate Binding Activity Assay for rhuMAb HER2 (Humanized Anti-$p185^{HER2}$ Monoclonal Antibody

The method of assaying rhuMAb HER2 described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of rhuMAb HER2 (Genentech, Inc., South San Francisco, Calif.), controls, and serum samples were diluted with Assay Diluent (PBS/0.5% BSA/0.05% Polysorbate 20/0.01% Thimerosal). The dilutions of standardized rhuMAb HER2 were prepared to span a range of concentrations useful for a standard curve. The samples were diluted to fall within the standard curve.

An aliquot of Coat Antigen in Coating buffer (recombinant $p185^{HER2}$ (Genentech, Inc.) in 0.05 M sodium carbonate buffer) was added to each well of a microtiter plate and incubated at 2–8° C. for 12–72 hours. The coatin solution was removed and each well was washed six times with water, then blotted to remove excess water.

An aliquot of Assay Diluent was added to each well and incubated for 1–2 hours at ambient temperature with agitation. The wells were washed as in the previous step.

Aliquots of diluted standard, control and sample solutions were added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish peroxidase; Organon Teknika catalog #55253 or equivalent) was diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution was added to each well and incubated at ambient temperature for 1 hour with agitation. The wells were washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in 12.5 ml 4 mM H_2O_2 in PBS) was added to each well and incubated for a sufficient period of time (approximately 8–10 minutes) in the dark at ambient temperature to allow color development. The reaction was stopped with an aliquot of 4.5 N sulfuric acid. Optical density was read at 490–492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; met al complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial growth factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

V. Treatment With the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as

intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

The treatment of the present invention involves the administration of an anti-ErbB2 antibody to an animal or human patient, followed at intervals by subsequent doses of equal or smaller doses such that a target serum concentration is achieved and maintained during treatment. Preferably, maintenance doses are delivered by bolus delivery, preferably by subcutaneous bolus administration, making treatment convenient and cost-effective for the patient and health care professionals.

Where combined administration of a chemotherapeutic agent (other than an antracycline) is desired, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF). Alternatively, or additionally, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. The ErbB2 antibody may be co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration, or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

In addition to the above therapeutic regimens, the patient may be subjected to surgical removal of cancer cells and/or-radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of anti-ErbB2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Where the treatment involves a series of treatments, the initial dose or initial doses are followed at daily or weekly intervals by maintenance doses. Each maintenance dose provides the same or a smaller amount of antibody compared to the amount of antibody administered in the initial dose or doses.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1–20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays.

According to the invention, dosage regimens may include an initial dose of anti-ErbB2 of 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous infusion, followed by subsequent weekly maintenance doses of 2 mg/kg by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

Alternatively, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

Another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

Still another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

As an alternative regimen, initial doses of 4 mg/kg anti-ErbB2 antibody may be administered on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

An additional regimen involves an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

Alternatively, the invention may include a cycle of dosing in which delivery of anti-ErbB2 antibody is 2–3 times per week for 3 weeks. The 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The invention further includes a cyclic dosage regimen in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms. Further information about suitable dosages is provided in the Examples below.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may comprise a package inserts with instruc-

tions for use, including, e.g., a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Preparation and Efficacy of HERCEPTIN® Anti-ErbB2 Antibody

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50: 1550–1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2–3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al., *Proc. Natl. Acad. Sci.* (*USA*) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN® anti-ErbB2 antibody). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285–4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of p_{185}^{HER2}, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® anti-ErbB2 antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria

Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffim-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for p_{185}^{HER2}].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women ≧18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria

Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer

Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® anti-ErbB2 antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered

intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® anti-ErbB2 antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3–5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease

Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response

Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response

A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response

A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease

No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed.2), New York, N.Y., Wiley, 1981, pp 13–17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN® anti-ErbB2 antibody, without increase in overall severe adverse events (AE):

TABLE 1

HERCEPTIN ® Anti-ErbB2 Antibody Efficacy

	Enrolled	TTP (months)	RR (%)	AE (%)
CRx	234	5.5	36.2	66
CRx + 14	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*$p < 0.001$ by log-rank test; ** $p < 0.01$ by X^2 test; CRx: chemotherapy; AC: anthracycline/cyclophosphamide treatment; H: HERCEPTIN ® anti-ErbB2 antibody; T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor treatment with HERCEPTIN® anti-ErbB2 antibody and paclitaxel (TAXOL®) where a combined treatment regimen is desired.

Example 2

Pharmacokinetic and Pharmacodynamic Properties of Anti-ErbB2 Antibody (HERCEPTIN®)

HERCEPTIN® anti-ErbB2 antibody was administered by intravenous, infusion to human patients selected according to the criteria provided in Example 1. An initial dose of 4 mg/kg HERCEPTIN® anti-ErbB2 antibody was delivered by intravenous infusion, followed by subsequent i.v. infusions of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody weekly for several weeks. Two hundred thirteen patients began this treatment regimen and serum drug concentration was obtained beyond 8 weeks for fewer than 90 patients as selective discontinuation of patients with rapidly progressing disease occurred. Of the 213 patients who began treatment, serum trough concentration data were available for 80 patients at Week 12, for 77 patients at Week 16, for 44 patients at Week 20, for 51 patients at Week 24, for 25 patients at Week 28, for 23 patients at Week 32, and for 37 patients at Week 36.

HERCEPTIN® anti-ErbB2 Antibody Trough Serum Concentrations for Weeks 0–36

The HERCEPTIN® anti-ErbB2 antibody trough serum concentrations (μg/ml, mean±SE) from Week 2 through Week 36 are plotted in FIG. **3** (dark circles). The number of patients was fairly constant because data from patients discontinued from the program due to rapidly progressing disease were excluded from this analysis. Trough serum concentrations tended to increase through Week 12 and tended to plateau after that time.

HERCEPTIN® anti-ErbB2 Antibody Trough and Peak Serum Concentrations for Weeks 1–8

Some HERCEPTIN® anti-ErbB2 antibody serum concentration data were available for 212 of the original 213 patients. Trough and peak serum concentration data reflecting the first HERCEPTIN® anti-ErbB2 antibody infusion

were available for 195 of the 212 patients. For the seventh infusion, trough serum concentration data were available for 137/212 patients and peak serum concentration data were available for 114/212 patients. Table 2 presents a summary of statistics from trough and peak serum concentrations for the first 8 weeks of treatment. Peak samples were drawn shortly after the end of HERCEPTIN® anti-ErbB2 antibody administration; trough samples were drawn prior to the subsequent dose (i.e., 1 week later). Serum concentrations of HERCEPTIN® anti-ErbB2 antibody were determined as disclosed herein.

TABLE 2

HERCEPTIN ® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for the First 8 Weeks of Treatment (μg/ml)

	Dose Number	n	Mean	SD	Minimum	Maximum
Peak	1	195	100.3	35.2	30.7	274.6
Trough		195	25.0	12.7	0.16	60.7
Peak	2	190	74.3	31.3	20.8	307.9
Trough		167	30.4	16.0	0.2	74.4
Peak	3	167	75.3	26.8	16.1	194.8
Trough		179	33.7	17.9	0.2	98.2
Peak	4	175	80.2	26.9	22.2	167
Trough		132	38.6	20.1	0.2	89.4
Peak	5	128	85.9	29.2	27.8	185.8
Trough		141	42.1	24.8	0.2	148.7
Peak	6	137	87.2	32.2	28.9	218.1
Trough		115	43.2	24.0	0.2	109.9
Peak	7	114	89.7	32.5	16.3	187.8
Trough		137	48.8	24.9	0.2	105.2
Peak	8	133	95.6	35.9	11.4	295.6

The data in Table 2 suggest that there was an increase in trough serum concentration over time. Of the many patients studied, there were 18 patients for whom the trough concentrations did not exceed 20 μg/ml from Week 2 through Week 8. A HERCEPTIN® anti-ErbB2 antibody trough serum concentration of 20 μg/ml was nominally targeted for these studies based on prior pharmacologic studies in animals and exploratory analyses in clinical trials.

Patient response status was evaluated relative to serum concentration of HERCEPTIN® anti-ErbB2 antibody. For this purpose, mean serum concentration (an average of troughs and peaks) was calculated for various times and patient response status (where the patient response status was determined by an independent Response Evaluation Committee). The increase in serum concentration between Weeks 2 and 8 appeared to be greater in responders than in nonresponders, suggesting that there is a relationship between response status and HERCEPTIN® anti-ErbB2 antibody serum concentration. A statistical analysis (analysis of variance) of trough serum concentration values at Week 2 and an average of Weeks 7 and 8 in relation to response status indicated a highly significant relationship between response status and average trough of Weeks 7 and 8 ($p<0.001$). The results indicated that there was a significant difference between the trough serum concentration (average troughs of Weeks 7 and 8) in the responders and nonresponders: trough concentrations were 60±20 μg/ml in the responders versus 44±25 μg/ml in the nonresponders (mean±SD). HER2 overexpression level and type of metastatic sites were associated with significant differences in trough serum concentrations. At Week 2, patients with 2+ HER2 overexpression had significantly higher trough serum concentrations (n=40, mean=28.8 μg/ml, SD=10.4) compared with patients with 3+ HER2 overexpression (n=155, mean=24.1 μg/ml, SD=13.1). This difference in the average trough serum concentrations for Weeks 7 and 8 was no longer statistically significant. Further, at Week 2, patients with superficial disease had significantly higher trough serum concentrations (n=12, mean 34.1 μg/ml, SD=12.0) compared with patients with visceral disease (n=183, mean=24.4 μg/ml, SD=12.6). This difference in the average trough serum concentrations for Weeks 7 and 8 was significant. These data indicate that the rise in trough serum concentrations between Weeks 2 and 7/8 occurs for human patients with various disease profiles.

In a subsequent, similarly designed study, human breast cancer patients were treated with a loading dose of 8 mg/kg followed by maintenance doses of 4 mg/kg weekly. The results of this preliminary human study indicated that an 8mg/kg load:4 mg/kg weekly maintenance regimen was efficacious in reducing tumor volume in the patients.

The data disclosed in this Example indicate that front loading of antibody, such that a target serum concentration is reached more quickly, may be associated with improved outcomes.

Example 3

I.V. Bolus Delivery and Subcutaneous Infusion of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

The efficacy of infusion or bolus delivery of humanized anti-ErbB2 antibody (HERCEPTIN®, see Example 1 for preparation), either by intravenous injection or subcutaneous injection, was examined. The purpose of the study was to ask whether subcutaneous delivery was feasible and whether the convenient subcutaneous bolus delivery was useful in treating metastatic breast cancer in animals inoculated with a cell line that overexpresses the HER2 gene. The results, detailed below, show that i.v. and s.c. infusion and bolus delivery are feasible treatment methodologies.

A study in a nude mouse xenograft model, which incorporates a human breast cancer cell line that naturally overexpresses the HER2 gene (BT-474MI, derived from BT-474 cells, ATCC Accession number HTB-20), comparing tumor volume as a function of i.v. bolus versus s.c. infusion was performed as follows. In the first study athymic nude nu nu 7–9 week old female mice were obtained from Taconic Inc (Germantown, N.Y.). To initiate tumor development, each mouse was inoculated subcutaneously with 3×10^6 BT474M 1 cells suspended in Matrigel™. When tumor nodules reached a volume of approximately 100 mm^3, animals were randomized to 4 treatment groups. The groups were treated according to Table 3.

TABLE 3

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Infusion

Group, Dose, Antibody	Target Serum Conc. μg/ml	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose
1-Control, rhuMAb E25	20	IV LD and SC infusion	2.20	0.250 mg/ml (infusate)
2-Low Dose SC rhuMAb HER2	1	IV LD and SC infusion	0.313	0.050 mg/ml (infusate)
3-High Dose SC rhuMAb HER2	20	IV LD and SC infusion	6.25	1.00 mg/ml (infusate)
4-IV Multi-Dose rhuMAb HER2	20 (trough)	IV LD and MD	4.00	2 mg/kg/week (IV bolus)

Serum Conc. = concentration in serum.
LD = loading dose.
MD = maintenance dose.
Infusate concentration was calculated to achieve targeted serum concentration using Alzet ® osmotic minipumps (Alza Corp., Palo Alto, CA).

Animals were exposed to estrogen by subcutaneous sustained release estrogen pellet 9 days before the start of

dosing to promote growth of grafted tumor cells. The animals were inoculated with the BT474M 1 cells 8 days before the beginning of treatment and tumors were allowed to grow. The animals were then treated with nonrelevant antibody E25 (non-specific for HER2 receptor, but a member of the monoclonal IgG class) or test antibody HERCEPTIN® anti-ErbB2 anitbody as indicated in Table 3. The dosage levels were selected to achieve target serum concentrations of HERCEPTIN®, either 1 μg/ml or 20 μg/ml, by subcutaneous pump infusion or by i.v. bolus delivery. The study groups were treated until day 35. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly (Oust prior to dosing for Group 4) using 3 mice/group/time point. The anti-ErbB2 antibody concentration was determined according to the method disclosed herein involving standard techniques. Tumor volumes were measured two days before dosing began and twice per week from day 6 to day 35 in the study for which data is tabulated below. Tumors were measured in three dimensions and volumes were expressed in mm^3. Efficacy was determined by a statistical comparison (ANOVA) of tumor volumes of test animals relative to untreated control animals.

As shown in Table 4, below, treatment of the BT474M 1 tumor-bearing mice with HERCEPTIN® anti-ErbB2 antibody by the indicated dosage methods significantly inhibited the growth of the tumors. All HERCEPTIN® -treated groups showed similar inhibition of tumor growth relative to the control group. No dose-response was observed.

TABLE 4

Comparison of S.C. Infusion and I.V. Bolus Delivery

Treatment Group	Tumor Volume (mm^3), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6–Day 35 (n = 13)	HERCEPT ® Serum Conc. (μg/ml), Day 27, (n = 3)
control s.c. infusion	764 ± 700	5650 ± 4700	4.16 ± 1.94
s.c. infusion (low dose)	80.6 ± 158	1610 ± 1250	2.11 ± 1.74
s.c. infusion (high dose)	31 ± 75.6	1440 ± 1140	22.1 ± 5.43
i.v. bolus dose*	49.7 ± 95.7	2150 ± 1480	21.7 ± 17.1**

s.c. = subcutaneous delivery; i.v. = intravenous delivery.
*4.0 mg/kg Loading Dose and 2.0 mg/kg/week Maintenance Dose.
**at predose (trough serum concentration immediately prior to a maintenance dose)

The results tabulated above indicate that maintenance of a serum concentration of approximately 2 μg/ml was as effective as a concentration of 20 μg/ml in this study. The results indicated that dosing by subcutaneous infusion was as effective as intravenous bolus dosing and achieved similar trough serum concentrations. The results also indicate that the dose levels studied are at the top of the dose-response curve in this model and that subcutaneous dosing is effective in treating breast cancer tumors. Thus, subcutaneous administration of maintenance doses is feasible as part of a HERCEPTIN® anti-ErbB2 antibody treatment regimen.

Example 4

I.V. Bolus and Subcutaneous Bolus Deliveries of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

Subcutaneous bolus delivery is convenient and cost-effective for the patient and health care professionals. The results of the study disclosed in this example indicate that subcutaneous bolus delivery was as effective as intravenous bolus delivery in reducing breast cell tumor size in a mouse.

This study was set up as disclosed herein in Example 3 for the comparison of intravenous bolus and subcutaneous infusion delivery. A sustained release estrogen implant was inserted subcutaneously one day before tumor cell innoculation as described in Example 3. Six days after tumor cell innoculation, the initial tumor measurement was performed. Seven days after tumor cell innoculation, the first dose of control antibody or HERCEPTIN® anti-ErbB2 antibody was delivered. The animal groups, type of delivery, loading dose and maintenance doses are provided in Table 4. Animals were dosed once weekly for 4 weeks.

TABLE 5

Animal Groups and Doses for Comparison of I.V. Bolus and S.C Bolus Delivery

Group	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose (mg/kg/week)	n
1-Control rbuMAb E25	IV	8	4	10
2-rhuMAb HER2	IV	2	1	10
3-rhuMAb HER2	IV	4	2	10
4-rhuMAb HER2	IV	8	4	10
5-rhuMAb HER2	SC	4	2	10

IV = intraveneous; SC = subcutaneous; n = number of animals per group.

The mice were treated according to the information in Table 4 and using the techniques disclosed in Example 3. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly before each weekly i.v. maintenance dose according to the procedure described herein and using standard techniques. The control E25 antibody serum concentration was determined according to standard immunoassay techniques. Table 6 shows the increase in HERCEPTIN® anti-ErbB2 antibody serum concentrations with time.

TABLE 6

IV versus SC Bolus Delivery: Serum HERCEPTIN ® Anti-ErbB2 Antibody Concentration Serum Concentration, μg/ml

Treatment Group (delivery, MD)	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
1-Control rhu MAb E25	0	25.9	34.6	38.5
(IV, 4mg/kg)	(0)	(8.29)	(11.2)	(14.4)
2-rhu MAb HER2	0	4.96	8.55	8.05
(IV, 1 mg/kg)	(0)	(3.79)	(5.83)	(4.67)
3-rhu MAb HER2	0	13.4	18.9	22.6
(IV, 2 mg/kg)	(0)	(9.24)	(12.0)	(9.21)
4-rhu MAb HER2	0	29.6	37.7	46.2
(IV, 4 mg/kg)	(0)	(13.5)	(14.4)	(13.8)
5-rhu MAb HER2	0	12.5	16.9	17.6
(SC, 2 mg/kg)	(0)	(7.33)	(10.2)	(10.7)

n = 10 for time points Days 0, 7 and 14.
N = 9 for Day 21.

Table 7 shows the relative efficacy of intravenous bolus delivery and subcutaneous bolus delivery for Groups 1–5 having achieved the serum antibody concentrations presented in Table 6. For this study, efficacy was measured as a decrease in tumor volume. Tumor volume was measured twice weekly.

TABLE 7

Efficacy of HERCEPTIN ® Anti-ErbB2 Antibody Measured as a Change in Tumor Volume Comparing Intravenous Bolus and Subcutaneous Bolus Delivery, Mean (SD)

Treatment Group (Delivery, MD)	Tumor Vol. Day 6, mm^3	Tumor Vol. Day 28 mm^3	Tumor Vol. Day 31, mm^3	Day 6–Day 31* Area Under Curve Tumor Vol., mm^3	Tumor Growth Rate on Log (TM + 1)
1-IV Control	321	1530	1630	13600	0.0660
	(190)	(1040)	(1170)	(7230)	(0.0200)
2-IV Herceptin	297	175	151	4690	–0.0505
1 mg/kg	(130)	(215)	(188)	(1400)	(0.142)
3-IV Herceptin	269	75.7	73.6	3510	–0.0608
2 mg/kg	(129)	(92.4)	(84.5)	(1220)	(0.110)
4-IV Herceptin	272	25.3	25.8	2880	–0.0810
4 mg/kg	(117)	(75.9)	(72.9)	(1230)	(0.0859)
5-SC Herceptin	268	76.2	90.4	3230	–0.0304
2 mg/kg	(117)	(98.8)	(105)	(1440)	(0.104)

N = 10 for each data point.
TM = tumor measurement.
IV = intravenous.
SC = subcutaneous.
MD = maintenance dose.
Tumor Vol. = tumor volume, mm^3.
*Day 17 excluded due to measurement error.
Tumor growth rate calculated on Day 21–Day 31 Log(TM + 1). Area under the curve is the area beneath a plot of tumor volume versus time.

FIGS. **4**A and **4**B are graphical plots of changes in tumor volume over time, some of which data is found in Table 7. FIG. **4**A is a linear plot of tumor volume versus time. FIG. **4**B is a semilogarithmic plot of the same data, allowing the test points be viewed more clearly. The data in Table 7 and FIGS. **4**A and **4**B indicate that, although a dose-related response was not observed between HERCEPTIN-treated groups, dosing by subcutaneous bolus was as effective as intravenous bolus dosing and achieved similar trough serum concentrations.

Example 5

Regimens for Intravenous and Subcutaneous Delivery of Anti-ErbB2 Antibody

According to the invention, methods of anti-ErbB2 antibody (e.g., HERCEPTIN®) delivery comprise greater front loading of the drug to achieve a target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less. According to the invention, this initial dosing is followed by dosing that maintains the target serum concentration by subsequent doses of equal or smaller amount. An advantage of the methods of the invention is that the maintenance dosing may be less frequent and/or delivered by subcutaneous injection, making the treatment regimens of the invention convenient and cost-effective for the patient and medical professionals administering the antibody. In addition, a subcutaneous maintenance dose regimen may be interrupted by intravenous dosing (such as infusion) when the patient's chemotherapy requires delivery of other drugs by intravenous injection.

To test the following dosage regimens, human subjects are selected according to the criteria disclosed in Example 1, above. The number of initial doses is one or more doses sufficient to achieve an efficacious target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The number of maintenance doses may be one or more doses sufficient to achieve suppression of disease symptoms, such as a decrease in tumor volume. The maintenance doses are equal to or smaller than the initial dose or doses, consistent with an object of the invention of administering HERCEPTIN® anti-ErbB2 antibody by regimens providing greater front loading. The specific drug delivery regimens disclosed herein are representative of the invention and are not meant to be limiting.

In one trial, an initial dose of 6 mg/kg, 8 mg/kg, or 12 mg/kg of HERCEPTIN® anti-ErbB2 antibody is delivered to human patients by intravenous or subcutaneous injection. Initial doses (loading doses) are delivered by intravenous infusion or bolus injection or preferably subcutaneous bolus injection. Preferably a target trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10–20 μg/ml is achieved.(averaged for all patients in the treatment group) and maintained by subsequent doses of anti-ErbB2 antibody that are equal to or smaller than the initial dose. In one method, a target trough serum concentration is achieved and maintained by once-per-week deliveries of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody by intravenous or subcutaneous injection for at least eight weeks. Alternatively, for this or any dosage regimen disclosed herein, subcutaneous continuous infusion by subcutaneous pump is used to delivery subsequent maintenance doses.

In another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10–20 μg/ml, averaged for an entire treatment group.

In another method, an initial (front loading) dose of 12 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10–20 μg/ml.

In yet another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous infusion or bolus injection, or preferably by subcutaneous bolus injection or infusion.

This is followed by administration of 8 mg/kg per week or 8 mg/kg per 2–3 weeks to maintain a trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10–20 μg/ml. Maintenance doses are delivered by intravenous infusion or bolus injection, or preferably by subcutaneous infusion or bolus injection.

In another method, the front loading initial dose is a series of intravenous or subcutaneous injections, for example, one on each of days 1, 2, and 3 of at least 1 mg/kg for each injection (where the amount of anti-ErbB2 antibody delivered by the sum of initial injections is more than 4 mg/kg), followed by maintenance doses of 6 mg/kg once each 3 week interval to maintain a target trough serum concentration (for example, approximately 10–20 μg/ml) of HERCEPTIN® anti-ErbB2 antibody. The maintenance doses are delivered by intravenous infusion or bolus injection or by subcutaneous infusion or subcutaneous bolus injection.

In yet another method, the front loading is by intravenous infusion of at least 1 mg/kg, preferably 4 mg/kg on each of five consecutive days, followed by repeats of this cycle a sufficient number of times to achieve suppression of disease symptoms. Following the initial dose or doses, subsequent doses may be delivered by subcutaneous infusion or bolus injection if tolerated by the patient. Such subcutaneous delivery is convenient and cost-effective for the patient and administering health care professionals.

In still another method, HERCEPTIN® anti-ErbB2 antibody is delivered initially as at least 2 intravenous infusions per week for three weeks, followed by repeats of this cycle to maintain an efficacious trough serum concentration of HERCEPTIN® 0 anti-ErbB2 antibody. The dose is at least 4 mg/kg of anti-ErbB2 antibody, preferably at least 5 mg/kg. The maintenance drug deliveries may be intravenous or subcutaneous.

Where the animal or patient tolerates the antibody during and after an initial dose, delivery of subsequent doses may be subcutaneous, thereby providing greater convenience and cost-effectiveness for the patient and health care professionals.

In animal studies, an initial dose of more than 4 mg/kg, preferably more than 5 mg/kg delivered by intravenous or subcutaneous injection, is followed by subcutaneous bolus injections of 2 mg/kg twice per week (separated by 3 days) to maintain a trough serum concentration of approximately 10–20 μg/ml. In addition, where the animal or patient is known to tolerate the antibody, an initial dose of HERCEPTIN® anti-ErbB2 antibody is optionally and preferably deliverable by subcutaneous bolus injection followed by subcutaneous maintenance injections.

While target serum concentrations are disclosed herein for the purpose of comparing animal studies and human trials, target serum concentrations in clinical uses may differ. The disclosure provided herein guides the user in selecting a front loading drug delivery regimen that provides an efficacious target trough serum concentration.

The methods of the invention disclosed herein optionally include the delivery of HERCEPTIN® anti-ErbB2 antibody in combination with a chemotherapeutic agent (other than an anthrocycline derivative) to achieve suppression of disease symptoms. The chemotherapeutic agent may be delivered with HERCEPTIN® anti-ErbB2 antibody or separately and according to a different dosing schedule. For example, subcutaneous delivery of HERCEPTIN® anti-ErbB2 antibody with TAXOL® is included in the invention. In addition, intravenous or subcutaneous injection of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody, followed by intravenous or subcutaneous injection of 6 mg/kg HERCEPTIN® anti-ErbB2 antibody every 3 weeks is administered in combination with a chemotherapeutic agent, such as a taxoid (e.g. paclitaxel 175mg/m^2 every 3 weeks) or an anthracycline derivative (e.g. doxorubicin 60 mg/m^2 or epirubicin 75 mg/m^2 every 3 weeks). Optionally, where an anthracycline derivative is administered, a cardioprotectant (e.g. 600 mg/m^2 cyclophosphamide every 3 weeks) is also administered. In another combination therapy, anti-ErbB2 antibody is administered in a loading dose of more than 4 mg/kg, preferably more than 5 mg/kg, and more preferably at least 8 mg/kg. The loading dose is followed by maintenance doses of at least 2 mg/kg weekly, preferably 6 mg/kg every 3 weeks. The combination therapy includes administration of a taxoid during treatment with anti-ErbB2 antibody. According to one embodiment of the invention, the taxoid is paclitaxel and is administered at a dose of 70–100 mg/m^2/week. According to another embodiment of the invention, the taxoid is docetaxel and is administered at a dose of 30–70 mg/m^2/week.

Example 6

HERCEPTIN® Administered Intravenously Every Three Weeks in Combination with Paclitaxel

Currently, the recommended dose of HERCEPTIN® is 2 mg/kg once weekly. Patients will be administered HERCEPTIN® every three weeks instead of weekly, along with paclitaxel (175 mg/m^2 every three weeks). Simulation of the proposed treatment regimen suggests that the trough serum concentrations will be 17 mcg/ml, in the range (10–20mcg/ml) of the targeted trough serum concentrations from previous HERCEPTIN® IV clinical trials. After the first 12 patients the PK parameters will be assessed, if exposure is felt inadequate, then the dose will be increased to 8 mg/kg every three weeks for the remaining 12 patients.

Inclusion Criteria

1) Females≧18 years of age
2) Histologically confirmed ErbB2 over-expressing metastatic breast cancer
3) Patients who have been newly diagnosed with metastatic disease
4) Have a Karnofsky performance status of≧70%
5) Give written informed consent prior to any study specific screening procedures with the understanding that the patient has the right to withdraw from the study at any time, without prejudice.

Exclusion Criteria

1) Pregnant or lactating women
2) Women of childbearing potential unless (1) surgically sterile or (2) using adequate measures of contraception such as oral contraceptive, intra-uterine device or barrier method of contraception in conjunction with spermicidal jelly.
3) Clinical or radiologic evidence of CNS metastases.
4) History of any significant cardiac disease
5) LVEF≦50%
6) No prior taxane therapy in any treatment setting.
7) Any of the following abnormal baseline hematologic values:
   Hb less than 9 g/dl
   WBC less than 3.0×10^9/l
   Granulocytes less than 1.5×109/l
   Platelets less than 100×109/l
8) Any of the following abnormal baseline liver function tests:
   Serum bilirubin greater than 1.5× ULN (upper normal limit)
   ALT and/or AST greater than 2.5× ULN (greater than 4.0× ULN if liver or bone metastasis)
   Alkaline phosphatase greater than 2.5× ULN (greater than 4.0× ULN if liver or bone metastasis)
9) The following abnormal baseline renal function tests: serum creatinine greater than 1.5× ULN
10) History of other serious medical conditions that would preclude patient participation in an investigational study.

HERCEPTIN® Loading dose and schedule: 8 mg/kg for first dose. Maintenance dose and schedule: 6 mg/kg every 3 weeks.

Paclitaxel—175 mg/m^2 IV every 3 weeks×6 cycles as a 3-hour infusion. NOTE: On the first cycle of treatment,

paclitaxel will be dosed 8 hours prior to HERCEPTIN® to determine the PK of paclitaxel alone. HERCEPTIN® will be administered 8 hours post-paclitaxel for the 1st cycle only. In subsequent treatment cycles, HERCEPTIN® will be administered prior to paclitaxel.

The total duration of this study is 18 weeks. Study subjects will receive up to 6 total HERCEPTIN® doses. After the last subject has received the last cycle of paclitaxel, data collection for safety and pharmacokinetic analysis will stop, and the study will close to protocol specified treatment. Study subjects may continue to receive the HERCEPTIN® +/− paclitaxel at the discretion of the investigator.

It is believed that the above treatment regimen will be effective in treating metastatic breast cancer, despite the infrequency with which HERCEPTIN® is administered to the patient.

While the particular aspects and embodiments of the invention as herein shown and disclosed in detail is fully capable of obtaining the objects and providing the advantages herein before stated, it is to be understood that it is merely illustrative of some of the presently preferred embodiments of the invention and that no limitations are intended to the details of methods and articles of manufacture shown other than as described in the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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Val Gln Gly Asn Leu Glu Leu Thr Tyr Leu Pro Thr Asn Ala Ser
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Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
50 55 60

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
65 70 75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
80 85 90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
95 100 105

Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
110 115 120

Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
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Ala

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Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
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Ile Gly Val Ala Trp Tyr Gln Gln Arg Pro Gly Gln Ser Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp
                 50                  55                  60

Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
                 65                  70                  75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
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Ile Lys
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Asp Tyr Thr Met Asp Trp Val Lys Gln Ser His Gly Lys Ser Leu
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Glu Trp Ile Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
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Asn Gln Arg Phe Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser
65 70 75

Ser Arg Ile Val Tyr Met Glu Leu Arg Ser Leu Thr Phe Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
95 100 105

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20 25 30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys

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Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
20 25 30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
50 55 60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
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                35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
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Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
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Ile Lys


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Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
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Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                95                 100                 105

Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115
```

What is claimed is:

1. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB2 antibody; and

administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose, wherein

the subsequent doses are separated in time from each other by at least two weeks.

2. The method of claim **1**, wherein the initial dose is at least approximately 6 mg/kg.

3. The method of claim **2**, wherein the initial dose is at least approximately 8 mg/kg.

4. The method of claim **3**, wherein the initial dose is at least approximately 12 mg/kg.

5. The method of claim **1**, wherein the subsequent doses are separated in time from each other by at least three weeks.

6. The method of claim **1**, wherein the initial dose is administered by intravenous injection, and wherein at least one subsequent dose is administered by subcutaneous injection.

7. The method of claim **1**, wherein the initial dose is administered by intravenous injection, wherein at least two subsequent doses are administered, and wherein each subsequent dose is administered by a method selected from the group consisting of intravenous injection and subcutaneous injection.

8. The method of claim **1**, wherein the initial dose and at least one subsequent dose are administered by subcutaneous injection.

9. The method of claim **1**, wherein the initial dose is selected from the group consisting of approximately 6 mg/kg, 8 mg/kg, or 12 mg/kg, wherein the plurality of subsequent doses are at least approximately 2 mg/kg.

10. The method of claim **9**, wherein the plurality of subsequent doses are separated in time from each other by at least three weeks.

11. The method of claim **10**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

12. The method of claim **10**, wherein the initial dose is approximately 12 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

13. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 8 mg/kg.

14. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, wherein at least one subsequent dose is 8 mg/kg, and wherein administration of the initial dose and subsequent doses are separated in time by at least 2 weeks.

15. The method of claim **14**, wherein the initial dose and subsequent doses are separated in time by at least 3 weeks.

16. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of the antibody, wherein the initial dose is a plurality of doses, wherein each of the plurality of initial doses is at least approximately 1 mg/kg and is administered on at least 3 consecutive days, and administering to the patient at least 1 subsequent dose of the antibody, wherein at least one subsequent dose is at least approximately 6 mg/kg, and wherein administration of the last initial dose and the first subsequent and additional subsequent doses are separated in time by at least 3 weeks.

17. The method of claim **1**, wherein said cancer is selected from the group consisting of breast cancer, leukemia, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

18. The method of claim **17**, wherein said cancer is breast cancer.

19. The method of claim **18**, wherein said cancer is metastatic breast carcinoma.

20. The method of claim **1**, wherein said antibody binds to the extracellular domain of the ErbB2 receptor.

21. The method of claim **20**, wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

22. The method of claim **21**, wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

23. The method of claim **1**, wherein efficacy is measured by determining the time to disease progression or the response rate.

24. A method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by two or more subsequent doses of the antibody, wherein the subsequent doses are separated in time from each other by at least two weeks.

25. The method of claim **24**, wherein the first dose and a first subsequent dose are separated from each other in time by at least about three weeks.

26. The method of claim **24**, wherein the first dose and subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

27. The method of claim **26**, wherein the first dose and subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

28. The method of claim **27**, wherein the first dose and subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

29. The method of claim **24**, wherein from about two to about ten subsequent doses of the antibody are administered to the patient.

30. The method of claim **24**, wherein the subsequent doses are separated in time from each other by at least about three weeks.

31. The method of claim **24**, wherein the two or more subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

32. The method of claim **24**, wherein the two or more subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

33. The method of claim **24**, wherein the two or more subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

* * * * *

EXHIBIT H

US007371379B2

(12) United States Patent
Baughman et al.

(10) Patent No.: US 7,371,379 B2
(45) Date of Patent: May 13, 2008

(54) **DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventors: **Sharon A. Baughman**, Ventura, CA (US); **Steven Shak**, Burlingame, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 540 days.

(21) Appl. No.: **10/600,152**

(22) Filed: **Jun. 20, 2003**

(65) **Prior Publication Data**

US 2004/0037824 A1 Feb. 26, 2004

Related U.S. Application Data

(62) Division of application No. 09/648,067, filed on Aug. 25, 2000, now Pat. No. 6,627,196.

(60) Provisional application No. 60/213,822, filed on Jun. 23, 2000, provisional application No. 60/151,018, filed on Aug. 27, 1999.

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.** **424/138.1**; 424/130.1; 424/133.1; 424/141.1; 424/142.1; 424/143.1; 424/155.1; 424/156.1; 424/174.1

(58) **Field of Classification Search** 424/130.1, 424/133.1, 138.1, 141.1, 142.1, 143.1, 155.1, 424/156.1, 174.1
See application file for complete search history.

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Primary Examiner—Alana M. Harris
Assistant Examiner—Anne L. Holleran
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with anti-ErbB2 antibody.

40 Claims, 5 Drawing Sheets

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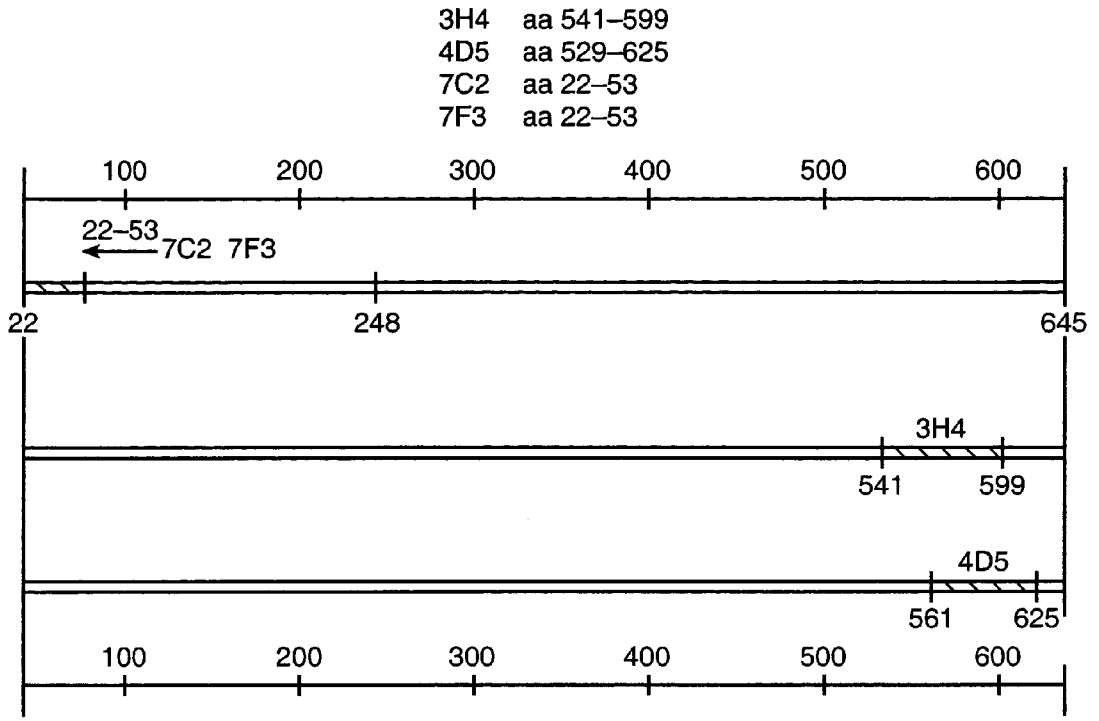

3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
541 **599**

4D5 epitope (SEQ ID NO:9) 64 residues

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP
561 **625**

FIG._1

```
1    MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPA
38   SPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFL
75   QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112  YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149  LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186  TNRSRA
```

FIG._2

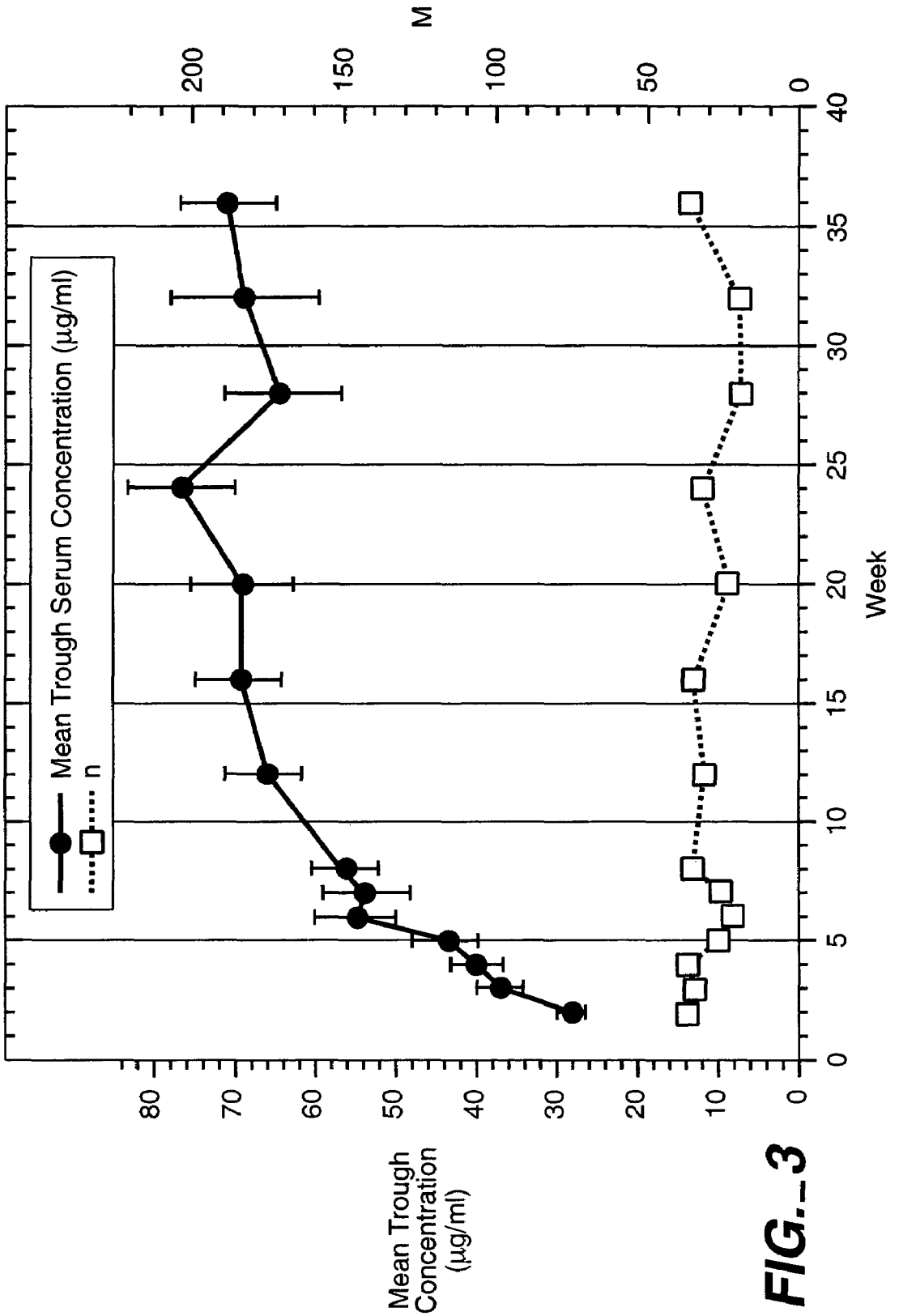
Mean Trough Serum Concentration (µg/ml)
n
Mean Trough Concentration (µg/ml)
0
10
20
30
40
50
60
70
80
n
0
50
100
150
200
Week
0
5
10
15
20
25
30
35
40

FIG._3

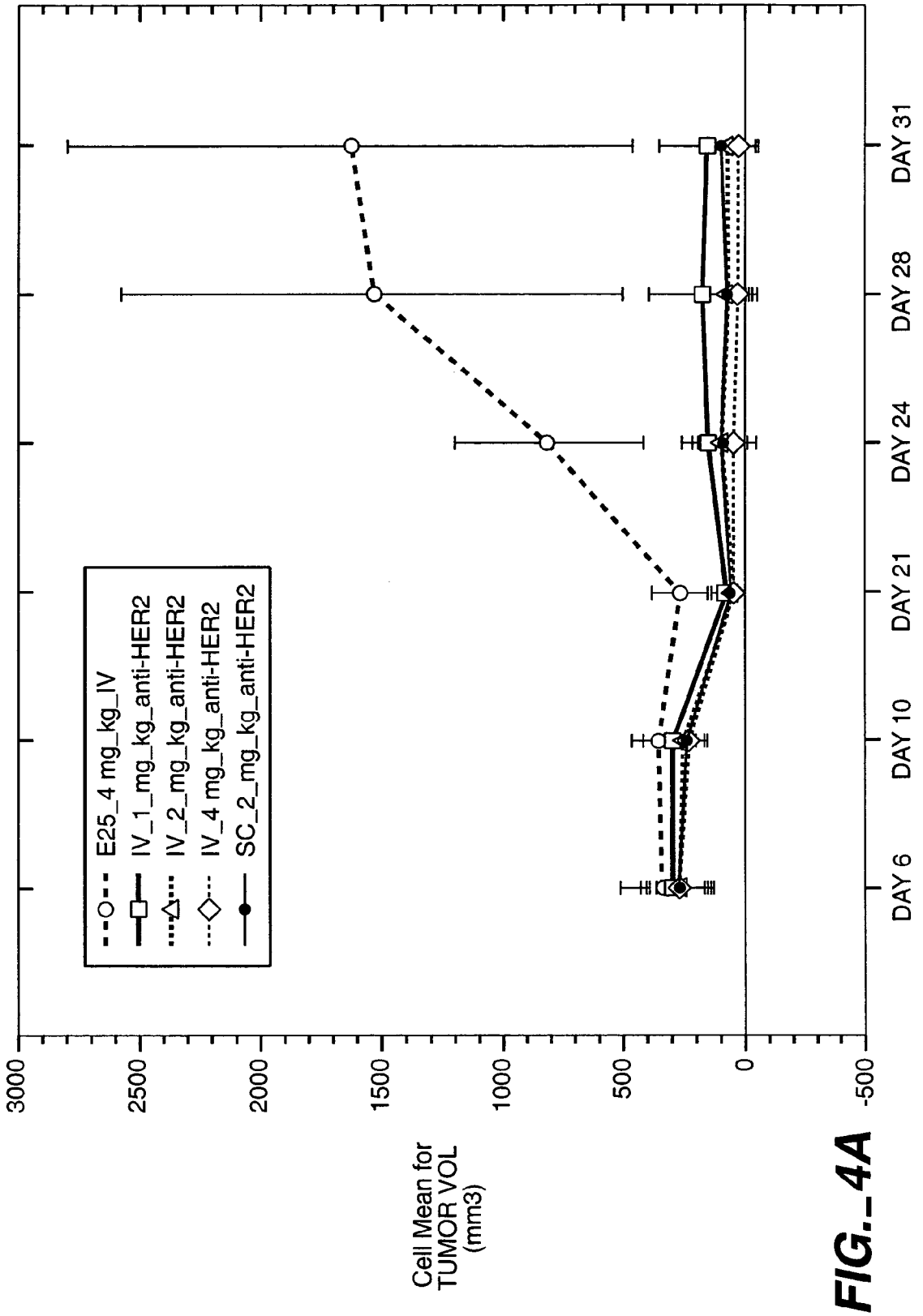
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
3000
2500
2000
1500
1000
500
0
-500
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4A

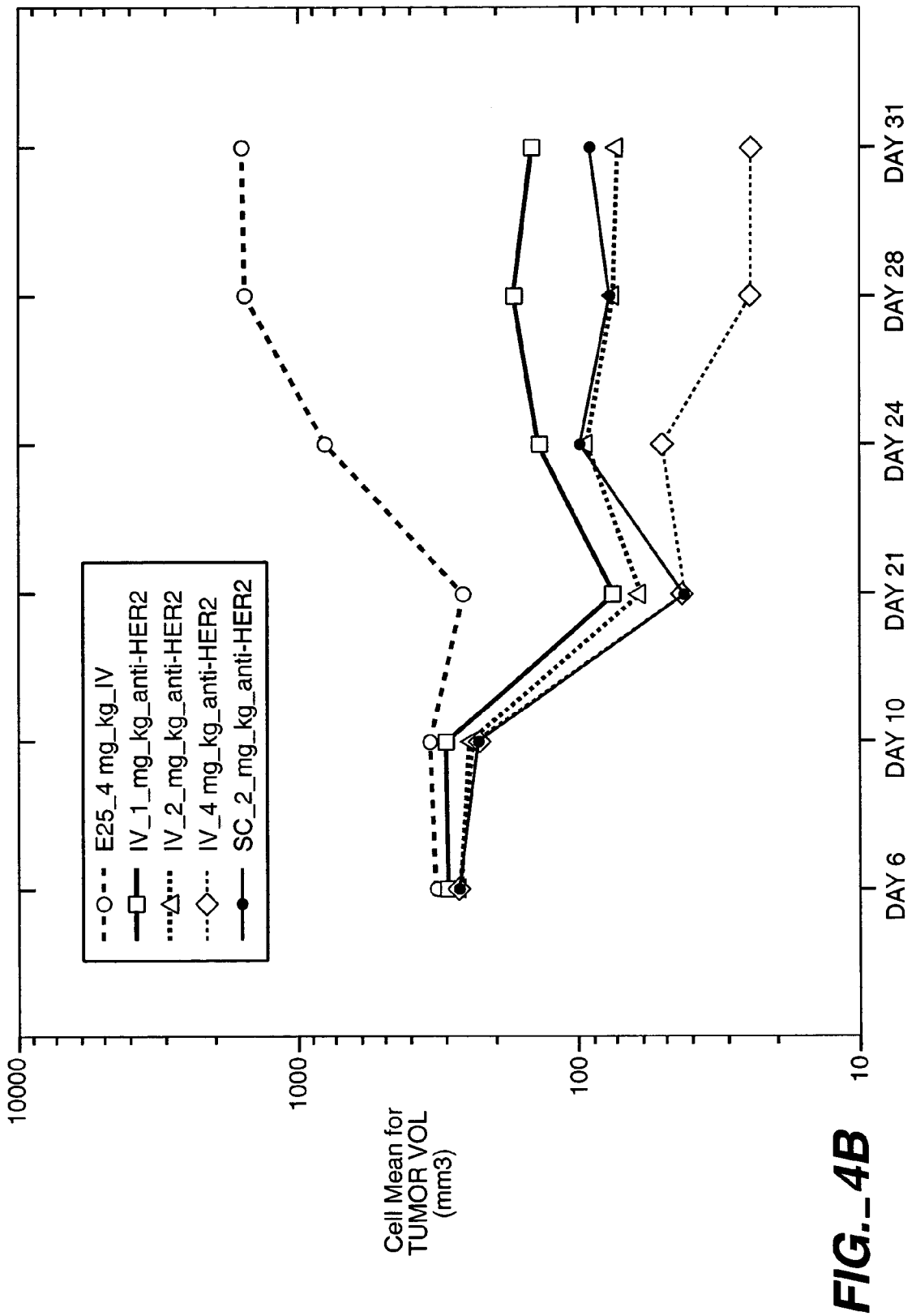
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
10000
1000
100
10
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4B

VARIABLE LIGHT

```
          1       10        20          30              40
2C4       DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA----] WYQQRP
           **    **** *      *                        *
574       DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA----] WYQQKP
                                   *   *  ****
hum kI    DIQMTQSPSSLSASVGDRVTITC [RASQSVSTSSYSYMH] WYQQKP

                    50          60        70        80
2C4       GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
           **                   *  *         *    * *
574       GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                     *  ****
hum kI    GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                   90          100
2C4       EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:10)
            * *                 *   *
574       EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:12)
                      ***
hum kI    EDFATYYC [QQYNSLPYT] FGQGTKVEIK (SEQ ID NO:14)
```

FIG._5A

VARIABLE HEAVY

```
          1       10        20          30          40
2C4       EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
              **  **  *  * ***  **                  *
574       EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                       ** * *
humIII    EVQLVESGGGSVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                    50        60          70        80
2C4       HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                     *** *    **** *
574       PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                   *  ******  ** ****       *. * *
humIII    PGKGLEWVS [VISGDGGSTYYADSVKG] RFTISRDDSKNTLYL

                     90         100           110
2C4       ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLVTSS (SEQ ID NO:11)
          ***  **                              *
574       QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:13)
                            ** ***
humIII    QMNSLRAEDTAVYYCAR [GRGGGS--DY] WGQGTLVTVSS (SEQ ID NO:15)
```

FIG._5B

DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES

RELATED APPLICATIONS

This application is divisional of U.S. Ser. No. 09/648,067 filed Aug. 25, 2000 (now U.S. Pat. No. 6,627,196), which claims priority under 35 USC 119(e) to provisional application Nos. 60/151,018, filed Aug. 27, 1999 and 60/213,822, filed Jun. 23, 2000, the contents of which are incorporated heroin by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2 or disorders expressing epidermal growth factor receptor (EGFR), comprising administering to a human or animal presenting the disorders a therapeutically effective amount of an antibody that binds ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 or expressing EGFR, where the treatment is with an anti-ErbB2 antibody administered by front loading the dose of antibody during treatment by intravenous and/or subcutaneous administration. The invention optionally includes treatment of cancer in a human patient with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, a taxoid. The taxoid may be, but is not limited to paclitaxel or docetaxel. The invention further includes treatment of cancer in a human patient with a combination of anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, an anthracycline derivative. Optionally, treatment with a combination of anti-ErbB2 and an anthracycline derivative includes treatment with an effective amount of a cardioprotectant. The present invention further concerns infrequent dosing of anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:78-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) a inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. Al of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994. Hudziak et al., *Mol. Cell. Biol.* 9(3): 1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells

was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth, whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2, HERCEPTIN®, or HERCEPTIN® anti-ErbB2 antibody) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]). The recommended initial loading dose for HERCEPTIN® is 4 mg/kg administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg and can be administered as a 30-minute infusion if the initial loading dose is well tolerated.

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Supp11):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the discovery that an early attainment of an efficacious target trough serum concentration by providing an initial dose or doses of anti-ErbB2 antibodies followed by subsequent doses of equal or smaller amounts of antibody (greater front loading) is more efficacious than conventional treatments. The efficacious target trough serum concentration is reached in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The target serum concentration is thereafter maintained by the administration of maintenance doses of equal or smaller amounts for the remainder of the treatment regimen or until suppression of disease symptoms is achieved.

The invention further concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of an anti-ErbB2 antibody subcutaneously. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance to the anti-ErbB2 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable.

According to the invention, the method of treatment involves administration of an initial dose of anti-ErbB2 antibody of more than approximately 4 mg/kg, preferably more than approximately 5 mg/kg. The maximum initial dose or a subsequent dose does not exceed 50 mg/kg, preferably does not exceed 40 mg/kg, and more preferably does not exceed 30 mg/kg. Administration is by intravenous or subcutaneous administration, preferably intravenous infusion or bolus injection, or more preferably subcutaneous bolus injection. The initial dose may be one or more administrations of drug sufficient to reach the target trough serum concentration in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less.

According to the invention, the initial dose or doses is/are followed by subsequent doses of equal or smaller amounts of antibody at intervals sufficiently close to maintain the

trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or subsequent dose does not exceed 50 mg/kg, and each subsequent dose is at least 0.01 mg/kg. Preferably the amount of drug administered is sufficient to maintain the target trough serum concentration such that the interval between administration cycles is at least one week. Preferably the trough serum concentration does not exceed 2500 μg/ml and does not fall below 0.01 μg/ml during treatment. The front loading drug treatment method of the invention has the advantage of increased efficacy by reaching a target serum drug concentration early in treatment. The subcutaneous delivery of maintenance doses according to the invention has the advantage of being convenient for the patient and health care professionals, reducing time and costs for drug treatment. Preferably, the initial dose (or the last dose within an initial dose series) is separated in time from the first subsequent dose by 4 weeks or less, preferably 3 weeks or less, more preferably 3 weeks or less, most preferably 1 week or less.

In an embodiment of the invention, the initial dose of anti-ErbB2 is 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous administration, such as intravenous infusion or subcutaneous bolus injection. The subsequent maintenance doses are 2 mg/kg delivered once per week by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated, the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In another embodiment, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In still another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

In yet another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

In another embodiment, the invention includes initial doses of at least 1 mg/kg, preferably 4 mg/kg, anti-ErbB2 antibody on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In another embodiment, the invention includes an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

In still another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. In one embodiment of the invention, each dose is approximately 25 mg/kg or less for a human patient, preferably approximately 10 mg/kg or less. This 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

In another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The method of the invention may further comprise administration of a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form. Other preferred ErbB2-binding antibodies include, but are not limited to, antibodies 7C2, 7F3, and 2C4, preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

The present application also provides a method of therapy involving infrequent dosing of an anti-ErbB2 antibody. In particular, the invention provides a method for the treatment of cancer (e.g. cancer characterized by overexpression of the ErbB2 receptor) in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). For instance, the antibody may be administered about every three weeks, about two to about 20 times, e.g. about six times. The first dose and subsequent dose may each be from about 2 mg/kg to about 16 mg/kg; e.g. from about 4 mg/kg to about 12 mg/kg; and optionally from about 6 mg/kg to about 12 mg/kg. Generally, two or more subsequent doses (e.g. from about two to about ten subsequent doses) of the antibody are administered to the patient, and those subsequent doses are preferably separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). The two or more subsequent doses may each be from about 2 mg/kg to about 16 mg/kg; or from about 4 mg/kg to about 12 mg/kg; or from about 6 mg/kg to about 12 mg/kg. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, and a package insert containing instructions to administer the antibody according to such methods.

The presently described dosing protocols may be applied to other anti-ErbB antibodies such as anti-epidermal growth factor receptor (EGFR), anti-ErbB3 and anti-ErbB4 antibodies. Thus, the invention provides a method for the treatment of cancer in a human patient, comprising administering an effective amount of an anti-ErbB antibody to the human patient, the method comprising administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose. Alternatively, or additionally, the invention pertains to a method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an

anti-ErbB antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB antibody, and a package insert containing instructions to administer the antibody according to such methods.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition. According to the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody at an initial dose of 5 mg/kg followed by the same or smaller subsequent dose or doses. In another embodiment of the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose, most preferably for all doses.

In a further aspect, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering to the patient effective amounts of an anti-ErbB2 antibody and a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is a taxoid including, but not limited to, paclitaxel and docetaxel. In another embodiment, the chemotherapeutic agent is an anthracyline derivative including, but not limited to, doxorubicin or epirubicin. In still another embodiment of the invention, treatment with an anti-ErbB2 antibody and an anthracycline derivative further includes administration of a cardioprotectant to the patient. In still another embodiment, an anthracycline derivative is not administered to the patient with the anti-ErbB2 antibody. One or more additional chemotherapeutic agents may also be administered to the patient. The cancer is preferably characterized by overexpression of ErbB2.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a chemotherapeutic agent to a patient. In another embodiment, the chemotherapeutic agent is other than an anthracycline, and is preferably a taxoid, such as TAXOL®. In still another embodiment, the chemotherapeutic agent is an anthracycline, including but not limited to, doxorubicin or epirubicin. In yet another embodiment, the chemotherapeutic agent is an anthracycline and the package insert further instructs the user to administer a cardioprotectant.

The methods and compositions of the invention comprise an anti-ErbB2 antibody and include a humanized anti-ErbB2 antibody. Thus, the invention further pertains to a composition comprising an antibody that binds ErbB2 and the use of the antibody for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. The invention also pertains to the use of the antibody for treating EGFR expressing cancer. Preferably the antibody is a monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5-8 (HERCEPTIN® anti-ErbB2 antibody); or monoclonal antibody 2C4, e.g., humanized 2C4. The antibody may be an intact antibody (e.g., an intact IgG_1 antibody) or an antibody fragment (e.g., a Fab, $F(ab')_2$, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-ErbB2 antibody 2C4 are shown in FIGS. **5**A and **5**B.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative Mabs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 24 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO:1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

FIG. **3** is a graph of anti-ErbB2 antibody (HERCEPTIN®) trough serum concentration (μg/ml, mean±SE, dark circles) by week from week 2 through week 36 for ErbB2 overexpressing patients treated with HERCEPTIN® anti-ErbB2 antibody at 4 mg/kg initial dose, followed by 2 mg/kg weekly. The number of patients at each time point is represented by "n" (white squares).

FIG. **4**A is a linear plot of tumor volume changes over time in mice treated with HERCEPTIN® anti-ErbB2 antibody. FIG. **4**B is a semi-logarithmic plot of the same data as in FIG. **4**A such that the variation in tumor volume for the treated animals is observed more readily.

FIGS. **5**A and **5**B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. **5**A) and variable heavy (V_H) (FIG. **5**B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 10 and 11, respectively); V_L and V_H domains of humanized Fab version 574 (SEQ ID Nos. 12 and 13, respectively), and human V_L and V_H consensus frameworks (hum κ1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 14 and 15, respectively). Asterisks identify differences between humanized Fab version 574 and murine monoclonal antibody 2C4 or between humanized Fab version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g. substituted as follows—IleL2Thr; ArgL54Leu; TyrL55Glu;

ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured in order to further improve or refine its affinity and/or other biological activities.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

An "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3 and ErbB4 receptors as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including variants thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS* (*USA*) 87:4207-4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.).

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS* (*USA*) 86:9193-9197 (1989), including variants thereof Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993), including variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein, and "erbB2," "c-erb-B2," and "her2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N-terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, E d Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO: 2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5

to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177-244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. 1, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these maybe further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ,

respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al., *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of

treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see Example 1, below). Therapeutically effective amount also refers to a target serum concentration, such as a trough serum concentration, that has been shown to be effective in suppressing disease symptoms when maintained for a period of time.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidaniine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The *Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W B Saunders:

Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions,* 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g.,an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum HERCEPTIN® anti-ErbB2 antibody concentration" refers to the concentration of a drug, such as HERCEPTIN® anti-ErbB2 antibody, in the blood serum of an animal or human patient being treated with the drug. Serum concentration of HERCEPTIN® anti-ErbB2 antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is an ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery

from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient, preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example, by pinching or drawing the skin up and away from underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration. According to the present invention, front loading is achieved by an initial dose or doses delivered over three weeks or less that causes the animal's or patient's serum concentration to reach a target serum trough concentration. Preferably, the initial front loading dose or series of doses is administered in two weeks or less, more preferably in 1 week or less, including 1 day or less. Most preferably, where the initial dose is a single dose and is not followed by a subsequent maintenance dose for at least 1 week, the initial dose is administered in 1 day or less. Where the initial dose is a series of doses, each dose is separated by at least 3 hours, but not more than 3 weeks or less, preferably 2 weeks or less, more preferably 1 week or less, most preferably 1 day or less. To avoid adverse immune reaction to an antibody drug such as an anti-ErbB2 antibody (e.g., HERCEPTIN® anti-ErbB2 antibody) in an animal or patient who has not previously been treated with the antibody, it may be preferable to deliver initial doses of the antibody by intravenous infusion. The present invention includes front loading drug delivery of initial and maintenance doses by infusion or bolus administration, intravenously or subcutaneously.

Published information related to anti-ErbB2 antibodies includes the following issued patents and published applications: PCT/US89/0005 1, published Jan. 5, 1989; PCT/US90/02697, published May 18, 1990; EU 0474727 issued Jul. 23, 1997; DE 69031120.6, issued Jul. 23, 1997; PCT/US97/18385, published Oct. 9, 1997; SA 97/9185, issued Oct. 14, 1997; U.S. Pat. No. 5,677,171, issued Oct. 14, 1997; U.S. Pat. No. 5,720,937, issued Feb. 24, 1998; U.S. Pat. No. 5,720,954, issued Feb. 24, 1998; U.S. Pat. No. 5,725,856, issued Mar. 10, 1998; U.S. Pat. No. 5,770,195, issued Jun. 23, 1998; U.S. Pat. No. 5,772,997, issued Jun. 30, 1998; PCT/US98/2626, published Dec. 10, 1998; and PCT/US99/06673, published Mar. 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., *PNAS* (*USA*) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl^2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypox-

anthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352: 624-628 (1991) and Marks et al., *J. Mol. Biol,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al. *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable

three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al. *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10: 163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO 89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as his (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl Acad. Sci. USA,* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al., *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature*

328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g. by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Determination of anti-ErbB2 Antibody Concentration in Serum

The following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific rhuMAb HER2 (humanized anti-$p185^{HER2}$ monoclonal antibody, including HERCEPTIN® anti-ErbB2 antibody) in a body fluid of a mammal including, but not limited to, serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids, and ocular vitreous gel.

Plate Binding Activity Assay for rhuMAb HER2 (Humanized Anti-p185^{HER2} Monoclonal Antibody

The method of assaying rhuMAb HER2 described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of rhuMAb HER2 (Genentech, Inc., South San Francisco, Calif.), controls, and serum samples were diluted with Assay Diluent (PBS/0.5% BSA/0.05% Polysorbate 20/0.01% Thimerosal). The dilutions of standardized rhuMAb HER2 were prepared to span a range of concentrations useful for a standard curve. The samples were diluted to fall within the standard curve.

An aliquot of Coat Antigen in Coating buffer (recombinant p185^{HER2} (Genentech, Inc.) in 0.05 M sodium carbonate buffer) was added to each well of a microtiter plate and incubated at 2-8° C. for 12-72 hours. The coating solution was removed and each well was washed six times with water, then blotted to remove excess water.

An aliquot of Assay Diluent was added to each well and incubated for 1-2 hours at ambient temperature with agitation. The wells were washed as in the previous step.

Aliquots of diluted standard, control and sample solutions were added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish peroxidase; Organon Teknika catalog #55253 or equivalent) was diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution was added to each well and incubated at ambient temperature for I hour with agitation. The wells were washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in 12.5 ml 4 mM H_2O_2 in PBS) was added to each well and incubated for a sufficient period of time (approximately 8-10 minutes) in the dark at ambient temperature to allow color development. The reaction was stopped with an aliquot of 4.5 N sulfuric acid. Optical density was read at 490-492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16 th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial growth factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16 th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

V. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disor-

ders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

The treatment of the present invention involves the administration of an anti-ErbB2 antibody to an animal or human patient, followed at intervals by subsequent doses of equal or smaller doses such that a target serum concentration is achieved and maintained during treatment. Preferably, maintenance doses are delivered by bolus delivery, preferably by subcutaneous bolus administration, making treatment convenient and cost-effective for the patient and health care professionals.

Where combined administration of a chemotherapeutic agent (other than an antracycline) is desired, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It maybe desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF). Alternatively, or additionally, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. The ErbB2 antibody may be co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration, or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

In addition to the above therapeutic regimens, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of anti-ErbB2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Where the treatment involves a series of treatments, the initial dose or initial doses are followed at daily or weekly intervals by maintenance doses. Each maintenance dose provides the same or a smaller amount of antibody compared to the amount of antibody administered in the initial dose or doses.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays.

According to the invention, dosage regimens may include an initial dose of anti-ErbB2 of 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous infusion, followed by subsequent weekly maintenance doses of 2 mg/kg by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

Alternatively, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

Another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

Still another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

As an alternative regimen, initial doses of 4 mg/kg anti-ErbB2 antibody may be administered on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

An additional regimen involves an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

Alternatively, the invention may include a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. The 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The invention further includes a cyclic dosage regimen in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms. Further information about suitable dosages is provided in the Examples below.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container

holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may comprise a package inserts with instructions for use, including, e.g., a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRT 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Preparation and Efficacy of HERCEPTIN® Anti-ErbB2 Antibody Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 5o:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al., *Proc. Natl. Acad. Sci.* (*USA*) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN® anti-ErbB2 antibody). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant $[K_d]$=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of $p185^{HER2}$, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® anti-ErbB2 antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

- Metastatic breast cancer
- Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].
- Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

- The ability to understand and willingness to sign a written informed consent form
- Women>18 years
- Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

- Prior cytotoxic chemotherapy for metastatic breast cancer
- Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.
- Concomitant malignancy that has not been curatively treated
- A performance status of <60% on the Kamofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® anti-ErbB2 antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® anti-ErbB2 antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN® anti-ErbB2 antibody, without increase in overall severe adverse events (AE):

TABLE 1

HERCEPTIN ® Anti-ErbB2 Antibody Efficacy

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test;
**p < 0.01 by X^2 test;
CRx: chemotherapy;
AC: anthracycline/cyclophosphamide treatment;
H: HERCEPTIN ® anti-ErbB2 antibody;
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor treatment with HERCEPTIN® anti-ErbB2 antibody and paclitaxel (TAXOL®) where a combined treatment regimen is desired.

Example 2

Pharmacokinetic and Pharmacodynamic Properties of Anti-ErbB2 Antibody (HERCEPTIN®)

HERCEPTIN® anti-ErbB2 antibody was administered by intravenous infusion to human patients selected according to the criteria provided in Example 1. An initial dose of 4 mg/kg HERCEPTIN® anti-ErbB2 antibody was delivered by intravenous infusion, followed by subsequent i.v. infu-

sions of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody weekly for several weeks. Two hundred thirteen patients began this treatment regimen and serum drug concentration was obtained beyond 8 weeks for fewer than 90 patients as selective discontinuation of patients with rapidly progressing disease occurred. Of the 213 patients who began treatment, serum trough concentration data were available for 80 patients at Week 12, for 77 patients at Week 16, for 44 patients at Week 20, for 51 patients at Week 24, for 25 patients at Week 28, for 23 patients at Week 32, and for 37 patients at Week 36.

HERCEPTIN® Anti-ErbB2 Antibody Trough Serum Concentrations for Weeks 0-36

The HERCEPTIN® anti-ErbB2 antibody trough serum concentrations (μg/ml, mean±SE) from Week 2 through Week 36 are plotted in FIG. **3** (dark circles). The number of patients was fairly constant because data from patients discontinued from the program due to rapidly progressing disease were excluded from this analysis. Trough serum concentrations tended to increase through Week 12 and tended to plateau after that time.

HERCEPTIN® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for Weeks 1-8

Some HERCEPTIN® anti-ErbB2 antibody serum concentration data were available for 212 of the original 213 patients. Trough and peak serum concentration data reflecting the first HERCEPTIN® anti-ErbB2 antibody infusion were available for 195 of the 212 patients. For the seventh infusion, trough serum concentration data were available for 137/212 patients and peak serum concentration data were available for 114/212 patients. Table 2 presents a summary of statistics from trough and peak serum concentrations for the first 8 weeks of treatment. Peak samples were drawn shortly after the end of HERCEPTIN® anti-ErbB2 antibody administration; trough samples were drawn prior to the subsequent dose (i.e., 1 week later). Serum concentrations of HERCEPTIN® anti-ErbB2 antibody were determined as disclosed herein.

TABLE 2

HERCEPTIN ® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for the First 8 Weeks of Treatment (μg/ml)

	Dose Number	n	Mean	SD	Minimun	Maximum
Peak	1	195	100.3	35.2	30.7	274.6
Trough		195	25.0	12.7	0.16	60.7
Peak	2	190	74.3	31.3	20.8	307.9
Trough		167	30.4	16.0	0.2	74.4
Peak	3	167	75.3	26.8	16.1	194.8
Trough		179	33.7	17.9	0.2	98.2
Peak	4	175	80.2	26.9	22.2	167
Trough		132	38.6	20.1	0.2	89.4
Peak	5	128	85.9	29.2	27.8	185.8
Trough		141	42.1	24.8	0.2	148.7
Peak	6	137	87.2	32.2	28.9	218.1
Trough		115	43.2	24.0	0.2	109.9
Peak	7	114	89.7	32.5	16.3	187.8
Trough		137	48.8	24.9	0.2	105.2
Peak	8	133	95.6	35.9	11.4	295.6

The data in Table 2 suggest that there was an increase in trough serum concentration over time. Of the many patients studied, there were 18 patients for whom the trough concentrations did not exceed 20 μg/ml from Week 2 through Week 8. A HERCEPTIN® anti-ErbB2 antibody trough serum concentration of 20 μg/ml was nominally targeted for these studies based on prior pharmacologic studies in animals and exploratory analyses in clinical trials.

Patient response status was evaluated relative to serum concentration of HERCEPTIN® anti-ErbB2 antibody. For this purpose, mean serum concentration (an average of troughs and peaks) was calculated for various times and patient response status (where the patient response status was determined by an independent Response Evaluation Committee). The increase in serum concentration between Weeks 2 and 8 appeared to be greater in responders than in nonresponders, suggesting that there is a relationship between response status and HERCEPTIN® anti-ErbB2 antibody serum concentration. A statistical analysis (analysis of variance) of trough serum concentration values at Week 2 and an average of Weeks 7 and 8 in relation to response status indicated a highly significant relationship between response status and average trough of Weeks 7 and 8 ($p<0.001$). The results indicated that there was a significant difference between the trough serum concentration (average troughs of Weeks 7 and 8) in the responders and nonresponders: trough concentrations were 60±20 μg/ml in the responders versus 44±25 μg/ml in the nonresponders (mean±SD). HER2 overexpression level and type of metastatic sites were associated with significant differences in trough serum concentrations. At Week 2, patients with 2+ HER2 overexpression had significantly higher trough serum concentrations (n=40, mean=28.8 μg/ml, SD=10.4) compared with patients with 3+ HER2 overexpression (n=155, mean=24.1 μg/ml, SD=13.1). This difference in the average trough serum concentrations for Weeks 7 and 8 was no longer statistically significant. Further, at Week 2, patients with superficial disease had significantly higher trough serum concentrations (n=12, mean 34.1 μg/ml, SD=12.0) compared with patients with visceral disease (n=183, mean=24.4 μg/ml, SD=12.6). This difference in the average trough serum concentrations for Weeks 7 and 8 was significant. These data indicate that the rise in trough serum concentrations between Weeks 2 and 7/8 occurs for human patients with various disease profiles.

In a subsequent, similarly designed study, human breast cancer patients were treated with a loading dose of 8 mg/kg followed by maintenance doses of4 mg/kg weekly. The results of this preliminary human study indicated that an 8 mg/kg load:4 mg/kg weekly maintenance regimen was efficacious in reducing tumor volume in the patients.

The data disclosed in this Example indicate that front loading of antibody, such that a target serum concentration is reached more quickly, may be associated with improved outcomes.

Example 3

I.V. Bolus Delivery and Subcutaneous Infusion of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

The efficacy of infusion or bolus delivery of humanized anti-ErbB2 antibody (HERCEPTIN®, see Example 1 for preparation), either by intravenous injection or subcutaneous injection, was examined. The purpose of the study was to ask whether subcutaneous delivery was feasible and whether the convenient subcutaneous bolus delivery was useful in treating metastatic breast cancer in animals inoculated with a cell line that overexpresses the HER2 gene. The results, detailed below, show that i.v. and s.c. infusion and bolus delivery are feasible treatment methodologies.

A study in a nude mouse xenograft model, which incorporates a human breast cancer cell line that naturally overexpresses the HER2 gene (BT-474M1, derived from BT-474 cells, ATCC Accession number HTB-20), comparing tumor volume as a function of i.v. bolus versus s.c. infusion was performed as follows. In the first study athymic nude nu nu

7-9 week old female mice were obtained from Taconic Inc (Germantown, N.Y.). To initiate tumor development, each mouse was inoculated subcutaneously with 3×10^6 BT474M1 cells suspended in Matrigel™. When tumor nodules reached a volume of approximately 100 mm^3, animals were randomized to 4 treatment groups. The groups were treated according to Table 3.

TABLE 3

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Infusion

Group, Dose, Antibody	Target Serum Conc. µg/ml	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose
1 - Control, rhuMAb E25	20	IV LD and SC infusion	2.20	0.250 mg/ml (infusate)
2 - Low Dose SC rhuMAb HER2	1	IV LD and SC infusion	0.313	0.050 mg/ml (infusate)
3 - High Dose SC rhuMAb HER2	20	IV LD and SC infusion	6.25	1.00 mg/ml (infusate)
4 - IV Multi-Dose rhuMAb HER2	20 (trough)	IV LD and MD	4.00	2 mg/kg/week (IV bolus)

Serum Conc. = concentration in serum. LD = loading dose. MD = maintenance dose.
Infusate concentration was calculated to achieve targeted serum concentration using Alzet ® osmotic minipumps (Alza Corp., Palo Alto, CA).

Animals were exposed to estrogen by subcutaneous sustained release estrogen pellet 9 days before the start of dosing to promote growth of grafted tumor cells. The animals were inoculated with the BT474M 1 cells 8 days before the beginning of treatment and tumors were allowed to grow. The animals were then treated with nonrelevant antibody E25 (non-specific for HER2 receptor, but a member of the monoclonal IgG class) or test antibody HERCEPTIN® anti-ErbB2 anitbody as indicated in Table 3. The dosage levels were selected to achieve target serum concentrations of HERCEPTIN®, either 1 µg/ml or 20 µg/ml, by subcutaneous pump infusion or by i.v. bolus delivery. The study groups were treated until day 35. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly (just prior to dosing for Group 4) using 3 mice/group/time point. The anti-ErbB2 antibody concentration was determined according to the method disclosed herein involving standard techniques. Tumor volumes were measured two days before dosing began and twice per week from day 6 to day 35 in the study for which data is tabulated below. Tumors were measured in three dimensions and volumes were expressed in mm^3. Efficacy was determined by a statistical comparison (ANOVA) of tumor volumes of test animals relative to untreated control animals.

As shown in Table 4, below, treatment of the BT474M 1 tumor-bearing mice with HERCEPTIN® anti-ErbB2 antibody by the indicated dosage methods significantly inhibited the growth of the tumors. All HERCEPTIN®-treated groups showed similar inhibition of tumor growth relative to the control group. No dose-response was observed.

TABLE 4

Comparison of S.C. Infusion and I.V. Bolus Delivery

Treatment Group	Tumor Volume (mm^3), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6-Day 35 (n = 13)	HERCEPTIN ® Serum Conc. (µg/ml), Day 27, (n = 3)
control s.c. infusion	764 ± 700	5650 ± 4700	4.16 ± 1.94

TABLE 4-continued

Comparison of S.C. Infusion and I.V. Bolus Delivery

Treatment Group	Tumor Volume (mm^3), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6-Day 35 (n = 13)	HERCEPTIN ® Serum Conc. (µg/ml), Day 27, (n = 3)
s.c. infusion (low dose)	80.6 ± 158	1610 ± 1250	2.11 ± 1.74
s.c. infusion (high dose)	31 ± 75.6	1440 ± 1140	22.1 ± 5.43
i.v. bolus dose*	49.7 ± 95.7	2150 ± 1480	21.7 ± 17.1**

s.c. = subcutaneous delivery; i.v. = intravenous delivery.
*4.0 mg/kg Loading Dose and 2.0 mg/kg/week Maintenance Dose.
**at predose (trough serum concentration immediately prior to a maintenance dose)

The results tabulated above indicate that maintenance of a serum concentration of approximately 2 µg/ml was as effective as a concentration of 20 µg/ml in this study. The results indicated that dosing by subcutaneous infusion was as effective as intravenous bolus dosing and achieved similar trough serum concentrations. The results also indicate that the dose levels studied are at the top of the dose-response curve in this model and that subcutaneous dosing is effective in treating breast cancer tumors. Thus, subcutaneous administration of maintenance doses is feasible as part of a HERCEPTIN® anti-ErbB2 antibody treatment regimen.

Example 4

I.V. Bolus and Subcutaneous Bolus Deliveries of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

Subcutaneous bolus delivery is convenient and cost-effective for the patient and health care professionals. The results of the study disclosed in this example indicate that subcutaneous bolus delivery was as effective as intravenous bolus delivery in reducing breast cell tumor size in a mouse.

This study was set up as disclosed herein in Example 3 for the comparison of intravenous bolus and subcutaneous infusion delivery. A sustained release estrogen implant was inserted subcutaneously one day before tumor cell innoculation as described in Example 3. Six days after tumor cell innoculation, the initial tumor measurement was performed. Seven days after tumor cell innoculation, the first dose of control antibody or HERCEPTIN® anti-ErbB2 antibody was delivered. The animal groups, type of delivery, loading dose and maintenance doses are provided in Table 4. Animals were dosed once weekly for 4 weeks.

TABLE 5

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Bolus Delivery

Group	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose (mg/kg/week)	n
1 - Control rhuMAb E25	IV	8	4	10
2 - rhuMAb HER2	IV	2	1	10
3 - rhuMAb HER2	IV	4	2	10
4 - rhuMAb HER2	IV	8	4	10
5 - rhuMAb HER2	SC	4	2	10

IV = intraveneous; SC = subcutaneous; n = number of animals per group.

The mice were treated according to the information in Table 4 and using the techniques disclosed in Example 3. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly before each weekly i.v. maintenance dose according to the procedure described herein and using standard techniques. The control E25 antibody serum concentration was determined according to standard immunoassay techniques. Table 6 shows the increase in HERCEPTIN® anti-ErbB2 antibody serum concentrations with time.

TABLE 6

IV versus SC Bolus Delivery: Serum HERCEPTIN ® Anti-ErbB2 Antibody Concentration

	Serum Concentration, μg/ml			
Treatment Group (delivery, MD)	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
1 - Control rhu MAb E25	0	25.9	34.6	38.5
(IV, 4 mg/kg)	(0)	(8.29)	(11.2)	(14.4)
2 - rhu MAb HER2	0	4.96	8.55	8.05
(IV, 1 mg/kg)	(0)	(3.79)	(5.83)	(4.67)
3 - rhu MAb HER2	0	13.4	18.9	22.6
(IV, 2 mg/kg)	(0)	(9.24)	(12.0)	(9.21)
4 - rhu MAb HER2	0	29.6	37.7	46.2
(IV, 4 mg/kg)	(0)	(13.5)	(14.4)	(13.8)
5 - rhu MAb HER2	0	12.5	16.9	17.6
(SC, 2 mg/kg)	(0)	(7.33)	(10.2)	(10.7)

n = 10 for time points Days 0, 7 and 14. N = 9 for Day 21.

Table 7 shows the relative efficacy of intravenous bolus delivery and subcutaneous bolus delivery for Groups 1-5 having achieved the serum antibody concentrations presented in Table 6. For this study, efficacy was measured as a decrease in tumor volume. Tumor volume was measured twice weekly.

TABLE 7

Efficacy of HERCEPTIN ® Anti-ErbB2 Antibody Measured as a Change in Tumor Volume Comparing Intravenous Bolus and Subcutaneous Bolus Delivery, Mean (SD)

Treatment Group (Delivery, MD)	Tumor Vol. Day 6, mm^3	Tumor Vol. Day 28, mm^3	Tumor Vol. Day 31, mm^3	Day 6-Day 31* Area Under Curve Tumor Vol., mm^3	Tumor Growth Rate on Log (TM + 1)
1-IV Control	321	1530	1630	13600	0.0660
	(190)	(1040)	(1170)	(7230)	(0.0200)
2-IV Herceptin	297	175	151	4690	−0.0505
1 mg/kg	(130)	(215)	(188)	(1400)	(0.142)
3-IV Herceptin	269	75.7	73.6	3510	−0.0608
2 mg/kg	(129)	(92.4)	(84.5)	(1220)	(0.110)
4-IV Herceptin	272	25.3	25.8	2880	−0.0810
4 mg/kg	(117)	(75.9)	(72.9)	(1230)	(0.0859)
5-SC Herceptin	268	76.2	90.4	3230	−0.0304
2 mg/kg	(117)	(98.8)	(105)	(1440)	(0.104)

N = 10 for each data point. TM = tumor measurement. IV = intravenous. SC = subcutaneous. MD = maintenance dose. Tumor Vol. = tumor volume, mm^3.
*Day 17 excluded due to measurement error.
Tumor growth rate calculated on Day 21-Day 31 Log (TM + 1). Area under the curve is the area beneath a plot of tumor volume versus time.

FIGS. **4**A and **4**B are graphical plots of changes in tumor volume over time, some of which data is found in Table 7. FIG. **4**A is a linear plot of tumor volume versus time. FIG. **4**B is a semilogarithmic plot of the same data, allowing the test points be viewed more clearly. The data in Table 7 and FIGS. **4**A and **4**B indicate that, although a dose-related response was not observed between HERCEPTIN-treated groups, dosing by subcutaneous bolus was as effective as intravenous bolus dosing and achieved similar trough serum concentrations.

Example 5

Regimens for Intravenous and Subcutaneous Delivery of Anti-ErbB2 Antibody

According to the invention, methods of anti-ErbB2 antibody (e.g., HERCEPTIN®) delivery comprise greater front loading of the drug to achieve a target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less. According to the invention, this initial dosing is followed by dosing that maintains the target serum concentration by subsequent doses of equal or smaller amount. An advantage of the methods of the invention is that the maintenance dosing may be less frequent and/or delivered by subcutaneous injection, making the treatment regimens of the invention convenient and cost-effective for the patient and medical professionals administering the antibody. In addition, a subcutaneous maintenance dose regimen may be interrupted by intravenous dosing (such as infusion) when the patient's chemotherapy requires delivery of other drugs by intravenous injection.

To test the following dosage regimens, human subjects are selected according to the criteria disclosed in Example 1, above. The number of initial doses is one or more doses sufficient to achieve an efficacious target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The number of maintenance doses may be one or more doses sufficient to achieve suppression of disease symptoms, such as a decrease in tumor volume. The maintenance doses are equal to or smaller than the initial dose or doses, consistent with an object of the invention of administering HERCEPTIN® anti-ErbB2 antibody by regimens providing greater front loading. The specific drug delivery regimens disclosed herein are representative of the invention and are not meant to be limiting.

In one trial, an initial dose of 6 mg/kg, 8 mg/kg, or 12 mg/kg of HERCEPTIN® anti-ErbB2 antibody is delivered to human patients by intravenous or subcutaneous injection. Initial doses (loading doses) are delivered by intravenous infusion or bolus injection or preferably subcutaneous bolus injection. Preferably a target trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 μg/ml is achieved (averaged for all patients in the treatment group) and maintained by subsequent doses of anti-ErbB2 antibody that are equal to or smaller than the initial dose. In one method, a target trough serum concentration is achieved and maintained by once-per-week deliveries of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody by intravenous or subcutaneous injection for at least eight weeks. Alternatively, for this or any dosage regimen disclosed herein, subcutaneous continuous infusion by subcutaneous pump is used to delivery subsequent maintenance doses.

In another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 μg/ml, averaged for an entire treatment group.

In another method, an initial (front loading) dose of 12 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 μg/ml.

In yet another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous infusion or bolus injection, or preferably by subcutaneous bolus injection or infusion. This is followed by administration of 8 mg/kg per week or 8 mg/kg per 2-3 weeks to maintain a trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 μg/ml. Maintenance doses are delivered by intravenous infusion or bolus injection, or preferably by subcutaneous infusion or bolus injection.

In another method, the front loading initial dose is a series of intravenous or subcutaneous injections, for example, one on each of days 1, 2, and 3 of at least 1 mg/kg for each injection (where the amount of anti-ErbB2 antibody delivered by the sum of initial injections is more than 4 mg/kg), followed by maintenance doses of 6 mg/kg once each 3 week interval to maintain a target trough serum concentration (for example, approximately 10-20 μg/ml) of HERCEPTIN® anti-ErbB2 antibody. The maintenance doses are delivered by intravenous infusion or bolus injection or by subcutaneous infusion or subcutaneous bolus injection.

In yet another method, the front loading is by intravenous infusion of at least 1 mg/kg, preferably 4 mg/kg on each of five consecutive days, followed by repeats of this cycle a sufficient number of times to achieve suppression of disease symptoms. Following the initial dose or doses, subsequent doses may be delivered by subcutaneous infusion or bolus injection if tolerated by the patient. Such subcutaneous delivery is convenient and cost-effective for the patient and administering health care professionals.

In still another method, HERCEPTIN® anti-ErbB2 antibody is delivered initially as at least 2 intravenous infusions per week for three weeks, followed by repeats of this cycle to maintain an efficacious trough serum concentration of HERCEPTIN® anti-ErbB2 antibody. The dose is at least 4 mg/kg of anti-ErbB2 antibody, preferably at least 5 mg/kg. The maintenance drug deliveries may be intravenous or subcutaneous.

Where the animal or patient tolerates the antibody during and after an initial dose, delivery of subsequent doses may be subcutaneous, thereby providing greater convenience and cost-effectiveness for the patient and health care professionals.

In animal studies, an initial dose of more than 4 mg/kg, preferably more than 5 mg/kg delivered by intravenous or subcutaneous injection, is followed by subcutaneous bolus injections of 2 mg/kg twice per week (separated by 3 days) to maintain a trough serum concentration of approximately 10-20 μg/ml. In addition, where the animal or patient is known to tolerate the antibody, an initial dose of HERCEPTIN® anti-ErbB2 antibody is optionally and preferably deliverable by subcutaneous bolus injection followed by subcutaneous maintenance injections.

While target serum concentrations are disclosed herein for the purpose of comparing animal studies and human trials, target serum concentrations in clinical uses may differ. The disclosure provided herein guides the user in selecting a front loading drug delivery regimen that provides an efficacious target trough serum concentration.

The methods of the invention disclosed herein optionally include the delivery of HERCEPTIN® anti-ErbB2 antibody in combination with a chemotherapeutic agent (other than an anthrocycline derivative) to achieve suppression of disease symptoms. The chemotherapeutic agent may be delivered with HERCEPTIN® anti-ErbB2 antibody or separately and according to a different dosing schedule. For example, subcutaneous delivery of HERCEPTIN® anti-ErbB2 antibody with TAXOL® is included in the invention. In addition, intravenous or subcutaneous injection of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody, followed by intravenous or subcutaneous injection of 6 mg/kg HERCEPTIN® anti-ErbB2 antibody every 3 weeks is administered in combination with a chemotherapeutic agent, such as a taxoid (e.g. paclitaxel 175 mg/m2 every 3 weeks) or an anthracycline derivative (e.g. doxorubicin 60 mg/m2 or epirubicin 75 mg/m2 every 3 weeks). Optionally, where an anthracycline derivative is administered, a cardioprotectant (e.g. 600 mg/m2 cyclophosphamide every 3 weeks) is also administered. In another combination therapy, anti-ErbB2 antibody is administered in a loading dose of more than 4 mg/kg, preferably more than 5 mg/kg, and more preferably at least 8 mg/kg. The loading dose is followed by maintenance doses of at least 2 mg/kg weekly, preferably 6 mg/kg every 3 weeks. The combination therapy includes administration of a taxoid during treatment with anti-ErbB2 antibody. According to one embodiment of the invention, the taxoid is paclitaxel and is administered at a dose of 70-100 mg/m^2/week. According to another embodiment of the invention, the taxoid is docetaxel and is administered at a dose of 30-70 mg/m^2/week.

Example 6

HERCEPTIN® Administered Intravenously Every Three Weeks in Combination with Paclitaxel

Currently, the recommended dose of HERCEPTIN® is 2 mg/kg once weekly. Patients will be administered HERCEPTIN® every three weeks instead of weekly, along with

paclitaxel (175 mg/m^2 every three weeks). Simulation of the proposed treatment regimen suggests that the trough serum concentrations will be 17 mcg/ml, in the range (10-20 mcg/ml) of the targeted trough serum concentrations from previous HERCEPTIN® IV clinical trials. After the first 12 patients the PK parameters will be assessed, if exposure is felt inadequate, then the dose will be increased to 8 mg/kg every three weeks for the remaining 12 patients.

Inclusion Criteria

1) Females24 18 years of age
2) Histologically confirmed ErbB2 over-expressing metastatic breast cancer
3) Patients who have been newly diagnosed with metastatic disease
4) Have a Karnofsky performance status of 24 70%
5) Give written informed consent prior to any study specific screening procedures with the understanding that the patient has the right to withdraw from the study at any time, without prejudice.

Exclusion Criteria

1) Pregnant or lactating women
2) Women of childbearing potential unless (1) surgically sterile or (2) using adequate measures of contraception such as oral contraceptive, intra-uterine device or barrier method of contraception in conjunction with spermicidal jelly.
3) Clinical or radiologic evidence of CNS metastases.
4) History of any significant cardiac disease
5) LVEF≦50%
6) No prior taxane therapy in any treatment setting.
7) Any of the following abnormal baseline hematologic values:
   Hb less than 9 g/dl
   WBC less than 3.0×10^9/l
   Granulocytes less than 1.5×10^9/l
   Platelets less than 100×10^9/l
8) Any of the following abnormal baseline liver function tests:
   Serum bilirubin greater than 1.5×ULN (upper normal limit)
   ALT and/or AST greater than 2.5×ULN (greater than 4.0×ULN if liver or bone metastasis)
   Alkaline phosphatase greater than 2.5×ULN (greater than 4.0×ULN if liver or bone metastasis)
9) The following abnormal baseline renal function tests:
   serum creatinine greater than 1.5×ULN
10) History of other serious medical conditions that would preclude patient participation in an investigational study.

HERCEPTIN® Loading dose and schedule: 8 mg/kg for first dose. Maintenance dose and schedule: 6 mg/kg every 3 weeks.

Paclitaxel—175 mg/m^2 IV every 3 weeks×6 cycles as a 3-hour infusion.

NOTE: On the first cycle of treatment, paclitaxel will be dosed 8 hours prior to HERCEPTIN® to determine the PK of paclitaxel alone. HERCEPTIN® will be administered 8 hours post-paclitaxel for the 1st cycle only. In subsequent treatment cycles, HERCEPTIN® will be administered prior to paclitaxel.

The total duration of this study is 18 weeks. Study subjects will receive up to 6 total HERCEPTIN® doses. After the last subject has received the last cycle of paclitaxel, data collection for safety and pharmacokinetic analysis will stop, and the study will close to protocol specified treatment. Study subjects may continue to receive the HERCEPTIN® +/− paclitaxel at the discretion of the investigator.

It is believed that the above treatment regimen will be effective in treating metastatic breast cancer, despite the infrequency with which HERCEPTIN® is administered to the patient.

While the particular aspects and embodiments of the invention as herein shown and disclosed in detail is fully capable of obtaining the objects and providing the advantages herein before stated, it is to be understood that it is merely illustrative of some of the presently preferred embodiments of the invention and that no limitations are intended to the details of methods and articles of manufacture shown other than as described in the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

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<223> OTHER INFORMATION: salvage receptor binding epitope

<400> SEQUENCE: 6

His Gln Asn Ile Ser Asp Gly Lys
1 5

<210> SEQ ID NO 7
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: salvage receptor binding epitope

<400> SEQUENCE: 7

Val Ile Ser Ser His Leu Gly Gln
1 5

<210> SEQ ID NO 8
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
1 5 10 15

Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
20 25 30

Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
35 40 45

Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg
50 55

<210> SEQ ID NO 9
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<213> ORGANISM: Homo sapiens

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Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr
1 5 10 15

Cys Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr
20 25 30

Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
35 40 45

Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
50 55 60

Gly Ala Cys Gln Pro
65

<210> SEQ ID NO 10
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Mus Musculus

-continued

<400> SEQUENCE: 10

Asp Thr Val Met Thr Gln Ser His Lys Ile Met Ser Thr Ser Val
1 5 10 15

Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
20 25 30

Ile Gly Val Ala Trp Tyr Gln Gln Arg Pro Gly Gln Ser Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp
50 55 60

Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
65 70 75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
80 85 90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu
95 100 105

Ile Lys

<210> SEQ ID NO 11
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 11

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Thr Phe Thr
20 25 30

Asp Tyr Thr Met Asp Trp Val Lys Gln Ser His Gly Lys Ser Leu
35 40 45

Glu Trp Ile Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
50 55 60

Asn Gln Arg Phe Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser
65 70 75

Ser Arg Ile Val Tyr Met Glu Leu Arg Ser Leu Thr Phe Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
95 100 105

Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
110 115

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1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
20 25 30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
50 55 60

-continued

```
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys


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<213> ORGANISM: Artificial sequence
<220> FEATURE:
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<400> SEQUENCE: 13

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  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
                20                  25                  30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
               110                 115


<210> SEQ ID NO 14
<211> LENGTH: 107
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<220> FEATURE:
<223> OTHER INFORMATION: VL consensus sequence

<400> SEQUENCE: 14

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  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
                20                  25                  30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys
```

-continued

<210> SEQ ID NO 15
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: VH consensus sequence

<400> SEQUENCE: 15

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                 20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                 95                 100                 105

Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115
```

The invention claimed is:

1. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB2 antibody; and

administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks; and

further comprising administering an effective amount of a chemotherapeutic agent to the patient.

2. The method of claim **1**, wherein the initial dose is at least approximately 6 mg/kg.

3. The method of claim **2**, wherein the initial dose is at least approximately 8 mg/kg.

4. The method of claim **3**, wherein the initial dose is at least approximately 12 mg/kg.

5. The method of claim **1**, wherein the subsequent doses are separated in time from each other by at least three weeks.

6. The method of claim **1**, wherein the initial dose is administered by intravenous injection, and wherein at least one subsequent dose is administered by subcutaneous injection.

7. The method of claim **1**, wherein the initial dose is administered by intravenous injection, wherein at least two subsequent doses are administered, and wherein each subsequent dose is administered by a method selected from the group consisting of intravenous injection and subcutaneous injection.

8. The method of claim **1**, wherein the initial dose and at least one subsequent dose are administered by subcutaneous injection.

9. The method of claim **1**, wherein the initial dose is selected from the group consisting of approximately 6 mg/kg, 8 mg/kg, or 12 mg/kg, wherein the plurality of subsequent doses are at least approximately 2 mg/kg.

10. The method of claim **9**, wherein the plurality of subsequent doses are separated in time from each other by at least three weeks.

11. The method of claim **10**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

12. The method of claim **10**, wherein the initial dose is approximately 12 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

13. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 8 mg/kg.

14. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, wherein at least one subsequent dose is 8 mg/kg, and wherein administration of the initial dose and subsequent doses are separated in time by at least 2 weeks.

15. The method of claim **14**, wherein the initial dose and subsequent doses are separated in time by at least 3 weeks.

16. The method of claim **1**, wherein said cancer is selected from the group consisting of breast cancer, leukemia, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

17. The method of claim **16**, wherein said cancer is breast cancer.

18. The method of claim 17, wherein said cancer is metastatic breast carcinoma.

19. The method of claim 1, wherein said antibody binds to the extracellular domain of the ErbB2 receptor.

20. The method of claim 19, wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

21. The method of claim 20, wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

22. The method of claim 1, wherein the chemotherapeutic agent is a taxoid.

23. The method of claim 22, wherein said taxoid is paclitaxel or docetaxel.

24. The method of claim 1, wherein the effective amount of the anti-ErbB2 antibody and the effective amount of the chemotherapeutic agent as a combination is lower than the sum of the effective amounts of said anti-ErbB2 antibody and said chemotherapeutic agent, when administered individually, as single agents.

25. The method of claim 1, wherein the chemotherapeutic agent is an anthracycline.

26. The method of claim 25, wherein the anthracycline is doxorubicin or epirubicin.

27. The method of claim 25, wherein the method further comprises administration of a cardioprotectant.

28. The method of claim 1, wherein efficacy is measured by determining the time to disease progression or the response rate.

29. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising: administering to the patient an initial dose of the antibody, wherein the initial dose is a plurality of doses, wherein each of the plurality of initial doses is at least approximately 1 mg/kg and is administered on at least 3 consecutive days, and administering to the patient at least one subsequent dose of the antibody, wherein at least one subsequent dose is at least approximately 6 mg/kg, and wherein administration of the last initial dose and the first subsequent and additional subsequent doses are separated in time by at least 3 weeks, and further comprising administering an effective amount of a chemotherapeutic agent to the patient.

30. A method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by two or more subsequent doses of the antibody, wherein the subsequent doses are separated from each other in time by at least about two weeks, and further comprising administering an effective amount of a chemotherapeutic agent to the patient.

31. The method of claim 30, wherein the first dose and a first subsequent dose are separated from each other in time by at least about three weeks.

32. The method of claim 30, wherein the first dose and subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

33. The method of claim 32, wherein the first dose and subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

34. The method of claim 33, wherein the first dose and subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

35. The method of claim 30, wherein from about two to about ten subsequent doses of the antibody are administered to the patient.

36. The method of claim 30, wherein the two or more subsequent doses are separated from each other in time by at least about three weeks.

37. The method of claim 30, wherein the two or more subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

38. The method of claim 30, wherein the two or more subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

39. The method of claim 30, wherein the two or more subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

40. The method of claim 30, wherein the chemotherapeutic agent is a taxoid.

* * * * *

EXHIBIT I

US006417335B1

(12) **United States Patent**
Basey et al.

(10) **Patent No.: US 6,417,335 B1**
(45) **Date of Patent: Jul. 9, 2002**

(54) **PROTEIN PURIFICATION**

(75) Inventors: **Carol D. Basey**, Winters; **Greg S. Blank**, Menlo Park, both of CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/680,148**

(22) Filed: **Oct. 3, 2000**

Related U.S. Application Data

(62) Division of application No. 09/304,465, filed on May 3, 1999.
(60) Provisional application No. 60/084,459, filed on May 6, 1998.

(51) **Int. Cl.**[7] **C07K 1/18**; C07K 16/00
(52) **U.S. Cl.** **530/387.1**; 530/387.3; 530/387.7; 530/388.85; 530/389.7; 530/416
(58) **Field of Search** 530/416, 387.1, 530/387.3, 387.7, 388.85, 389.7

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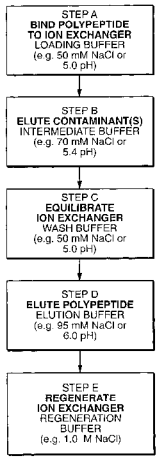
Primary Examiner—David Saunders
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

A method for purifying a polypeptide by ion exchange chromatography is described which involves changing the conductivity and/or pH of buffers in order to resolve a polypeptide of interest from one or more contaminants.

9 Claims, 7 Drawing Sheets



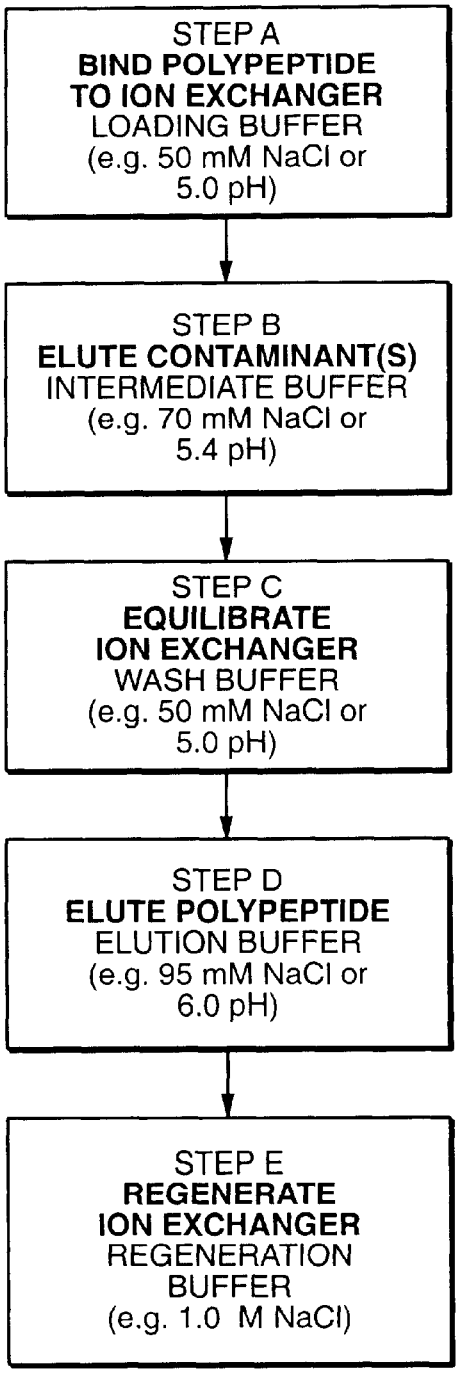

STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
5.4 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
6.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._1

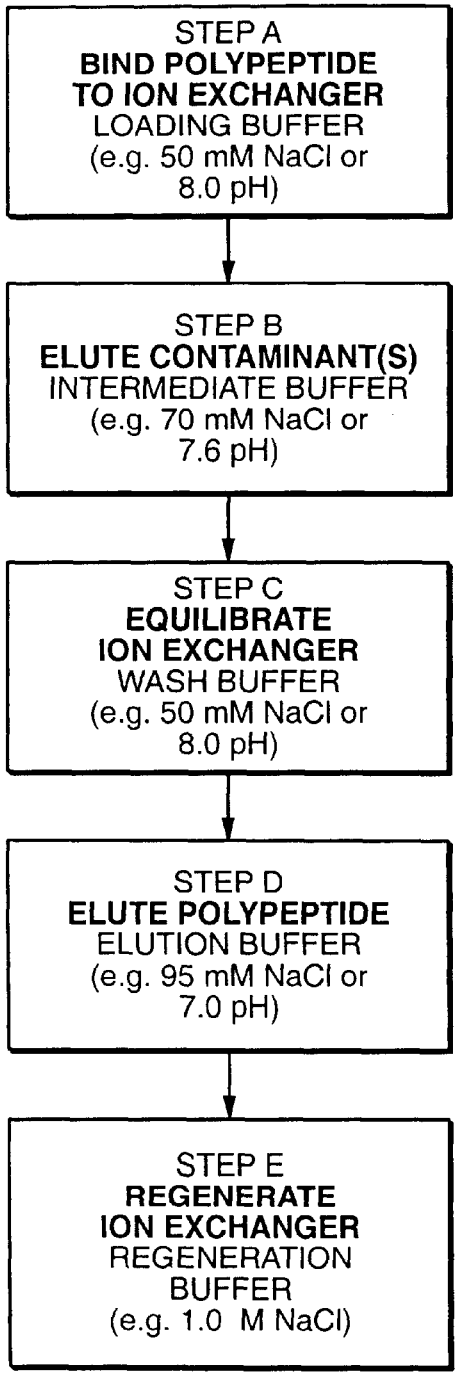
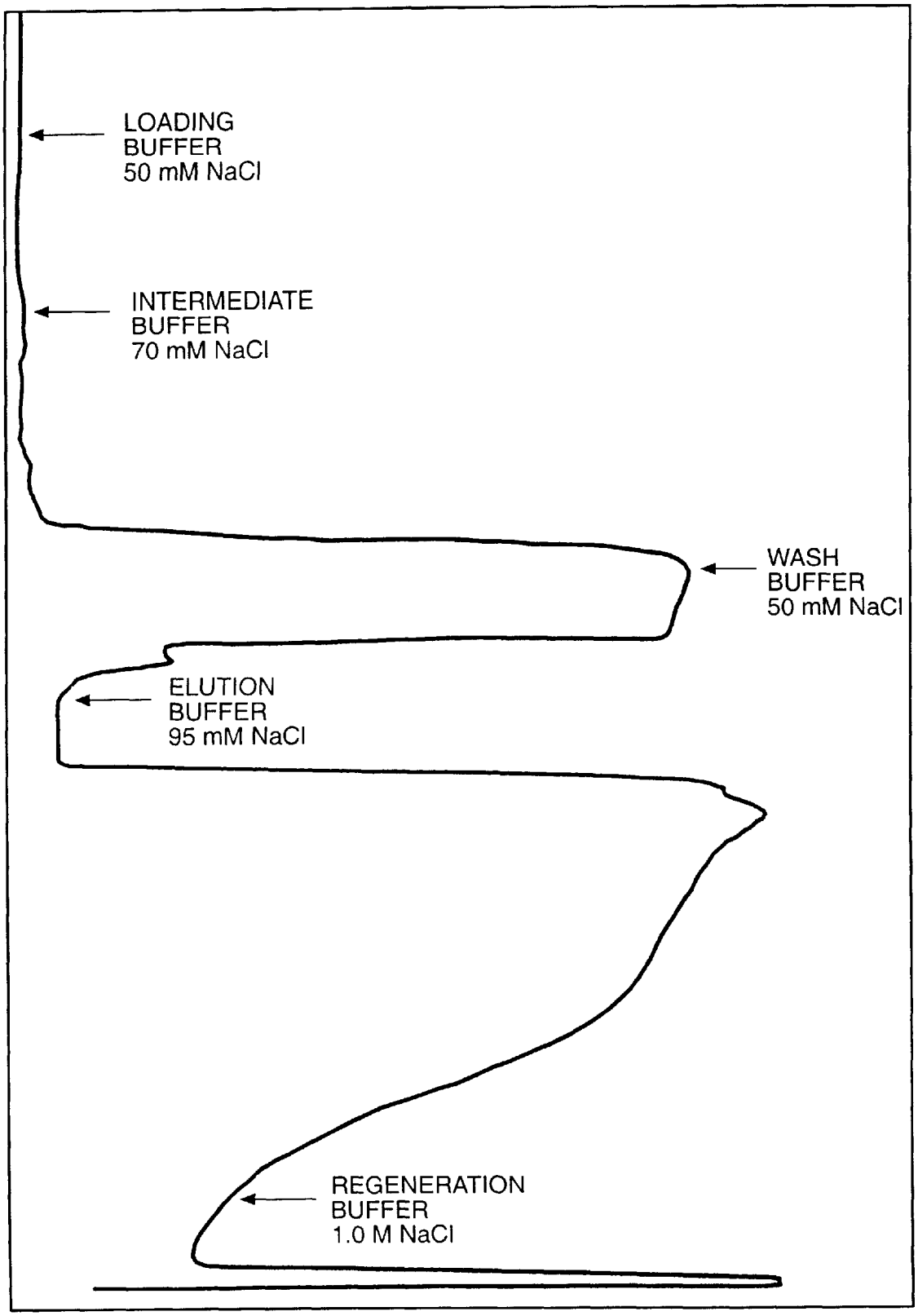

STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
7.6 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
7.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._2


LOADING
BUFFER
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INTERMEDIATE
BUFFER
70 mM NaCl
WASH
BUFFER
50 mM NaCl
ELUTION
BUFFER
95 mM NaCl
REGENERATION
BUFFER
1.0 M NaCl

FIG._3

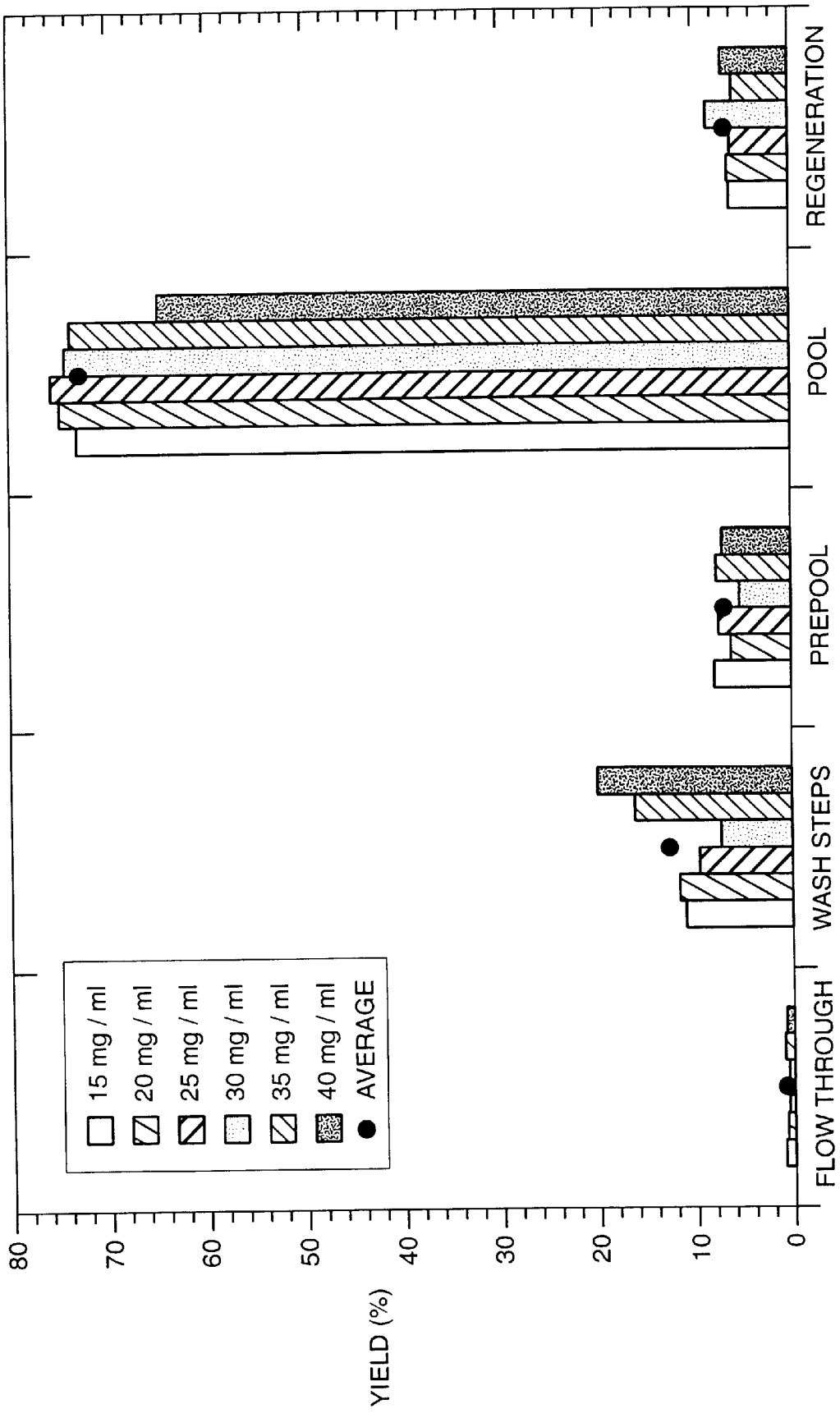

YIELD (%)
0
10
20
30
40
50
60
70
80
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
AVERAGE
FLOW THROUGH
WASH STEPS
PREPOOL
POOL
REGENERATION
CHROMATOGRAPHY FRACTION

FIG._4

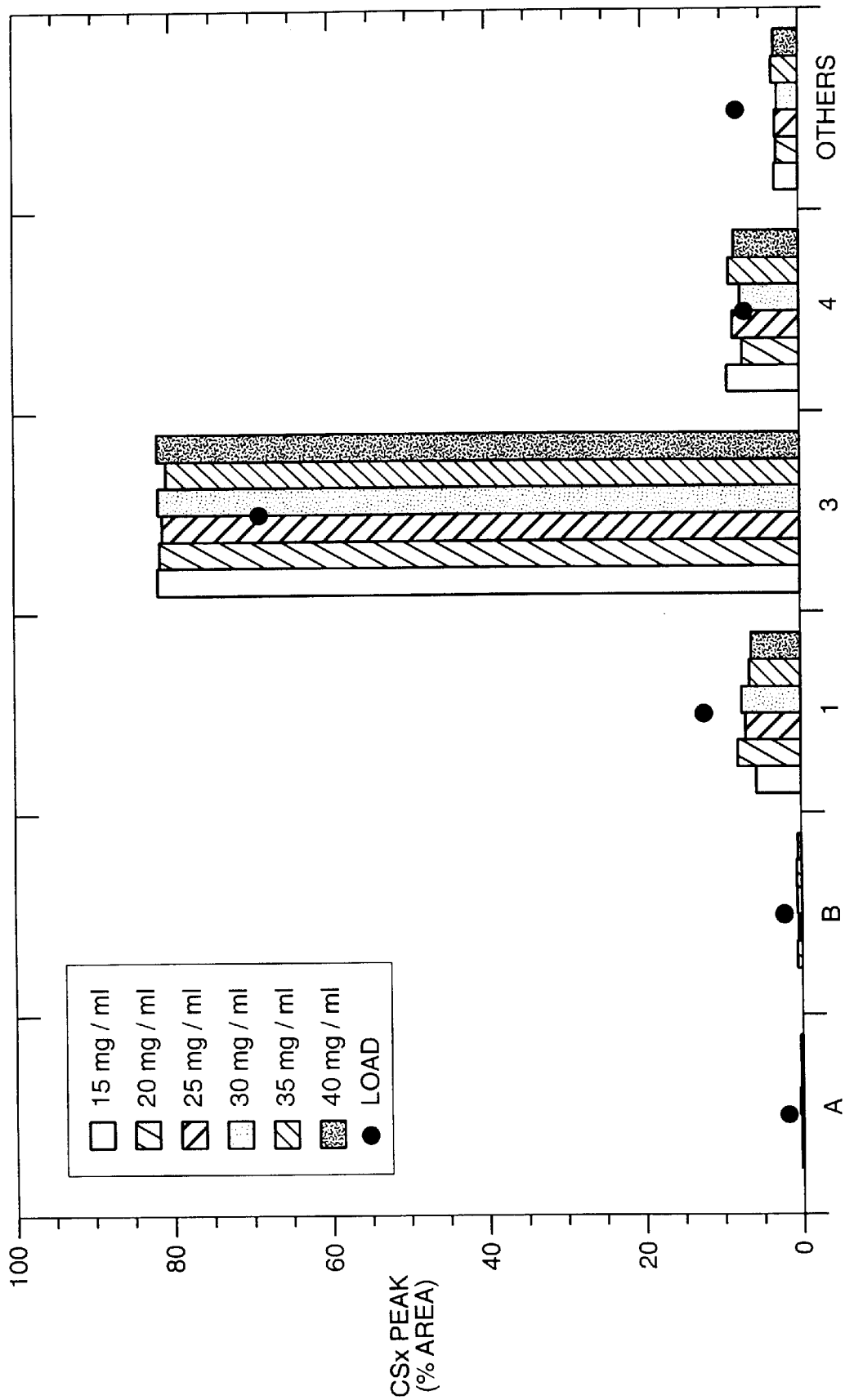

CSx PEAK (% AREA)
100
80
60
40
20
0
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
LOAD
A
B
1
3
4
OTHERS
CSx PEAK

FIG._5

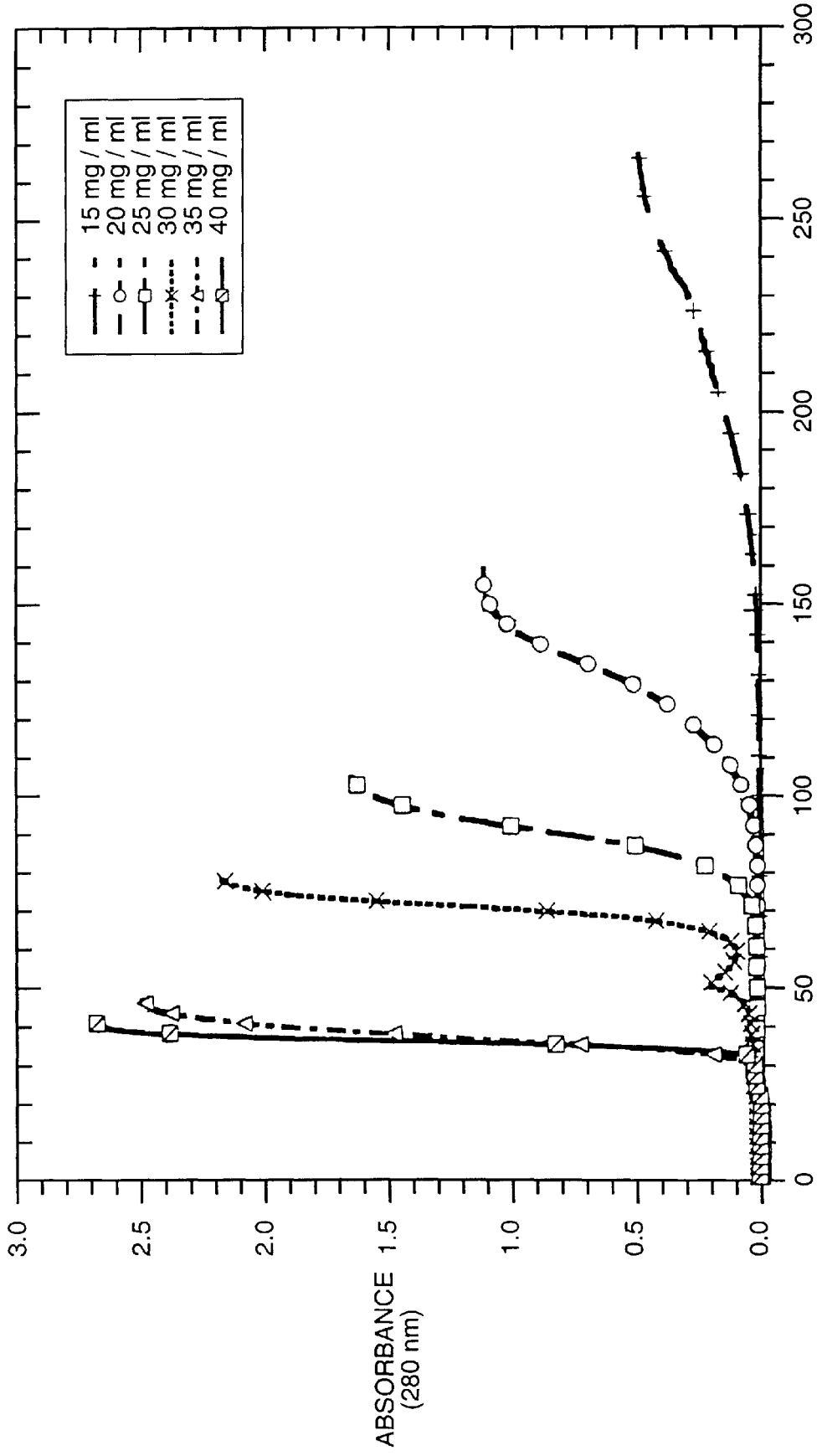
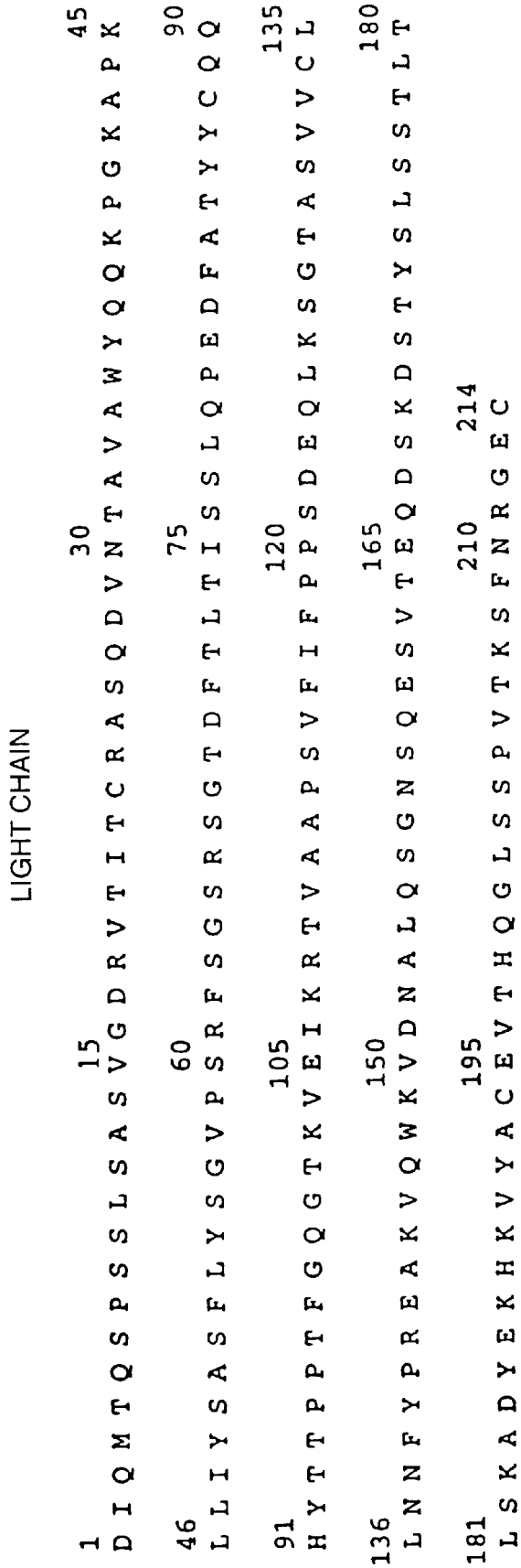

15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
ABSORBANCE (280 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
TIME (MINUTES)

FIG._6


LIGHT CHAIN
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91 H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
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30
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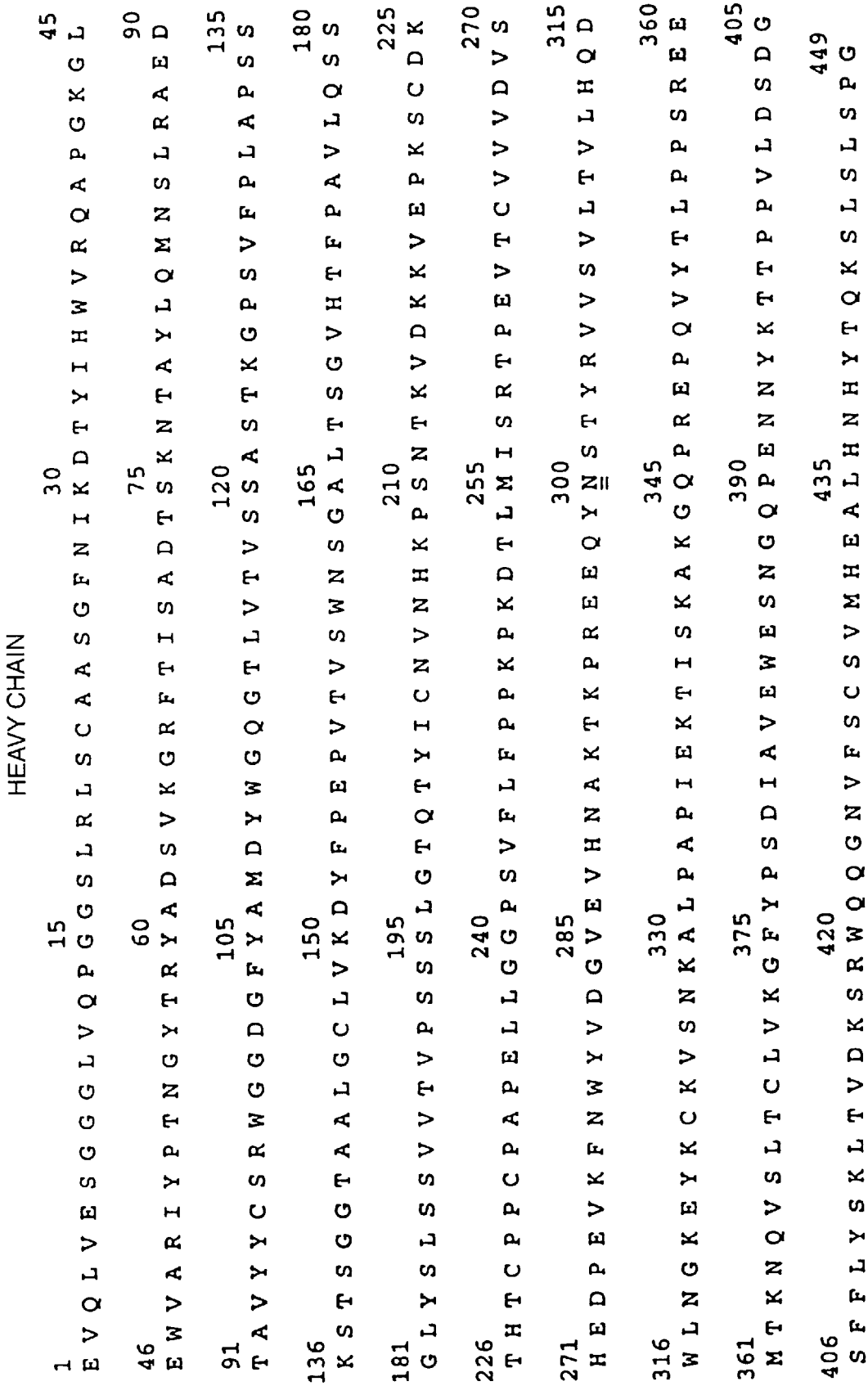
75
90
105
120
135
150
165
180
195
210
214

FIG._7A


HEAVY CHAIN
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46 E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D
91 T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S
136 K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S
181 G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K
226 T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S
271 H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D
316 W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E
361 M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G
406 S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375 390 405 420 435 449

FIG._7B

PROTEIN PURIFICATION

This is a divisional of application Ser. No. 09/304,465 filed May 3, 1999 which claims priority under 35 USC §119 to provisional application Ser. No. 60/084,459 filed May 6, 1998, both disclosures of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to protein purification. In particular, the invention relates to a method for purifying a polypeptide (e.g. an antibody) from a composition comprising the polypeptide and at least one contaminant using the method of ion exchange chromatography.

2. Description of Related Art

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through".

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins.

In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

SUMMARY OF THE INVENTION

The present invention provides an ion exchange chromatographic method wherein a polypeptide of interest is bound to the ion exchange material at an initial conductivity or pH and then the ion exchange material is washed with an intermediate buffer at a different conductivity or pH, or both. At a specific point following this intermediate wash, and contrary to ion exchange chromatography standard practice, the ion exchange material is washed with a wash buffer where the change in conductivity or pH, or both, from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity or pH, or both, achieved in the previous steps. Only after washing with the wash buffer, is the ion exchange material prepared for the polypeptide molecule of interest to be eluted by the application of the elution buffer having a conductivity or pH, or both, which differ from the conductivity or pH, or both, of the buffers used in previous steps.

This novel approach to ion exchange chromatography is particularly useful in situations where a product molecule must be separated from a very closely related contaminant molecule at full manufacturing scale, where both purity and high recovery of polypeptide product are desired.

Accordingly, the invention provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to an ion exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the ion exchange material with an intermediate buffer at a second conductivity and/or pH so as to elute the contaminant from the ion exchange material;

(c) washing the ion exchange material with a wash buffer which is at a third conductivity and/or pH, wherein the change in conductivity and/or pH from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity and/or pH from the loading buffer to the intermediate buffer; and

(d) washing the ion exchange material with an elution buffer at a fourth conductivity and/or pH so as to elute the polypeptide from the ion exchange material. The first conductivity and/or pH may be the same as the third conductivity and/or pH.

Where the ion exchange material comprises a cation exchange resin, the conductivity and/or pH of the intermediate buffer is/are preferably greater than the conductivity and/or pH of the loading buffer; the conductivity and/or pH of the wash buffer is/are preferably less than the conductivity and/or pH of the intermediate buffer; and the conductivity and/or pH of the elution buffer is/are preferably greater than the conductivity and/or pH of the intermediate buffer. Preferably, the conductivity and/or pH of the wash buffer is/are about the same as the conductivity and/or pH of the loading buffer.

Preferably elution of the contaminant and of the polypeptide is achieved by modifying the conductivity of the intermediate buffer and of the elution buffer, respectively, while keeping the pH of these buffers approximately the same.

The invention also provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to a cation exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the cation exchange material with an intermediate buffer at a second conductivity and/or pH which is greater than that of the loading buffer so as to elute the contaminant from the ion exchange material;

(c) washing the cation exchange material with a wash buffer which is at a third conductivity and/or pH which is less than that of the intermediate buffer; and

(d) washing the cation exchange material with an elution buffer at a fourth conductivity and/or pH which is greater than that of the intermediate buffer so as to elute the polypeptide from the ion exchange material.

In addition, the invention provides a method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin and, optionally, further comprising eluting the antibody from the cation exchange resin. The method preferably further comprises an intermediate wash step for eluting one or more contaminants from the ion exchange resin. This intermediate wash step usually precedes the step of eluting the antibody.

The invention further provides a composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof, wherein the amount of the acidic variant(s) in the composition is less than about 25% and preferably less than about 20%, e.g. in the range from about 1% to about 18%. Optionally, the composition further comprises a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram showing how one could perform cation exchange chromatography by altering conductivity (e.g. to the NaCl concentrations of Example 1 below) or by altering pH (e.g. to the pH values as shown in the flow diagram).

FIG. **2** is a flow diagram showing how one could perform anion exchange chromatography by altering conductivity (e.g. to the NaCl concentrations as depicted in the figure) or by altering pH (e.g. to the pH values as shown).

FIG. **3** is an absorbance trace from a cation exchange chromatography run of Example 1 at full manufacturing scale. Points at which the column is washed with the different buffers described herein are marked with arrows.

FIG. **4** depicts recombinant humanized anti-HER2 monoclonal antibody (rhuMAb HER2) recovered in each chromatography fraction (calculated as the percentage of the sum total of all fractions of the relevant chromatography). Flow through, wash steps, and prepool fractions are all effluent samples collected from the onset of load to the initiation of pooling. The pool fraction is the five column volume effluent sample of elution starting at the leading shoulder's inflection point. The regeneration fraction contains effluent captured from the end of pooling to the end of regeneration.

FIG. **5** shows the quality of rhuMAb HER2 in each cation exchange chromatography pool sample as evaluated by carboxy sulfon cation exchange high pressure liquid chromatography (CSx HPIEX). Peaks a, b, and 1 are deamidated forms of rhuMAb HER2. Peak 3 is nondeamidated rhuMAb HER2. Peak 4 is a combination of C-terminal Lysine containing and iso-aspartate variants of rhuMAb HER2.

FIG. **6** shows the absorbance (280 nm) profiles of the 0.025 M MES/0.070 M NaCl, pH 5.6 wash for each chromatography. The mass of rhuMAb HER2 applied to the cation exchange resin effects the peak's absorbance level at the apex as well as the amount of buffer required to reach the apex. Due to minor peaks which occur (as best seen in the 30 mg/mL load) in this wash, the apex is defined as absorbance levels of at least 0.5 absorbance units (AU).

FIGS. **7**A and **7**B show the amino acid sequences of humMAb4D5-8 light chain (SEQ ID NO:1) and humMAb4D5-8 heavy chain (SEQ ID NO:2), respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The "composition" to be purified herein comprises the polypeptide of interest and one or more contaminants. The composition may be "partially purified" (i.e. having been subjected to one or more purification steps, such as Protein A Chromatography as in Example 1 below) or may be obtained directly from a host cell or organism producing the polypeptide (e.g. the composition may comprise harvested cell culture fluid).

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β1, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor

binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides. Most preferred is a full length antibody that binds human HER2.

A "contaminant" is a material that is different from the desired polypeptide product. The contaminant may be a variant of the desired polypeptide (e.g. a deamidated variant or an amino-aspartate variant of the desired polypeptide) or another polypeptide, nucleic acid, endotoxin etc.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch et al., *Proc. Natl. Acad. Sci. USA* 80:1382–1386 (1983), version of the algorithm described by Needleman et al., *J. Mol. Biol.* 48:443–453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide may be prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

An "acidic variant" is a variant of a polypeptide of interest which is more acidic (e.g. as determined by cation exchange chromatography) than the polypeptide of interest. An example of an acidic variant is a deamidated variant.

A "deamidated" variant of a polypeptide molecule is a polypeptide wherein one or more asparagine residue(s) of the original polypeptide have been converted to aspartate, i.e. the neutral amide side chain has been converted to a residue with an overall acidic character. Deamidated humMAb4D5 antibody from the Example below has Asn30 in CDR1 of either or both of the V_L regions thereof converted to aspartate. The term "deamidated human DNase" as used herein means human DNase that is deamidated at the asparagine residue that occurs at position 74 in the amino acid sequence of native mature human DNase (U.S. Pat. No. 5,279,823; expressly incorporated herein by reference).

The term "mixture" as used herein in reference to a composition comprising an anti-HER2 antibody, means the presence of both the desired anti-HER2 antibody and one or more acidic variants thereof. The acidic variants may comprise predominantly deamidated anti-HER2 antibody, with minor amounts of other acidic variant(s). It has been found, for example, that in preparations of anti-HER2 antibody obtained from recombinant expression, as much as about 25% of the anti-HER2 antibody is deamidated.

In preferred embodiments of the invention, the polypeptide is a recombinant polypeptide. A "recombinant polypeptide" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the polypeptide, or produces the polypeptide as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in vitro cell culture as well a cell within a host animal. Methods for recombinant production of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

The antibody herein is directed against an "antigen" of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and av/b3 integrin including either a or b subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations

which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990). Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24–34 (L1), 50–56 (L2) and 89–97 (L3) in the light chain variable domain and 31–35 (H1), 50–65 (H2) and 95–102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Polypeptides of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26–32 (L1), 50–52 (L2) and 91–96 (L3) in the light chain variable domain and 26–32 (H1), 53–55 (H2) and 96–101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901–917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the rhuMAb HER2 antibody of the example below (humAb4D5–8) are identified in Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107–117 (1992) and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163–167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185. "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269–315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90:6444–6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Polypeptide Eng.* 8(10):1057–1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

"Multispecific antibodies" have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell adhesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-$p185^{HER2}$/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-RP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-D3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537–539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO 96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'—SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody F(ab')2 molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547–1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444–15 6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites.

Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

The phrase "ion exchange material" refers to a solid phase which is negatively charged (i.e. a cation exchange resin) or positively charged (i.e. an anion exchange resin). The charge may be provided by attaching one or more charged ligands to the solid phase, e.g. by covalent linking. Alternatively, or in addition, the charge may be an inherent property of the solid phase (e.g. as is the case for silica, which has an overall negative charge).

By "solid phase" is meant a non-aqueous matrix to which one or more charged ligands can adhere. The solid phase may be a purification column, a discontinuous phase of discrete particles, a membrane, or filter etc. Examples of materials for forming the solid phase include polysaccharides (such as agarose and cellulose); and other mechanically stable matrices such as silica (e.g. controlled pore glass), poly(styrenedivinyl)benzene, polyacrylamide, ceramic particles and derivatives of any of the above.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, BAKERBOND ABX™, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia).

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in Buffers. *A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., Ed. Calbiochem Corporation (1975). In one

embodiment, the buffer has a pH in the range from about 5 to about 7 (e.g. as in Example 1 below). Examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more contaminants onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more contaminants) is/are bound to the ion exchange resin.

The "intermediate buffer" is used to elute one or more contaminants from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that the contaminant is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all contaminants and the polypeptide of interest from the ion exchange resin.

The term "conductivity" refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution will have a higher conductivity. The unit of measurement for conductivity is mmhos (mS/cm), and can be measured using a conductivity meter sold, e.g., by Orion. The conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a buffering agent and/or concentration of a salt (e.g. NaCl or KCl) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers is modified to achieve the desired conductivity as in the Example below.

By "purifying" a polypeptide from a composition comprising the polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in a "homogeneous" y composition, which is used herein to refer to a composition comprising at least about 70% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 80% by weight.

Unless indicated otherwise, the term "HER2" when used herein refers to human HER2 protein and "HER2" refers to human HER2 gene. The human HER2 gene and HER2 protein are described in Semba et al., *PNAS (USA)* 82:6497–6501 (1985) and Yamamoto et al. *Nature* 319:230–234 (1986) (Genebank accession number X03363), for example.

The term "humMAb4D5–8" when used herein refers to a humanized anti-HER2 antibody comprising the light chain amino acid sequence of SEQ ID NO:1 and the heavy chain amino acid sequence of SEQ ID NO:2 or amino acid sequence variants thereof which retain the ability to bind HER2 and inhibit growth of tumor cells which overexpress HER2 (see U.S. Pat. No. 5,677,171; expressly incorporated herein by reference).

The "pI" or "isoelectric point" of a polypeptide refer to the pH at which the polypeptide's positive charge balances its negative charge. pI can be calculated from the net charge of the amino acid residues of the polypeptide or can be determined by isoelectric focussing (e.g. using CSx chromatography as in the Example below).

By "binding" a molecule to an ion exchange material is meant exposing the molecule to the ion exchange material under appropriate conditions (pH/conductivity) such that the molecule is reversibly immobilized in or on the ion exchange material by virtue of ionic interactions between the molecule and a charged group or charged groups of the ion exchange material.

By "washing" the ion exchange material is meant passing an appropriate buffer through or over the ion exchange material.

To "elute" a molecule (e.g. polypeptide or contaminant) from an ion exchange material is meant to remove the molecule therefrom by altering the ionic strength of the buffer surrounding the ion exchange material such that the buffer competes with the molecule for the charged sites on the ion exchange material.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the polypeptide purified as described herein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL™, Bristol-Myers Squibb Oncology, Princeton, N.J.), and doxetaxel, toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

MODES FOR CARRYING OUT THE INVENTION

The invention herein provides a method for purifying a polypeptide from a composition (e.g. an aqueous solution) comprising the polypeptide and one or more contaminants. The composition is generally one resulting from the recombinant production of the polypeptide, but may be that resulting from production of the polypeptide by peptide synthesis (or other synthetic means) or the polypeptide may be purified from a native source of the polypeptide. Preferably the polypeptide is an antibody, e.g. one which binds the HER2 antigen.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., *E. coli*, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., *Salmonella typhimurium*, Serratia, e.g., *Serratia marcescans*, and Shigella, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as *P. aeruginosa*, and Streptomyces. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; Kluyveromyces hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; yarrowia (EP 402,226); *Pichia pastoris* (EP 183,070); Candida; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; Schwanniomyces such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243–251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44–68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifuga-

tion or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The polypeptide is then subjected to one or more purification steps, including the ion exchange chromatography method as claimed herein. Examples of additional purification procedures which may be performed prior to, during, or following the ion exchange chromatography method include fractionation on a hydrophobic interaction chromatography (e.g. on phenyl sepharose), ethanol precipitation, isoelectric focusing, Reverse Phase HPLC, chromatography on silica, chromatography on HEPARIN SEPHAROSE™, further anion exchange chromatography and/or further cation exchange chromatography, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (e.g. using protein A, protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

Ion exchange chromatography is performed as claimed herein. A decision is first made as to whether an anion or cation exchange resin is to be employed. In general, a cation exchange resin may be used for polypeptides with pIs greater than about 7 and an anion exchange resin may be used for polypeptides with pIs less than about 7.

The anion or cation exchange resin is prepared according to known methods. Usually, an equilibration buffer is passed through the ion exchange resin prior to loading the composition comprising the polypeptide and one or more contaminants onto the resin. Conveniently, the equilibration buffer is the same as the loading buffer, but this is not required.

The various buffers used for the chromatography depend, for example, on whether a cation or anion exchange resin is employed. This is shown more clearly in the flow diagrams of FIGS. **1** and **2**.

With particular reference to FIG. **1**, which shows exemplary steps to be performed where a cation exchange resin is used, the pH and/or conductivity of each buffer is/are increased relative to the preceding buffer, except for the wash buffer where the conductivity and/or pH is/are less than the conductivity and/or pH of the preceding intermediate buffer. The aqueous solution comprising the polypeptide of interest and contaminant(s) is loaded onto the cation exchange resin using the loading buffer that is at a pH and/or conductivity such that the polypeptide and the contaminant bind to the cation exchange resin. As in the Example below, the loading buffer may be at a first low conductivity (e.g. from about 5.2 to about 6.6 mmhos). An exemplary pH for the loading buffer may be about 5.0 (see FIG. **1**). From about 20 mg/mL to about 35 mg/mL of the polypeptide (e.g. of a full length antibody) may, for example, be loaded on the ion exchange resin.

The cation exchange resin is then washed with an intermediate buffer which is at a second conductivity and/or pH so as to essentially elute the contaminant, but not a substantial amount of the polypeptide of interest. This may be achieved by increasing the conductivity or pH, or both, of the intermediate buffer. The change from loading buffer to intermediate buffer may be step-wise or gradual as desired. In the Example herein, the intermediate buffer had a greater conductivity than that of the loading buffer (i.e. the intermediate buffer's conductivity was in the range from about 7.3 to about 8.4 mmhos). Alternatively, as shown in FIG. **1**, the pH of the intermediate buffer may exceed that of the loading buffer in this embodiment of the invention, where a cation exchange resin is used. For example, the intermediate buffer may have a pH of about 5.4.

Following washing with the intermediate buffer, the cation exchange resin is washed or re-equilibrated with the wash buffer which has a conductivity or pH, or both, which is/are less than that of the intermediate buffer (i.e. the conductivity, or pH, or both, is/are changed in an opposite, i.e. reverse, direction to the preceding step, unlike ion exchange chromatography steps in the literature). In the Example below, the wash buffer had about the same conductivity as the loading buffer (i.e. in the range from about 5.2 to about 6.6 mmhos) and its conductivity was, therefore, less than that of the intermediate buffer. In another embodiment, one may reduce the conductivity of the wash buffer to a conductivity that is less than, or greater than, that of the loading buffer, provided the conductivity of the wash buffer is less than that of the intermediate buffer. In another embodiment, the pH of the wash buffer may be less than the pH of the intermediate buffer (e.g. the pH of the wash buffer may about 5.0). The change in conductivity and/or pH of the wash buffer compared to the intermediate buffer may be achieved by step-wise or gradual change of either or both of these parameters.

After the wash step of the preceding paragraph, the cation exchange resin is prepared for elution of the desired polypeptide molecule therefrom. This is achieved using an elution buffer that has a pH and/or conductivity such that the desired polypeptide no longer binds to the cation exchange resin and therefore is eluted therefrom. The pH and/or conductivity of the elution buffer generally exceed(s) the pH and/or conductivity of the loading buffer, the intermediate buffer and the wash buffer used in the previous steps. In the Example below, the conductivity of the elution buffer was in the range from about 10.0 to about 11.0 mmhos. Alternatively, or in addition, the pH of the elution buffer may be increased relative to the wash buffer and to the intermediate buffer (for example, the pH of the elution buffer may about 6.0). The change in conductivity and/or pH may be step-wise or gradual, as desired. Hence, the desired polypeptide is retrieved from the cation exchange resin at this stage in the method.

In an alternative embodiment, the ion exchange material comprises an anion exchange resin. This embodiment of the invention is depicted in FIG. **2** herein. As illustrated in this figure, the changes in conductivity are generally as described above with respect to a cation exchange resin. However, the direction of change in pH is different for an anion exchange resin. For example, if elution of the contaminant(s) and polypeptide are to be achieved by altering pH, the loading buffer has a first pH and the pH is decreased in the intermediate buffer so as to elute the contaminant or contaminants. In the third step, the column is washed/re-equilibrated with the wash buffer and the change in conductivity or pH, or both, is in the opposite direction to that of the previous step. Hence, the pH may be

increased in the wash buffer, compared to the intermediate buffer. Following this step, the polypeptide of interest is eluted from the anion exchange resin using an elution buffer at a fourth conductivity and/or pH. If pH is altered, it will normally be less than the pH of the loading buffer, the intermediate buffer and the wash buffer. The change in pH and/or conductivity in progressive buffers can, as explained above, be step-wise or gradual.

In the preferred embodiment of the invention, a single parameter (i.e. either conductivity or pH) is changed to achieve elution of both the polypeptide and contaminant, while the other parameter (i.e. pH or conductivity, respectively) remains about constant. For example, while the conductivity of the various buffers (loading buffer, intermediate buffer, wash buffer and/or elution buffer) may differ, the pH's thereof may be essentially the same.

In an optional embodiment of the invention, the ion exchange resin is regenerated with a regeneration buffer after elution of the polypeptide, such that the column can be re-used. Generally, the conductivity and/or pH of the regeneration buffer is/are such that substantially all contaminants and the polypeptide of interest are eluted from the ion exchange resin. Generally, the regeneration buffer has a very high conductivity for eluting contaminants and polypeptide from the ion exchange resin.

The method herein is particularly useful for resolving a polypeptide molecule of interest from at least one contaminant, where the contaminant and polypeptide molecule of interest differ only slightly in ionic charge. For example, the pIs of the polypeptide and contaminant may be only "slightly different", for example they may differ by only about 0.05 to about 0.2 pI units. In the Example below, this method-could be used to resolve an anti-HER2 antibody having a pI of 8.87, from a singly-deamidated variant thereof having a pI of 8.79. Alternatively, the method may be used to resolve a deamidated DNase, for example, from nondeamidated DNase. In another embodiment, the method may be used. to resolve a polypeptide from a glycosylation variant thereof, e.g. for resolving a variant of a polypeptide having. a different distribution of sialic acid compared to the nonvariant polypeptide.

The polypeptide preparation obtained according to the ion exchange chromatography method herein may be subjected to additional purification steps, if necessary. Exemplary further purification steps have been discussed above.

Optionally, the polypeptide is conjugated to one or more heterologous molecules as desired. The heterologous molecule may, for example, be one which increases the serum half-life of the polypeptide (e.g. polyethylene glycol, PEG), or it may be a label (e.g. an enzyme, fluorescent label and/or radionuclide) or a cytotoxic molecule (e.g. a toxin, chemotherapeutic drug, or radioactive isotope etc).

A therapeutic formulation comprising the polypeptide, optionally conjugated with a heterologous molecule, may be prepared by mixing the polypeptide having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalqse or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). The humMAb4D5–8 antibody of particular interest herein may be prepared as a lyophilized formulation, e.g. as described in WO 97/04801; expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. For example, for an anti-HER2 antibody a chemotherapeutic agent, such as a taxoid or tamoxifen, may be added to the formulation.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulation to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly (vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The polypeptide purified as disclosed herein or the composition comprising the polypeptide and a pharmaceutically acceptable carrier is then used for various diagnostic, thera-

peutic or other uses known for such polypeptides and compositions. For example, the polypeptide may be used to treat a disorder in a mammal by administering a therapeutically effective amount of the polypeptide to the mammal.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

Full length human-IgG rhuMAb HER2 (humAb4D5–8 in Carter et al. *Proc. Natl. Acad. Sci.* 89: 4285–4289 (1992) comprising the light chain amino acid sequence of SEQ ID NO:1 and heavy chain amino acid sequence of SEQ ID NO:2) was produced recombinantly in CHO cells. Following protein production and secretion to the cell culture medium, the CHO cells were separated from the cell culture medium by tangential flow filtration (PROSTACK™). Protein A chromatography was then performed by applying the Harvested Cell Culture Fluid (HCCF) from the CHO cells directly to an equilibrated PROSEP A™ column (Bioprocessing, Ltd).

Following Protein A chromatography, cation exchange chromatography was performed using a sulphopropyl (SP)-SEPHAROSE FAST FLOW™ (SPSFF) column (Pharmacia) to further separate the desired anti-HER2 antibody molecule. The chromatography operation was performed in bind and elute mode.

The SPSFF column was prepared for load by sequential washes with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6) followed by equilibration buffer (0.025 M MES/50 mM NaCl, pH 5.6). The column was then loaded with Protein A pool adjusted to a pH of 5.60±0.05 and a conductivity of 5.8±0.2 mmhos. Prior to elution, the column was washed in three steps: (1) loading buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1 column volume; (2) intermediate buffer (0.025 M MES/70 mM NaCl, pH 5.6) until an apex of a 280 nm peak was reached; and (3) wash buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1.2 column volumes. rhuMAb HER2 was then eluted from the column with elution buffer (0.025 M MES/95 mM NaCl, pH 5.6). The elution 280 nm profile has a shoulder on the leading edge (FIG. **3**). At the inflection point of this shoulder, pooling starts and continues for an additional 5 column volumes. The column was. then regenerated with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6).

MATERIALS AND METHODS

Column and Load Preparation: A reduced-scale SPSFF column was packed. The dimensions were: 27.0 mL volume, 1.0 cm diameter and 34.5 cm bed height. The pH of an aliquot of Protein A pool was titered to 5.6 with 1.5 M Tris base. The conductivity of the pool was reduced by the addition of an equal volume of sterile water for injection (SWFI).

Chromatography: The chromatography runs for this study were performed with Pharmacia's UNICORN™ FPLC system. The equilibration, load, and initial wash steps were performed at a linear flow rate of 200 cm/h. All chromatography steps were performed at a linear flow rate of 100 cm/h.

The sequence of chromatography steps are defined in Table 1. A total of six chromatography runs were performed with load densities of 15, 20, 25, 30, 35, and 40 mg of rhuMAb HER2 per mL of SPSFF resin.

TABLE 1

Chromatography Steps[1]

Chromatography Step	Buffer	Approximate Endpoint
Equilibration: Part 1	0.025 M MES/1.0 M NaCl, pH 5.6	2 CV[2]
Equilibration: Part 2	0.025 M MES/0.05 M NaCl, pH 5.6	pH: 5.6 ± 0.1 Cond.: 5.8 ± 0.2 mmhos
Load	Adjusted Protein A Pool	As Required
Wash 1	0.025 M MES/0.05 M NaCl, pH 5.6	1.5 CV
Wash 2	0.025 M MES/0.07 M NaCl, pH 5.6	Apex of Peak
Wash 3	0.025 M MES/0.05 M NaCl, pH 5.6	2 CV
Elution: Prepool	0.025 M MES/0.095 M NaCl, pH 5.6	To Leading Shoulder's Inflection Point (~1.2 CV)
Elution: Pool	0.025 M MES/0.095 M NaCl, pH 5.6	5 CV
Regeneration	0.025 M MES/1.0 M NaCl, pH 5.6	2 CV

[1]The equilibration of the resin was performed in manual mode; the remaining steps were executed from a Pharmacia Unicom Program.
[2]CV = column volume(s).

Total Protein: The protein concentration of each chromatography fraction (flow through, wash steps, elution prepool, elution pool, and regeneration) was determined by spectrophometric scans of each sample. The results were used to calculate product recovery yields. The extinction coefficient for rhuMAb HER2 is 1.45. Calculations used to derive the results (FIG. **4**) are:

$$\text{Protein Concentration (mg/mL)} = \frac{280\text{ nm}}{1.45} \times \text{Dilution Factor}$$

Protein Mass (mg) in Each Fraction=Protein Concentration (mg/mL)×Fraction Volume (mL)

$$\text{Yield (\%)} = \frac{\text{Fraction Mass (mg)}}{\text{Total Mass (mg)}} \times 100$$

Determination of rhuMAb HER2 Antibody Variants (CSx HPIEX): The rhuMAb HER2 SPSFF chromatography column resolves antibody variants. Fractions from each of the study chromatographies were tested for the relative amount of variant antibody by CSx HPIEX chromatography. A BAKERBOND WIDE-PORE™ CSx HPIEX column (4.6× 250 mm) was run at 1 mL/min at 55° C. The mobile phase was formed from a tertiary gradient (Table 2).

TABLE 2

Gradient Scheme

Time (min)	% A	% B	% C
0-Initial Conditions	49	1	50
10.0	40	10	50
50.0	33	17	50

TABLE 2-continued

Gradient Scheme

Time (min)	% A	% B	% C
50.2	49	1	50
70.0	49	1	50

The column is run at 1 mL/min at 55° C.

The A buffer was 0.025 M MES, pH 5.9; the B buffer was 1 M Ammonium Acetate, pH 7.0; and the C solution was sterile water for injection. The column was equilibrated with the gradient's initial conditions (49% A; 1% B; and 50% C) and 200 μl of sample, diluted with SWFI and containing<300 μg protein, was injected. Each resulting chromatogram was integrated to determine the percent area of each peak for each fraction (Table 3 and FIG. **5**).

TABLE 3

CSx HPIEX analysis of rhuMAb HER2

CSx Peak	rhuMAb HER2 Variant
a & b	Light Chain: Asn → Asp^{30} deamidation and Other unidentifiable variation by tryptic map
1	Light Chain: Asn → Asp^{30} deamidation
3	Fully Processed Antibody
4	Heavy Chain: Asp → $Iso\text{-}Asp^{102}$ and/or Heavy Chain: An Additional Lys^{450}
Others	Heavy Chain: Asp → Succinimide and/or Multiple permulations found in Peaks 1 and 4

Chromatograms Compared: The absorbance data (AU 280 nm) from each chromatography file was exported from Unicorn in ASCII format. The data from the 0.025 M MES/0.07 M NaCl, pH 5.6 wash was translated into Excel format and copied into KALEIDAGRAPH™. Using KALEIDAGRAPH™, the wash profiles were overlaid (FIG. **6**) and compared to each other.

RESULTS AND DISCUSSION

Deamidated and other acidic variants of rhuMAb HER2 were produced when the antibody was made by recombinant DNA technology (see e.g., CSx peaks a, b and 1 in FIG. **5**). The deamidated and other acidic variants constituted about 25% (calculated as area under the integrated curve or profile obtained by CSx chromatography) of the composition obtained from the initial Protein A chromatography step. It was discovered that the ion exchange method described herein could be used to substantially reduce the amount of deamidated and other acidic variants in the anti-HER2 composition, i.e. to about 13% or less (i.e. the amount of acidic variants in the preparation subjected to cation exchange chromatography as described herein was decreased by about 50% or more).

An absorbance trace from a cation exchange column run performed as described above is shown in FIG. **3**. This method resolved a deamidated variant of anti-HER2 antibody that differed only slightly from nondeamidated anti-HER2 antibody. The increase in conductivity from the initial conditions to the intermediate wash began to elute the deamidated anti-HER2 antibody. However, continued washing at this conductivity was found to elute nondeamidated anti-HER2 antibody, resulting in a loss of product. Proceeding directly from the intermediate buffer to the elution buffer was observed to result in either an unacceptably low removal of deamidated anti-HER2 antibody from the product if pooling began early or unacceptably low yields of anti-HER2 antibody product if pooling was delayed until the deamidated anti-HER2 antibody was reduced. It was discovered that by going back to lower conductivity as used initially, the elution of deamidated anti-HER2 antibody continued, without significant anti-HER2 antibody product elution.

The effect of rhuMAb HER2 load on (a) buffer requirements, (b) product recovery in the pool, and (c) product quality in the pool was evaluated.

At load densities of 15 mg/mL up to 35 mg/mL, the product yield in the elution pool is approximately 75%. For the load density of 40 mg/mL, the product yield in the pool dropped to 65% (FIG. **4**). This reduced recovery in the pool is largely attributed to an increased antibody in the two wash steps (at 70 mM NaCl and 50 mM NaCl, respectively).

The quality of rhuMAb HER2 in all the elution pools is equivalent as determined by CSx HPIEX analysis (FIG. **5**). Compared to the load material; there is an enrichment of the nondeamidated antibody (Peak 3), no change in the amount $Iso\text{-}Asp^{102}$ or Lys^{450} antibody (Peak 4), and a reduction of the amount of Asp^{30} deamidated antibody (Peaks a, b, 1 and others).

The quality of rhuMAb HER2 in these cation pools is improved through the intermediate wash step. As the mass of rhuMAb HER2 bound to the resin increases, the intermediate buffer volume consumption needed to reach the apex of the 280 nm peak decreases. The buffer volume required for a 40 mg/mL load density is approximately 2.5 column volumes. The buffer volume required for a 15 mg/mL load density is approximately 15 column volumes. The exact increase of buffer requirement is not linear with the 5 mg/mL incremental changes between these two extremes. The greatest increase is seen between the load densities of 20 mg/mL and 15 mg/mL. Here the requirement doubles from 7.5 column volumes to the previously mentioned 15 column volumes of buffer. If the apex of the 70 mM NaCl wash peak is reached, however, the product quality is equivalent for any of load densities examined.

This study determined how much rhuMAb HER2 can be loaded onto the SPSFF resin. Between the ranges of 15 to 40 mg of antibody per mL of resin, there is no difference in the quality of rhuMAb HER2 recovered in the elution pool. The quantity of rhuMAb HER2 recovered, however, is reduced by approximately 10% when the resin is loaded with greater than 35 mg/mL. For consistent yields it is recommended that 35 mg/mL be set as the maximum load for manufacture of rhuMAb HER2. Furthermore, due to the substantial increase in the 70 mM NaCl wash volume requirement between the 20 and 15 mg/mL; it is recommended that 20 mg/mL be set as the minimal load for manufacture of rhuMAb HER2.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

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<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

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  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                 20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
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<210> SEQ ID NO 2
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<213> ORGANISM: Artificial sequence
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<223> OTHER INFORMATION: Sequence is synthesized.

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                 20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                 50                  55                  60
```

-continued

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Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                 65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                 95                 100                 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
                125                 130                 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
                140                 145                 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                155                 160                 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
                170                 175                 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
                185                 190                 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
                200                 205                 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
                215                 220                 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
                275                 280                 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
                290                 295                 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
                305                 310                 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                320                 325                 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                335                 340                 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
                350                 355                 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
                365                 370                 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                380                 385                 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                410                 415                 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                425                 430                 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445
```

What is claimed is:

1. A method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises:

(a) loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin; and

(b) eluting the antibody from the cation exchange resin.

2. The method of claim **1** wherein the cation exchange resin comprises sulphopropyl immobilized on agarose.

3. The method of claim **1** further comprising eluting the contaminant from the cation exchange resin in an intermediate wash step prior to eluting the antibody from the cation exchange resin.

4. A method for purifying an anti-HER2 antibody from a composition comprising the antibody and a contaminant, which method comprises:

(a) loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin; and

(b) eluting the antibody from the cation exchange resin.

5. The method of claim **4** wherein the antibody is humMAb4D5–8.

6. The method of claim **4** further comprising eluting the contaminant from the cation exchange resin in an intermediate wash step prior to eluting the antibody from the cation exchange resin.

7. A method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises:

(a) loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from 20 mg to 35 mg of the antibody per mL of cation exchange resin;

(b) eluting the contaminant from the cation exchange resin in an intermediate wash step; and

(c) eluting the antibody from the cation exchange resin.

8. The method of claim **7** wherein the antibody is an anti-HER2 antibody.

9. The method of claim **8** wherein the antibody is humMAb4D5–8.

* * * * *

EXHIBIT J

US009249218B2

(12) **United States Patent**
Basey et al.

(10) **Patent No.: US 9,249,218 B2**
(45) **Date of Patent: *Feb. 2, 2016**

(54) **PROTEIN PURIFICATION**

(75) Inventors: **Carol D. Basey**, Winters, CA (US); **Greg S. Blank**, Menlo Park, CA (US)

(73) Assignee: **GENENTECH, INC.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 214 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/313,931**

(22) Filed: **Dec. 7, 2011**

(65) **Prior Publication Data**

US 2014/0018523 A1 Jan. 16, 2014

Related U.S. Application Data

(60) Continuation of application No. 12/418,905, filed on Apr. 6, 2009, now abandoned, which is a continuation of application No. 11/398,447, filed on Apr. 5, 2006, now Pat. No. 7,531,645, which is a continuation of application No. 10/949,683, filed on Sep. 24, 2004, now Pat. No. 7,074,404, which is a continuation of application No. 10/253,366, filed on Sep. 24, 2002, now abandoned, which is a division of application No. 09/304,465, filed on May 3, 1999, now Pat. No. 6,489,447.

(60) Provisional application No. 60/084,459, filed on May 6, 1998.

(51) **Int. Cl.**

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A61K 39/00	(2006.01)
A61K 39/40	(2006.01)
C07K 16/28	(2006.01)
C07K 1/18	(2006.01)
C07K 16/06	(2006.01)
C07K 16/32	(2006.01)
A61K 38/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 16/2863*** (2013.01); ***C07K 1/18*** (2013.01); ***C07K 16/065*** (2013.01); ***C07K 16/32*** (2013.01); *A61K 38/00* (2013.01); *C07K 2317/24* (2013.01); *C07K 2317/56* (2013.01)

(58) **Field of Classification Search**

USPC 424/130.1, 133.1, 138.1, 141.1, 152.1

See application file for complete search history.

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(57) **ABSTRACT**

A method for purifying a polypeptide by ion exchange chromatography is described which involves changing the conductivity and/or pH of buffers in order to resolve a polypeptide of interest from one or more contaminants.

7 Claims, 7 Drawing Sheets

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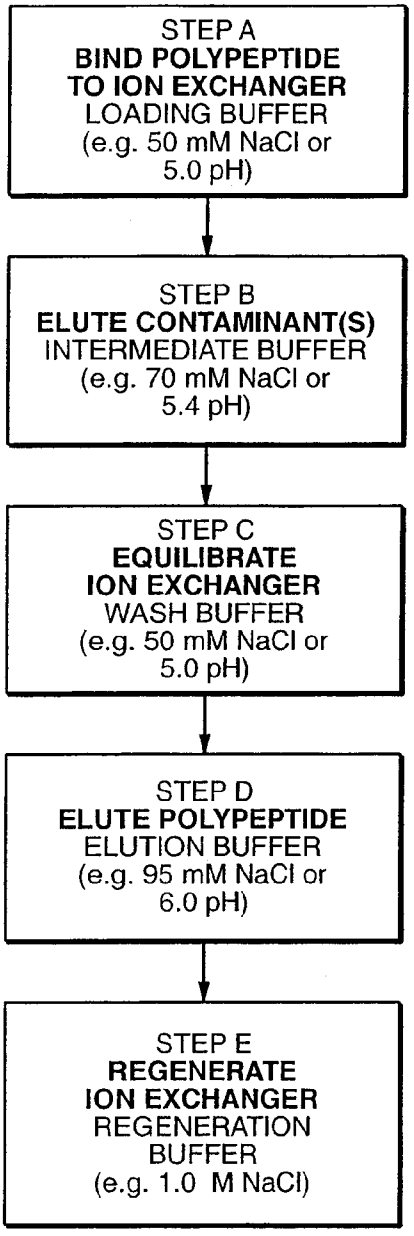
STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
5.4 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
6.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._1

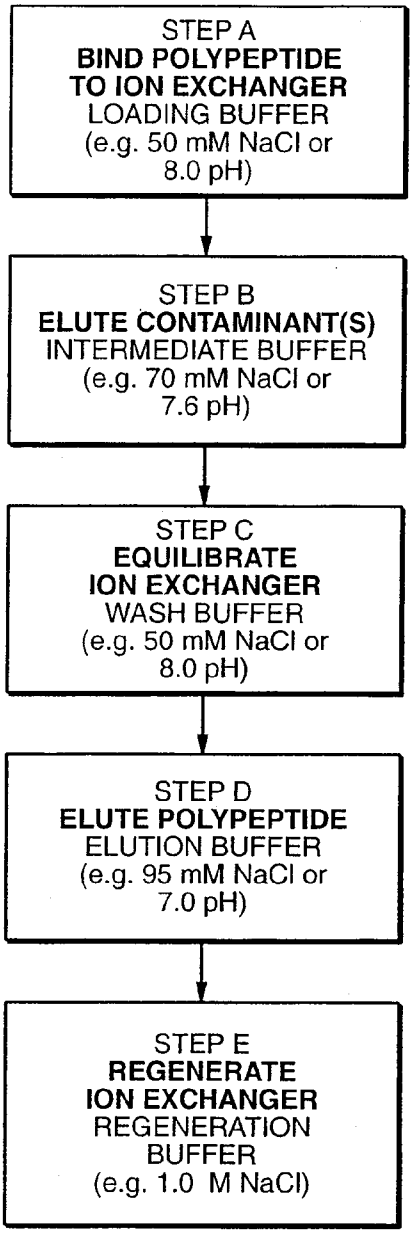
STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
7.6 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
7.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._2

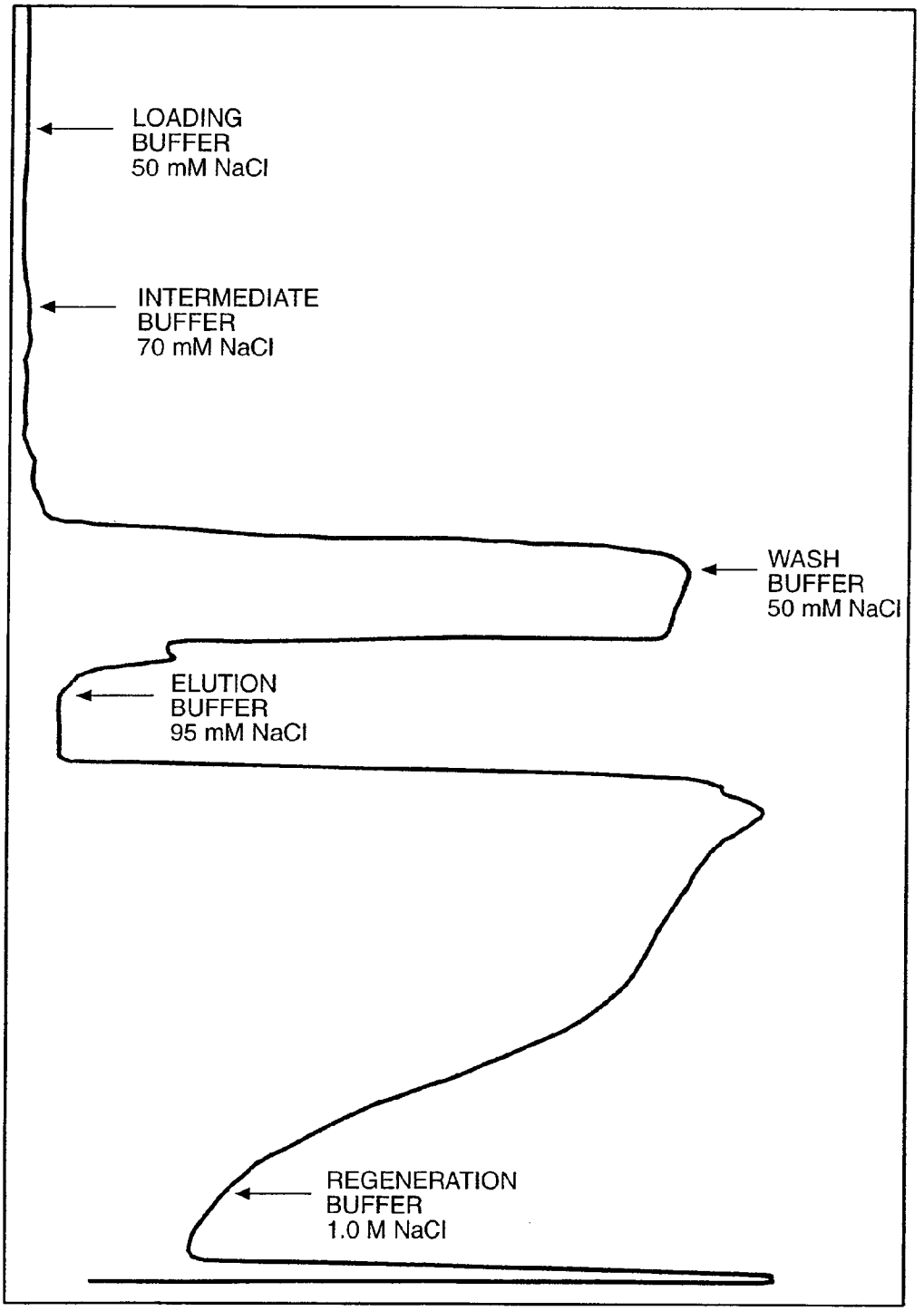
LOADING
BUFFER
50 mM NaCl
INTERMEDIATE
BUFFER
70 mM NaCl
WASH
BUFFER
50 mM NaCl
ELUTION
BUFFER
95 mM NaCl
REGENERATION
BUFFER
1.0 M NaCl

FIG._3

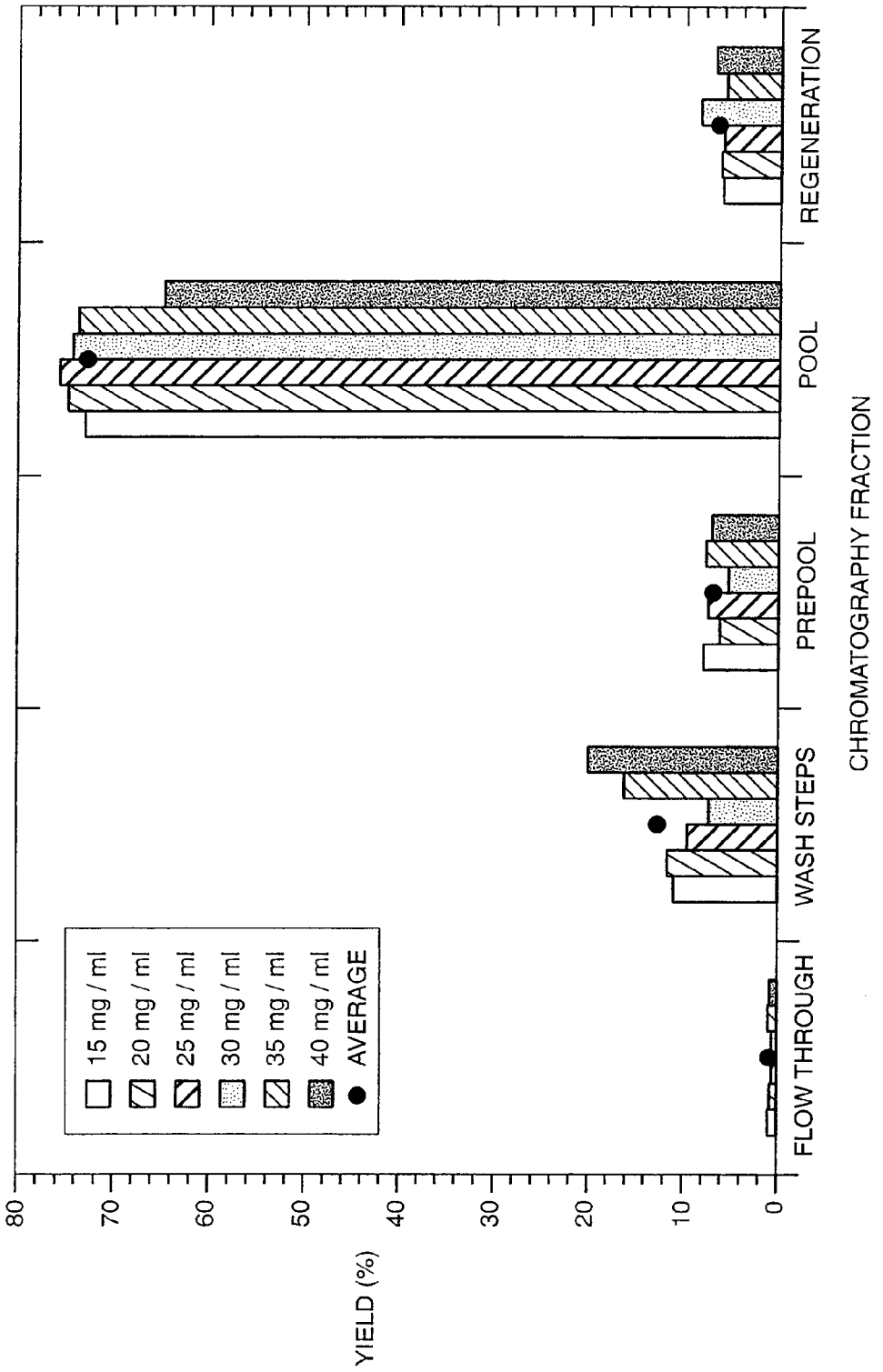
YIELD (%)
0
10
20
30
40
50
60
70
80
FLOW THROUGH
WASH STEPS
PREPOOL
POOL
REGENERATION
CHROMATOGRAPHY FRACTION
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
AVERAGE

FIG._4

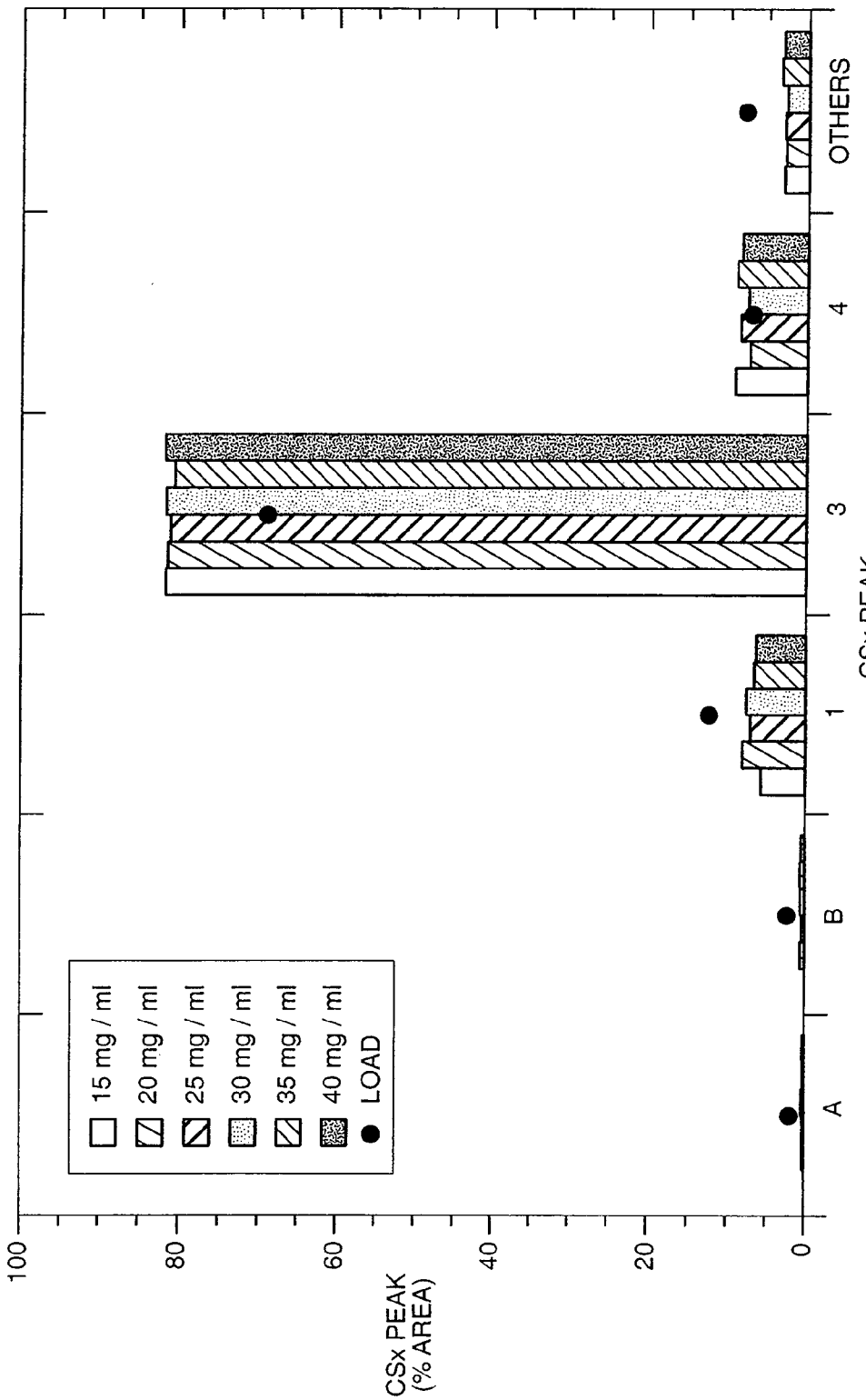
CSx PEAK (% AREA)
100
80
60
40
20
0
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
LOAD
A
B
1
3
4
OTHERS
CSx PEAK

FIG._5

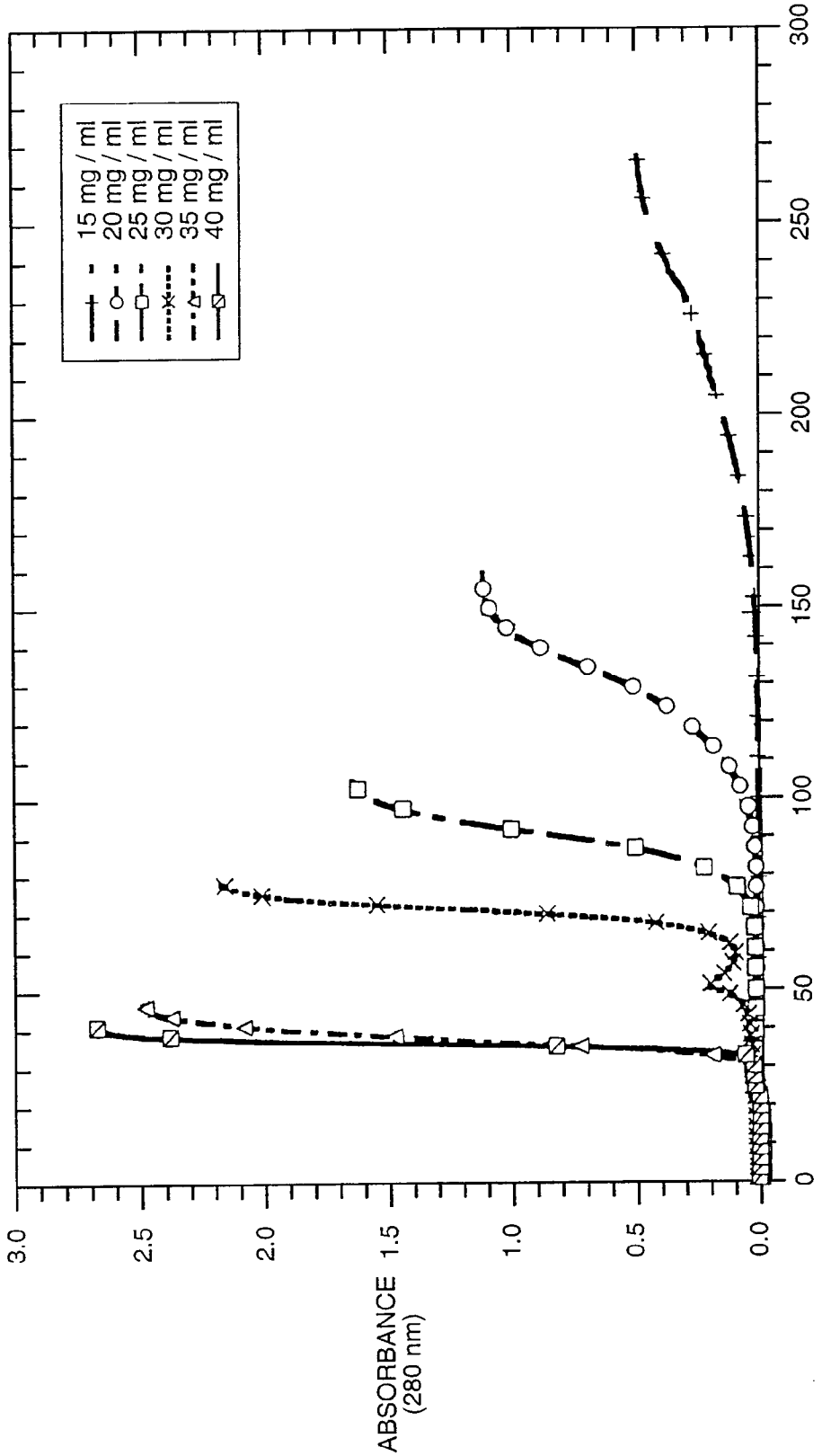
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
ABSORBANCE (280 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
TIME (MINUTES)

FIG._6

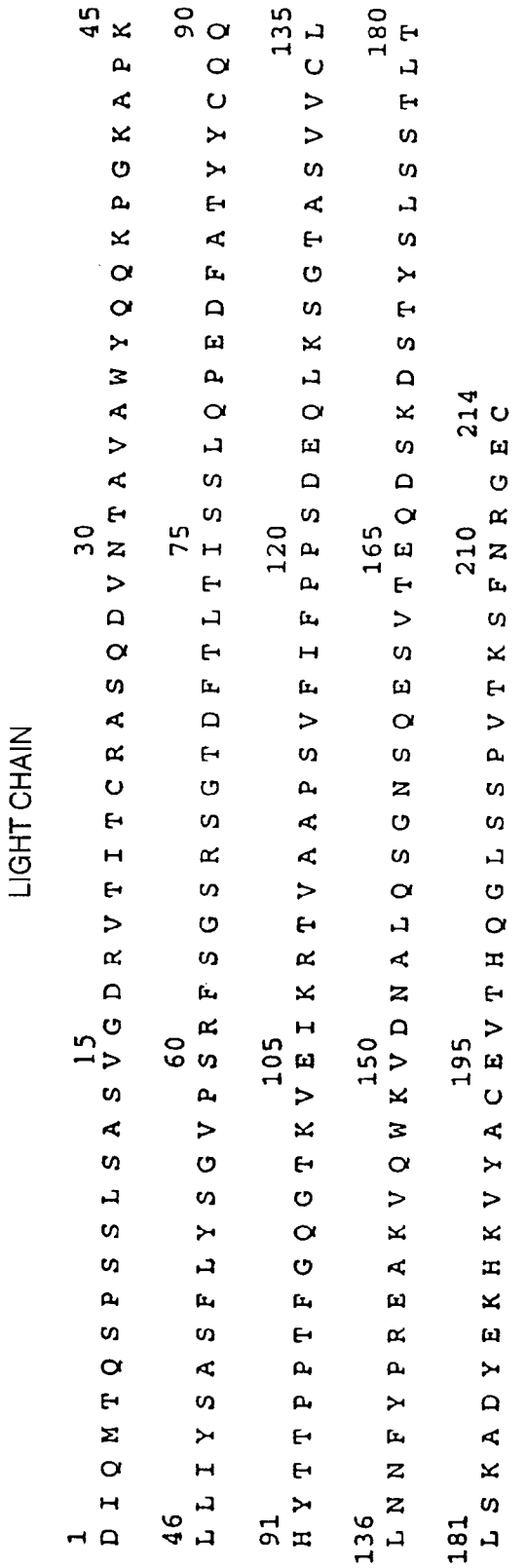
LIGHT CHAIN
1 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPK 45
46 LLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQ 90
91 HYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCL 135
136 LNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT 180
181 LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC 214

FIG._7A

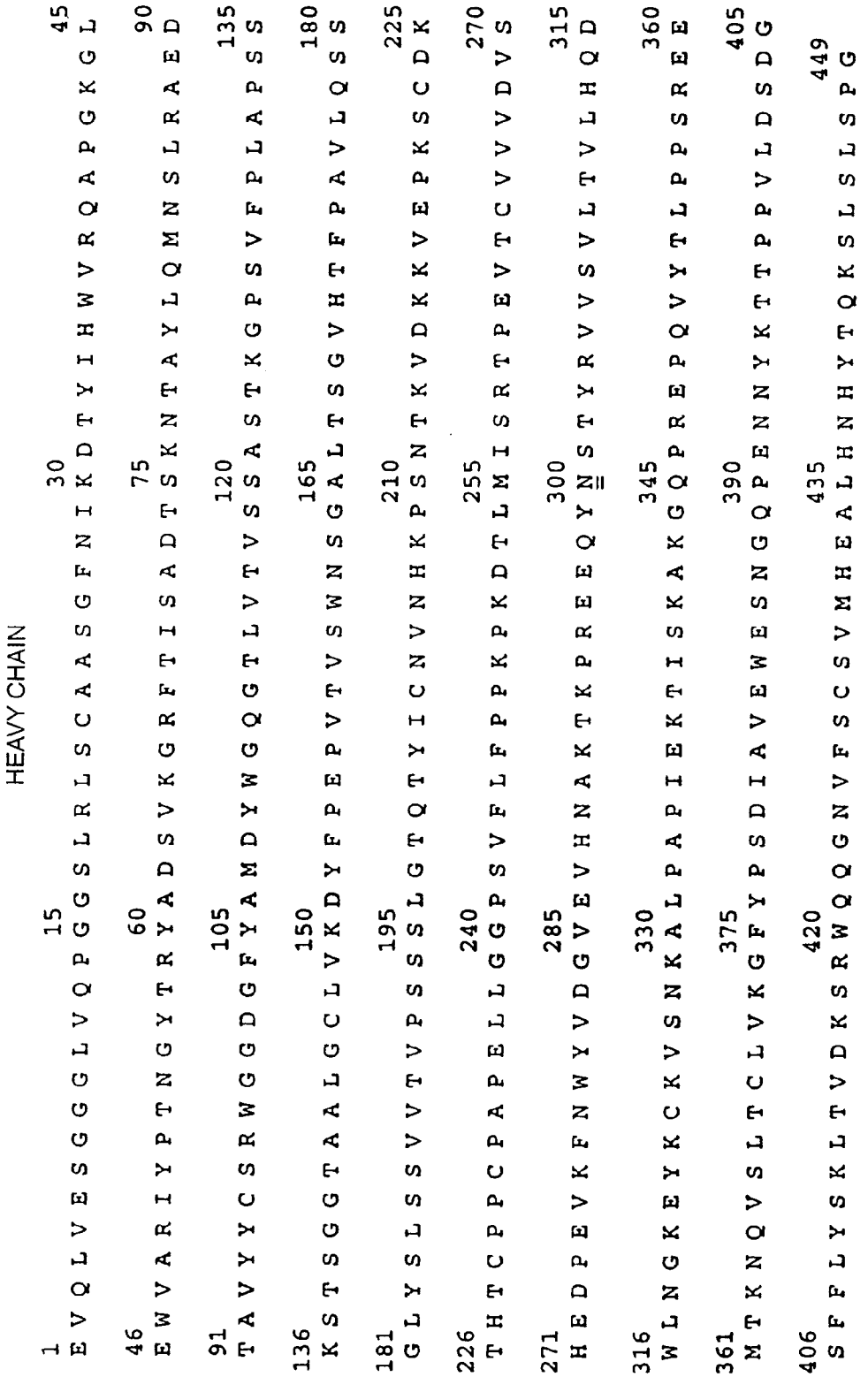
HEAVY CHAIN
1 E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L
15 30 45
46 E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D
60 75 90
91 T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S
105 120 135
136 K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S
150 165 180
181 G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K
195 210 225
226 T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S
240 255 270
271 H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D
285 300 315
316 W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E
330 345 360
361 M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G
375 390 405
406 S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
420 435 449

FIG._7B

PROTEIN PURIFICATION

This application is a continuation of U.S. application Ser. No. 12/418,905 filed Apr. 6, 2009 (now abandoned), which is a continuation of U.S. application Ser. No. 11/398,447, filed Apr. 5, 2006, (now U.S. Pat. No. 7,531,645 issued May 12, 2009) which is a continuation of and claims priority under 35 U.S.C. §120 to U.S. patent application Ser. No. 10/949,683, filed Sep. 24, 2004, (now U.S. Pat. No. 7,074,404 issued Jul. 11, 2006), which is a continuation of and which claims priority to under 35 USC §120 to U.S. application Ser. No. 10/253,366 filed Sep. 24, 2002 (now abandoned), which claims priority to U.S. application Ser. No. 09/304,465 filed May 3, 1999 (now U.S. Pat. No. 6,489,447 issued Dec. 3, 2002), which claims priority under 35 U.S.C. §119 to provisional application No. 60/084,459 filed May 6, 1998, the entire disclosures of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to protein purification. In particular, the invention relates to a method for purifying a polypeptide (e.g. an antibody) from a composition comprising the polypeptide and at least one contaminant using the method of ion exchange chromatography.

2. Description of Related Art

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through".

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

SUMMARY OF THE INVENTION

The present invention provides an ion exchange chromatographic method wherein a polypeptide of interest is bound to the ion exchange material at an initial conductivity or pH and then the ion exchange material is washed with an intermediate buffer at a different conductivity or pH, or both. At a specific point following this intermediate wash, and contrary to ion exchange chromatography standard practice, the ion exchange material is washed with a wash buffer where the change in conductivity or pH, or both, from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity or pH, or both, achieved in the previous steps. Only after washing with the wash buffer, is the ion exchange material prepared for the polypeptide molecule of interest to be eluted by the application of the elution buffer having a conductivity or pH, or both, which differ from the conductivity or pH, or both, of the buffers used in previous steps.

This novel approach to ion exchange chromatography is particularly useful in situations where a product molecule must be separated from a very closely related contaminant molecule at full manufacturing scale, where both purity and high recovery of polypeptide product are desired.

Accordingly, the invention provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to an ion exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the ion exchange material with an intermediate buffer at a second conductivity and/or pH so as to elute the contaminant from the ion exchange material;

(c) washing the ion exchange material with a wash buffer which is at a third conductivity and/or pH, wherein the change in conductivity and/or pH from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity and/or pH from the loading buffer to the intermediate buffer; and

(d) washing the ion exchange material with an elution buffer at a fourth conductivity and/or pH so as to elute the

polypeptide from the ion exchange material. The first conductivity and/or pH may be the same as the third conductivity and/or pH.

Where the ion exchange material comprises a cation exchange resin, the conductivity and/or pH of the intermediate buffer is/are preferably greater than the conductivity and/or pH of the loading buffer; the conductivity and/or pH of the wash buffer is/are preferably less than the conductivity and/or pH of the intermediate buffer; and the conductivity and/or pH of the elution buffer is/are preferably greater than the conductivity and/or pH of the intermediate buffer. Preferably, the conductivity and/or pH of the wash buffer is/are about the same as the conductivity and/or pH of the loading buffer.

Preferably elution of the contaminant and of the polypeptide is achieved by modifying the conductivity of the intermediate buffer and of the elution buffer, respectively, while keeping the pH of these buffers approximately the same.

The invention also provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to a cation exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the cation exchange material with an intermediate buffer at a second conductivity and/or pH which is greater than that of the loading buffer so as to elute the contaminant from the ion exchange material;

(c) washing the cation exchange material with a wash buffer which is at a third conductivity and/or pH which is less than that of the intermediate buffer; and

(d) washing the cation exchange material with an elution buffer at a fourth conductivity and/or pH which is greater than that of the intermediate-buffer so as to elute the polypeptide from the ion exchange material.

In addition, the invention provides a method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin and, optionally, further comprising eluting the antibody from the cation exchange resin. The method preferably further comprises an intermediate wash step for eluting one or more contaminants from the ion exchange resin. This intermediate wash step usually precedes the step of eluting the antibody.

The invention further provides a composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof, wherein the amount of the acidic variant(s) in the composition is less than about 25% and preferably less than about 20%, e.g. in the range from about 1% to about 18%. Optionally, the composition further comprises a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram showing how one could perform cation exchange chromatography by altering conductivity (e.g. to the NaCl concentrations of Example 1 below) or by altering pH (e.g. to the pH values as shown in the flow diagram).

FIG. **2** is a flow diagram showing how one could perform anion exchange chromatography by altering conductivity (e.g. to the NaCl concentrations as depicted in the figure) or by altering pH (e.g. to the pH values as shown).

FIG. **3** is an absorbance trace from a cation exchange chromatography run of Example 1 at full manufacturing scale. Points at which the column is washed with the different buffers described herein are marked with arrows.

FIG. **4** depicts recombinant humanized anti-HER2 monoclonal antibody (rhuMAb HER2) recovered in each chromatography fraction (calculated as the percentage of the sum total of all fractions of the relevant chromatography). Flow through, wash steps, and prepool fractions are all effluent samples collected from the onset of load to the initiation of pooling. The pool fraction is the five column volume effluent sample of elution starting at the leading shoulder's inflection point. The regeneration fraction contains effluent captured from the end of pooling to the end of regeneration.

FIG. **5** shows the quality of rhuMAb HER2 in each cation exchange chromatography pool sample as evaluated by carboxy sulfon cation exchange high pressure liquid chromatography (Csx HPIEX). Peaks A, B, and 1 are deamidated forms of rhuMAb HER2. Peak 3 is nondeamidated rhuMAb HER2. Peak 4 is a combination of C-terminal Lysine containing and iso-aspartate variants of rhuMAb HER2.

FIG. **6** shows the absorbance (280 nm) profiles of the 0.025 M MES/0.070 M NaCl, pH 5.6 wash for each chromatography. The mass of rhuMAb HER2 applied to the cation exchange resin effects the peak's absorbance level at the apex as well as the amount of buffer required to reach the apex. Due to minor peaks which occur (as best seen in the 30 mg/mL load) in this wash, the apex is defined as absorbance levels of at least 0.5 absorbance units (AU).

FIGS. **7**A and **7**B show the amino acid sequences of humMAb4D5-8 light chain (SEQ ID NO:1) and humMAb4D5-8 heavy chain (SEQ ID NO:2), respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The "composition" to be purified herein comprises the polypeptide of interest and one or more contaminants. The composition may be "partially purified" (i.e. having been subjected to one or more purification steps, such as Protein A Chromatography as in Example 1 below) or may be obtained directly from a host cell or organism producing the polypeptide (e.g. the composition may comprise harvested cell culture fluid).

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-

lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides. Most preferred is a full length antibody that binds human HER2.

A "contaminant" is a material that is different from the desired polypeptide product. The contaminant may be a variant of the desired polypeptide (e.g. a deamidated variant or an amino-aspartate variant of the desired polypeptide) or another polypeptide, nucleic acid, endotoxin etc.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch et al., *Proc. Natl. Acad. Sci. USA* 80:1382-1386 (1983), version of the algorithm described by Needleman et al., *J. Mol. Biol.* 48:443-453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide may be prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

An "acidic variant" is a variant of a polypeptide of interest which is more acidic (e.g. as determined by cation exchange chromatography) than the polypeptide of interest. An example of an acidic variant is a deamidated variant.

A "deamidated" variant of a polypeptide molecule is a polypeptide wherein one or more asparagine residue(s) of the original polypeptide have been converted to aspartate, i.e. the neutral amide side chain has been converted to a residue with an overall acidic character. Deamidated humMAb4D5 antibody from the Example below has Asn30 in CDR1 of either or both of the V_L regions thereof converted to aspartate. The term "deamidated human DNase" as used herein means human DNase that is deamidated at the asparagine residue that occurs at position 74 in the amino acid sequence of native mature human DNase (U.S. Pat. No. 5,279,823; expressly incorporated herein by reference).

The term "mixture" as used herein in reference to a composition comprising an anti-HER2 antibody, means the presence of both the desired anti-HER2 antibody and one or more acidic variants thereof.

The acidic variants may comprise predominantly deamidated anti-HER2 antibody, with minor amounts of other acidic variant(s). It has been found, for example, that in preparations of anti-HER2 antibody obtained from recombinant expression, as much as about 25% of the anti-HER2 antibody is deamidated.

In preferred embodiments of the invention, the polypeptide is a recombinant polypeptide. A "recombinant polypeptide" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the polypeptide, or produces the polypeptide as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in in vitro cell culture as well a cell within a host animal. Methods for recombinant production of polypeptides are described in U.S. Pat. No. 5,534,615; expressly incorporated herein by reference, for example.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

The antibody herein is directed against an "antigen" of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and av/b3 integrin including either a or b subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mp1 receptor; CTLA-4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may

be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Polypeptides of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the rhuMAb HER2 antibody of the example below (humAb4D5-8) are identified in Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these dis-

plays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185. "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Polypeptide Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

"Multispecific antibodies" have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell ahesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-$p185^{HER2}$/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal

ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

The phrase "ion exchange material" refers to a solid phase which is negatively charged (i.e. a cation exchange resin) or positively charged (i.e. an anion exchange resin). The charge may be provided by attaching one or more charged ligands to the solid phase, e.g. by covalent linking. Alternatively, or in addition, the charge may be an inherent property of the solid phase (e.g. as is the case for silica, which has an overall negative charge).

By "solid phase" is meant a non-aqueous matrix to which one or more charged ligands can adhere. The solid phase may be a purification column, a discontinuous phase of discrete particles, a membrane, or filter etc. Examples of materials for forming the solid phase include polysaccharides (such as agarose and cellulose); and other mechanically stable matrices such as silica (e.g. controlled pore glass), poly(styrenedivinyl)benzene, polyacrylamide, ceramic particles and derivatives of any of the above.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, BAKERBOND ABX™, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia).

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the

desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., Ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 5 to about 7 (e.g. as in Example 1 below). Examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more contaminants onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more contaminants) is/are bound to the ion exchange resin.

The "intermediate buffer" is used to elute one or more contaminants from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that the contaminant is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all contaminants and the polypeptide of interest from the ion exchange resin.

The term "conductivity" refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution will have a higher conductivity. The unit of measurement for conductivity is mmhos (mS/cm), and can be measured using a conductivity meter sold, e.g., by Orion. The conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a buffering agent and/or concentration of a salt (e.g. NaCl or KCl) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers is modified to achieve the desired conductivity as in the Example below.

By "purifying" a polypeptide from a composition comprising the polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in a "homogeneous" composition, which is used herein to refer to a composition comprising at least about 70% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 80% by weight.

Unless indicated otherwise, the term "HER2" when used herein refers to human HER2 protein and "HER2" refers to human HER2 gene. The human HER2 gene and HER2 protein are described in Semba et al., *PANAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363), for example.

The term "humMAb4D5-8" when used herein refers to a humanized anti-HER2 antibody comprising the light chain amino acid sequence of SEQ ID NO:1 and the heavy chain amino acid sequence of SEQ ID NO:2 or amino acid sequence variants thereof which retain the ability to bind HER2 and inhibit growth of tumor cells which overexpress HER2 (see U.S. Pat. No. 5,677,171; expressly incorporated herein by reference).

The "pI" or "isoelectric point" of a polypeptide refer to the pH at which the polypeptide's positive charge balances its negative charge. pI can be calculated from the net charge of the amino acid residues of the polypeptide or can be determined by isoelectric focussing (e.g. using CSx chromatography as in the Example below).

By "binding" a molecule to an ion exchange material is meant exposing the molecule to the ion exchange material under appropriate conditions (pH/conductivity) such that the molecule is reversibly immobilized in or on the ion exchange material by virtue of ionic interactions between the molecule and a charged group or charged groups of the ion exchange material.

By "washing" the ion exchange material is meant passing an appropriate buffer through or over the ion exchange material.

To "elute" a molecule (e.g. polypeptide or contaminant) from an ion exchange material is meant to remove the molecule therefrom by altering the ionic strength of the buffer surrounding the ion exchange material such that the buffer competes with the molecule for the charged sites on the ion exchange material.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the polypeptide purified as described herein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL™, Bristol-Myers Squibb Oncology, Princeton, N.J.), and doxetaxel, toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, caminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

MODES FOR CARRYING OUT THE INVENTION

The invention herein provides a method for purifying a polypeptide from a composition (e.g. an aqueous solution)

comprising the polypeptide and one or more contaminants. The composition is generally one resulting from the recombinant production of the polypeptide, but may be that resulting from production of the polypeptide by peptide synthesis (or other synthetic means) or the polypeptide may be purified from a native source of the polypeptide. Preferably the polypeptide is an antibody, e.g. one which binds the HER2 antigen.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella*, e.g., *Salmonella typhimurium, Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 Apr. 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe; Kluyveromyces* hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183, 070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/−DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The polypeptide is then subjected to one or more purification steps, including the ion exchange chromatography method as claimed herein. Examples of additional purifica-

tion procedures which may be performed prior to, during, or following the ion exchange chromatography method include fractionation on a hydrophobic interaction chromatography (e.g. on phenyl sepharose), ethanol precipitation, isoelectric focusing, Reverse Phase HPLC, chromatography on silica, chromatography on HEPARIN SEPHAROSE™, further anion exchange chromatography and/or further cation exchange chromatography, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (e.g. using protein A, protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

Ion exchange chromatography is performed as claimed herein. A decision is first made as to whether an anion or cation exchange resin is to be employed. In general, a cation exchange resin may be used for polypeptides with pIs greater than about 7 and an anion exchange resin may be used for polypeptides with pIs less than about 7.

The anion or cation exchange resin is prepared according to known methods. Usually, an equilibration buffer is passed through the ion exchange resin prior to loading the composition comprising the polypeptide and one or more contaminants onto the resin. Conveniently, the equilibration buffer is the same as the loading buffer, but this is not required.

The various buffers used for the chromatography depend, for example, on whether a cation or anion exchange resin is employed. This is shown more clearly in the flow diagrams of FIGS. **1** and **2**.

With particular reference to FIG. **1**, which shows exemplary steps to be performed where a cation exchange resin is used, the pH and/or conductivity of each buffer is/are increased relative to the preceding buffer, except for the wash buffer where the conductivity and/or pH is/are less than the conductivity and/or pH of the preceding intermediate buffer. The aqueous solution comprising the polypeptide of interest and contaminant(s) is loaded onto the cation exchange resin using the loading buffer that is at a pH and/or conductivity such that the polypeptide and the contaminant bind to the cation exchange resin. As in the Example below, the loading buffer may be at a first low conductivity (e.g. from about 5.2 to about 6.6 mmhos). An exemplary pH for the loading buffer may be about 5.0 (see FIG. **1**). From about 20 mg/mL to about 35 mg/mL of the polypeptide (e.g. of a full length antibody) may, for example, be loaded on the ion exchange resin.

The cation exchange resin is then washed with an intermediate buffer which is at a second conductivity and/or pH so as to essentially elute the contaminant, but not a substantial amount of the polypeptide of interest. This may be achieved by increasing the conductivity or pH, or both, of the intermediate buffer. The change from loading buffer to intermediate buffer may be step-wise or gradual as desired. In the Example herein, the intermediate buffer had a greater conductivity than that of the loading buffer (i.e. the intermediate buffer's conductivity was in the range from about 7.3 to about 8.4 mmhos). Alternatively, as shown in FIG. **1**, the pH of the intermediate buffer may exceed that of the loading buffer in this embodiment of the invention, where a cation exchange resin is used. For example, the intermediate buffer may have a pH of about 5.4.

Following washing with the intermediate buffer, the cation exchange resin is washed or re-equilibrated with the wash buffer which has a conductivity or pH, or both, which is/are less than that of the intermediate buffer (i.e. the conductivity, or pH, or both, is/are changed in an opposite, i.e. reverse, direction to the preceding step, unlike ion exchange chromatography steps in the literature). In the Example below, the wash buffer had about the same conductivity as the loading buffer (i.e. in the range from about 5.2 to about 6.6 mmhos) and its conductivity was, therefore, less than that of the intermediate buffer. In another embodiment, one may reduce the conductivity of the wash buffer to a conductivity that is less than, or greater than, that of the loading buffer, provided the conductivity of the wash buffer is less than that of the intermediate buffer. In another embodiment, the pH of the wash buffer may be less than the pH of the intermediate buffer (e.g. the pH of the wash buffer may about 5.0). The change in conductivity and/or pH of the wash buffer compared to the intermediate buffer may be achieved by step-wise or gradual change of either or both of these parameters.

After the wash step of the preceding paragraph, the cation exchange resin is prepared for elution of the desired polypeptide molecule therefrom. This is achieved using an elution buffer that has a pH and/or conductivity such that the desired polypeptide no longer binds to the cation exchange resin and therefore is eluted therefrom. The pH and/or conductivity of the elution buffer generally exceed(s) the pH and/or conductivity of the loading buffer, the intermediate buffer and the wash buffer used in the previous steps. In the Example below, the conductivity of the elution buffer was in the range from about 10.0 to about 11.0 mmhos. Alternatively, or in addition, the pH of the elution buffer may be increased relative to the wash buffer and to the intermediate buffer (for example, the pH of the elution buffer may about 6.0). The change in conductivity and/or pH may be step-wise or gradual, as desired. Hence, the desired polypeptide is retrieved from the cation exchange resin at this stage in the method.

In an alternative embodiment, the ion exchange material comprises an anion exchange resin. This embodiment of the invention is depicted in FIG. **2** herein. As illustrated in this figure, the changes in conductivity are generally as described above with respect to a cation exchange resin. However, the direction of change in pH is different for an anion exchange resin. For example, if elution of the contaminant(s) and polypeptide are to be achieved by altering pH, the loading buffer has a first pH and the pH is decreased in the intermediate buffer so as to elute the contaminant or contaminants. In the third step, the column is washed/re-equilibrated with the wash buffer and the change in conductivity or pH, or both, is in the opposite direction to that of the previous step. Hence, the pH may be increased in the wash buffer, compared to the intermediate buffer. Following this step, the polypeptide of interest is eluted from the anion exchange resin using an elution buffer at a fourth conductivity and/or pH. If pH is altered, it will normally be less than the pH of the loading buffer, the intermediate buffer and the wash buffer. The change in pH and/or conductivity in progressive buffers can, as explained above, be step-wise or gradual.

In the preferred embodiment of the invention, a single parameter (i.e. either conductivity or pH) is changed to achieve elution of both the polypeptide and contaminant, while the other parameter (i.e. pH or conductivity, respectively) remains about constant. For example, while the conductivity of the various buffers (loading buffer, intermediate buffer, wash buffer and/or elution buffer) may differ, the pH's thereof may be essentially the same.

In an optional embodiment of the invention, the ion exchange resin is regenerated with a regeneration buffer after elution of the polypeptide, such that the column can be re-used. Generally, the conductivity and/or pH of the regeneration buffer is/are such that substantially all contaminants and the polypeptide of interest are eluted from the ion exchange resin. Generally, the regeneration buffer has a very high conductivity for eluting contaminants and polypeptide from the ion exchange resin.

The method herein is particularly useful for resolving a polypeptide molecule of interest from at least one contaminant, where the contaminant and polypeptide molecule of interest differ only slightly in ionic charge. For example, the pIs of the polypeptide and contaminant may be only "slightly different", for example they may differ by only about 0.05 to about 0.2 pI units. In the Example below, this method could be used to resolve an anti-HER2 antibody having a pI of 8.87, from a singly-deamidated variant thereof having a pI of 8.79. Alternatively, the method may be used to resolve a deamidated DNase, for example, from nondeamidated DNase. In another embodiment, the method may be used to resolve a polypeptide from a glycosylation variant thereof, e.g. for resolving a variant of a polypeptide having a different distribution of sialic acid compared to the nonvariant polypeptide.

The polypeptide preparation obtained according to the ion exchange chromatography method herein may be subjected to additional purification steps, if necessary. Exemplary further purification steps have been discussed above.

Optionally, the polypeptide is conjugated to one or more heterologous molecules as desired. The heterologous molecule may, for example, be one which increases the serum half-life of the polypeptide (e.g. polyethylene glycol, PEG), or it may be a label (e.g. an enzyme, fluorescent label and/or radionuclide) or a cytotoxic molecule (e.g. a toxin, chemotherapeutic drug, or radioactive isotope etc).

A therapeutic formulation comprising the polypeptide, optionally conjugated with a heterologous molecule, may be prepared by mixing the polypeptide having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). The humMAb4D5-8 antibody of particular interest herein may be prepared as a lyophilized formulation, e.g. as described in WO 97/04801; expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. For example, for an anti-HER2 antibody a chemotherapeutic agent, such as a taxoid or tamoxifen, may be added to the formulation.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulation to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The polypeptide purified as disclosed herein or the composition comprising the polypeptide and a pharmaceutically acceptable carrier is then used for various diagnostic, therapeutic or other uses known for such polypeptides and compositions. For example, the polypeptide may be used to treat a disorder in a mammal by administering therapeutically effective amount of the polypeptide to the mammal.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

Example 1

Full length human IgG rhuMAb HER2 (humAb4D5-8 in Carter et al. *Proc. Natl. Acad. Sci.* 89: 4285-4289 (1992) comprising the light chain amino acid sequence of SEQ ID NO:1 and heavy chain amino acid sequence of SEQ ID NO:2) was produced recombinantly in CHO cells. Following protein production and secretion to the cell culture medium, the CHO cells were separated from the cell culture medium by tangential flow filtration (PROSTACK™). Protein A chromatography was then performed by applying the Harvested Cell Culture Fluid (HCCF) from the CHO cells directly to an equilibrated PROSEP A™ column (Bioprocessing, Ltd).

Following Protein A chromatography, cation exchange chromatography was performed using a sulphopropyl (SP)-SEPHAROSE FAST FLOW™ (SPSFF) column (Pharmacia) to further separate the desired anti-HER2 antibody molecule. The chromatography operation was performed in bind and elute mode.

The SPSFF column was prepared for load by sequential washes with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6) followed by equilibration buffer (0.025 M MES/50 mM NaCl, pH 5.6). The column was then loaded with Protein A pool adjusted to a pH of 5.60±0.05 and a conductivity of 5.8±0.2 mmhos. Prior to elution, the column was washed in three steps: (1) loading buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1 column volume; (2) intermediate buffer (0.025 M MES/70 mM NaCl, pH 5.6) until an apex of a 280 nm peak was reached; and (3) wash buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1.2 column volumes. rhuMAb HER2 was then eluted from the column

with elution buffer (0.025 M MES/95 mM NaCl, pH 5.6). The elution 280 nm profile has a shoulder on the leading edge (FIG. **3**). At the inflection point of this shoulder, pooling starts and continues for an additional 5 column volumes. The column was then regenerated with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6).

Materials and Methods

Column and Load Preparation:

A reduced-scale SPSFF column was packed. The dimensions were: 27.0 mL volume, 1.0 cm diameter and 34.5 cm bed height. The pH of an aliquot of Protein A pool was titered to 5.6 with 1.5 M Tris base. The conductivity of the pool was reduced by the addition of an equal volume of sterile water for injection (SWFI).

Chromatography:

The chromatography runs for this study were performed with Pharmacia's UNICORN™ FPLC system. The equilibration, load, and initial wash steps were performed at a linear flow rate of 200 cm/h. All chromatography steps were performed at a linear flow rate of 100 cm/h. The sequence of chromatography steps are defined in Table 1. A total of six chromatography runs were performed with load densities of 15, 20, 25, 30, 35, and 40 mg of rhuMAb HER2 per mL of SPSFF resin.

TABLE 1

Chromatography Steps[1]

Chromatography Step	Buffer	Approximate Endpoint
Equilibration: Part 1	0.025M MES/1.0M NaCl, pH 5.6	2 CV[2]
Equilibration: Part 2	0.025M MES/0.05M NaCl, pH 5.6	pH: 5.6 ± 0.1 Cond.: 5.8 ± 0.2 mmhos
Load	Adjusted Protein A Pool	As Required
Wash 1	0.025M MES/0.05M NaCl, pH 5.6	1.5 CV
Wash 2	0.025M MES/0.07M NaCl, pH 5.6	Apex of Peak
Wash 3	0.025M MES/0.05M NaCl, pH 5.6	2 CV
Elution: Prepool	0.025M MES/0.095M NaCl, pH 5.6	To Leading Shoulder's Inflection Point (−1.2 CV)
Elution: Pool	0.025M MES/0.095M NaCl, pH 5.6	5 CV
Regeneration	0.025M MES/1.0M NaCl, pH 5.6	2 CV

[1]The equilibration of the resin was performed in manual mode; the remaining steps were executed from a Pharmacia Unicorn Program.
[2]CV = column volume(s).

Total Protein:

The protein concentration of each chromatography fraction (flow through, wash steps, elution prepool, elution pool, and regeneration) was determined by spectrophometric scans of each sample. The results were used to calculate product recovery yields. The extinction coefficient for rhuMAb HER2 is 1.45. Calculations used to derive the results (FIG. **4**) are:

$$\text{Protein Concentration (mg/mL)} = \frac{280\text{ nm}}{1.45} \times \text{Dilution Factor}$$

$$\text{Protein Mass (mg) in Each Fraction} = \text{Protein Concentration (mg/mL)} \times \text{Fraction Volume (mL)}$$

$$\text{Yield (\%)} = \frac{\text{Fraction Mass (mg)}}{\text{Total Mass (mg)}} \times 100$$

Determination of rhuMAb HER2 Antibody Variants (CSx HPIEX):

The rhuMAb HER2 SPSFF chromatography column resolves antibody variants. Fractions from each of the study chromatographies were tested for the relative amount of variant antibody by CSx HPIEX chromatography. A BAKERBOND WIDE-PORE™ CSx HPIEX column (4.6×250 mm) was run at 1 mL/min at 55° C. The mobile phase was formed from a tertiary gradient (Table 2).

TABLE 2

Gradient Scheme

Time (min)	% A	% B	% C
0 - Initial Conditions	49	1	50
10.0	40	10	50
50.0	33	17	50
50.2	49	1	50
70.0	49	1	50

The column is run at 1 mL/min at 55° C.

The A buffer was 0.025 M MES, pH 5.9; the B buffer was 1 M Ammonium Acetate, pH 7.0; and the C solution was sterile water for injection. The column was equilibrated with the gradient's initial conditions (49% A; 1% B; and 50% C) and 200 µl of sample, diluted with SWFI and containing <300 µg protein, was injected. Each resulting chromatogram was integrated to determine the percent area of each peak for each fraction (Table 3 and FIG. **5**).

TABLE 3

CSx HPIEX analysis of rhuMAb HER2

CSx Peak	rhuMAb HER2 Variant
A & B	Light Chain: Asn →Asp[30] deamidation - and - Other unidentifiable variation by tryptic map
1	Light Chain: Asn → Asp[30] deamidation
3	Fully Processed Antibody
4	Heavy Chain: Asp → Iso-Asp[102] - and/or - Heavy Chain: An Additional Lys[450]
Others	Heavy Chain: Asp → Succinimide[102] - and/or - Multiple permutations found in Peaks 1 and 4

Chromatograms Compared:

The absorbance data (AU 280 nm) from each chromatography file was exported from Unicorn in ASCII format. The data from the 0.025 M MES/0.07 M NaCl, pH 5.6 wash was translated into Excel format and copied into KALEIDAGRAPH™. Using KALEIDAGRAPH™, the wash profiles were overlaid (FIG. **6**) and compared to each other.

Results and Discussion

Deamidated and other acidic variants of rhuMAb HER2 were produced when the antibody was made by recombinant DNA technology (see e.g., CSx peaks a, b and 1 in FIG. **5**). The deamidated and other acidic variants constituted about 25% (calculated as area under the integrated curve or profile obtained by CSx chromatography) of the composition obtained from the initial Protein A chromatography step. It was discovered that the ion exchange method described herein could be used to substantially reduce the amount of deamidated and other acidic variants in the anti-HER2 composition, i.e. to about 13% or less (i.e. the amount of acidic

variants in the preparation subjected to cation exchange chromatography as described herein was decreased by about 50% or more).

An absorbance trace from a cation exchange column run performed as described above is shown in FIG. **3**. This method resolved a deamidated variant of anti-HER2 antibody that differed only slightly from nondeamidated anti-HER2 antibody. The increase in conductivity from the initial conditions to the intermediate wash began to elute the deamidated anti-HER2 antibody. However, continued washing at this conductivity was found to elute nondeamidated anti-HER2 antibody, resulting in a loss of product. Proceeding directly from the intermediate buffer to the elution buffer was observed to result in either an unacceptably low removal of deamidated anti-HER2 antibody from the product if pooling began early or unacceptably low yields of anti-HER2 antibody product if pooling was delayed until the deamidated anti-HER2 antibody was reduced. It was discovered that by going back to lower conductivity as used initially, the elution of deamidated anti-HER2 antibody continued, without significant anti-HER2 antibody product elution.

The effect of rhuMAb HER2 load on (a) buffer requirements, (b) product recovery in the pool, and (c) product quality in the pool was evaluated.

At load densities of 15 mg/mL up to 35 mg/mL, the product yield in the elution pool is approximately 75%. For the load density of 40 mg/mL, the product yield in the pool dropped to 65% (FIG. **4**). This reduced recovery in the pool is largely attributed to an increased antibody in the two wash steps (at 70 mM NaCl and 50 mM NaCl, respectively).

The quality of rhuMAb HER2 in all the elution pools is equivalent as determined by CSx HPIEX analysis (FIG. **5**). Compared to the load material; there is an enrichment of the nondeamidated antibody (Peak 3), no change in the amount Iso-Asp^{102} or Lys^{450} antibody (Peak 4), and a reduction of the amount of Asp^{30} deamidated antibody (Peaks a, b, 1 and others).

The quality of rhuMAb HER2 in these cation pools is improved through the intermediate wash step. As the mass of rhuMAb HER2 bound to the resin increases, the intermediate buffer volume consumption needed to reach the apex of the 280 nm peak decreases. The buffer volume required for a 40 mg/mL load density is approximately 2.5 column volumes. The buffer volume required for a 15 mg/mL load density is approximately 15 column volumes. The exact increase of buffer requirement is not linear with the 5 mg/mL incremental changes between these two extremes. The greatest increase is seen between the load densities of 20 mg/mL and 15 mg/mL. Here the requirement doubles from 7.5 column volumes to the previously mentioned 15 column volumes of buffer. If the apex of the 70 mM NaCl wash peak is reached, however, the product quality is equivalent for any of load densities examined.

This study determined how much rhuMAb HER2 can be loaded onto the SPSFF resin. Between the ranges of 15 to 40 mg of antibody per mL of resin, there is no difference in the quality of rhuMAb HER2 recovered in the elution pool. The quantity of rhuMAb HER2 recovered, however, is reduced by approximately 10% when the resin is loaded with greater than 35 mg/mL. For consistent yields it is recommended that 35 mg/mL be set as the maximum load for manufacture of rhuMAb HER2. Furthermore, due to the substantial increase in the 70 mM NaCl wash volume requirement between the 20 and 15 mg/mL; it is recommended that 20 mg/mL be set as the minimal load for manufacture of rhuMAb HER2.

SEQUENCE LISTING

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<220> FEATURE:
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Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                 20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
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                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys


<210> SEQ ID NO 2
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  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                95                 100                 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
                125                 130                 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
                140                 145                 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                155                 160                 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
                170                 175                 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
                185                 190                 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
                200                 205                 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
                215                 220                 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255
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-continued

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Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
                275                 280                 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
                290                 295                 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
                305                 310                 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                320                 325                 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                335                 340                 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
                350                 355                 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
                365                 370                 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                380                 385                 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                410                 415                 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                425                 430                 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445
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The invention claimed is:

1. A therapeutic composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof,

wherein the amount of the acidic variant(s) is less than about 25%,

and wherein the acidic variant(s) are predominantly deamidated variants wherein one or more asparagine residues of the anti-HER2 antibody have been deamidated,

and wherein the anti-HER2 antibody is humMAb4D5-8,

and wherein the deamidated variants have Asn30 in CDR1 of either or both V_L regions of humMAb4D5-8 converted to aspartate,

and a pharmaceutically acceptable carrier.

2. The therapeutic composition of claim **1**, wherein the amount of the acidic variant(s) is less than about 20%.

3. The therapeutic composition of claim **2**, wherein the amount of the acidic variant(s) is less than about 13%.

4. The therapeutic composition of claim **2**, wherein the amount of the acidic variant(s) is in the range of about 1 to 18%.

5. The therapeutic composition of any one of claims **1** to **4**, wherein the anti-HER2 antibody comprises the light chain amino acid sequence of SEQ ID NO 1 and the heavy chain amino acid sequence of SEQ ID NO: 2.

6. The therapeutic composition of any one of claims **1** to **4**, which is in the form of a lyophilized formulation or an aqueous solution.

7. The therapeutic composition of claim **5**, which is in the form of a lyophilized formulation or an aqueous solution.

* * * * *

EXHIBIT K

US007993834B2

(12) **United States Patent**
Mass

(10) **Patent No.: US 7,993,834 B2**
(45) **Date of Patent: Aug. 9, 2011**

(54) **DETECTION OF ERBB2 GENE AMPLIFICATION TO INCREASE THE LIKELIHOOD OF THE EFFECTIVENESS OF ERBB2 ANTIBODY BREAST CANCER THERAPY**

(75) Inventor: **Robert D. Mass**, Mill Valley, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 276 days.

(21) Appl. No.: **11/942,449**

(22) Filed: **Nov. 19, 2007**

(65) **Prior Publication Data**

US 2008/0112958 A1 May 15, 2008

Related U.S. Application Data

(60) Continuation of application No. 11/441,995, filed on May 26, 2006, now abandoned, which is a division of application No. 09/863,101, filed on May 18, 2001, now abandoned.

(60) Provisional application No. 60/205,754, filed on May 19, 2000.

(51) **Int. Cl.**
C12Q 1/68 (2006.01)
G01N 33/574 (2006.01)

(52) **U.S. Cl.** **435/6**; 435/7.23

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner — Laura B Goddard
(74) *Attorney, Agent, or Firm* — Arnold & Porter LLP; Diane Marschang; Ginger R. Dreger

(57) **ABSTRACT**

The invention provides a method for more effective treatment of patients susceptible to or diagnosed with tumors overexpressing ErbB, as determined by a gene amplification assay, with an ErbB antagonist. Such method comprises administering a cancer-treating dose of the ErbB antagonist, preferably in addition to chemotherapeutic agents, to a subject in whose tumor cells ErbB has been found to be amplified e.g., by fluorescent in situ hybridization. ErbB antagonists described include an anti-HER2 antibody. Pharmaceutical packaging for providing the components for such treatment is also provided.

6 Claims, No Drawings

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DETECTION OF ERBB2 GENE AMPLIFICATION TO INCREASE THE LIKELIHOOD OF THE EFFECTIVENESS OF ERBB2 ANTIBODY BREAST CANCER THERAPY

This is a continuation of application Ser. No. 11/441,995 filed on May 26, 2006 (now abandoned), which is a divisional application of non-provisional application Ser. No. 09/863,101 filed on May 18, 2001 (now abandoned), which claims priority under 35U.S.C.§119(e) to provisional application Ser. No. 60/205,754, filed on May 19, 2000, the entire disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of cancers characterized by the overexpression of of a tumor antigen, such as an ErbB receptor, particularly HER2. More specifically, the invention concerns more effective treatment of human patients susceptible to or diagnosed with cancer, in which the tumor cells overexpress ErbB as determined by a gene amplification assay, with an ErbB antagonist, e.g., an anti-ErbB antibody. The invention further provides pharmaceutical packages for such treatment.

BACKGROUND OF THE INVENTION

Advancements in the understanding of genetics and developments in technology and epidemiology have allowed for the correlation of genetic abnormalities with certain malignancies as well as risk assessment of an individual for developing certain malignancies. However, most of the methodologies available for evaluation of tissue for the presence of genes associated with or predisposing an individual to a malignancy have well-known drawbacks. For example, methods that require disaggregation of the tissue, such as Southern, Northern, or Western blot analysis, are rendered less accurate by dilution of the malignant cells by the normal or otherwise non-malignant cells that are present in the same tissue. Furthermore, the resulting loss of tissue architecture precludes the ability to correlate malignant cells with the presence of genetic abnormalities in a context that allows morphological specificity. This issue is particularly problematic in tissue types known to be heterogeneous, such as in human breast carcinoma, where a significant percentage of the cells present in any area may be non-malignant.

The her2/neu gene encodes a protein product, often identified as p185HER2. The native p185HER2 protein is a membrane receptor-like molecule with homology to the epidermal growth factor receptor (EGFR). Amplification and overexpression of HER2 in human breast cancer has been correlated with shorter disease-free interval and shorter overall survival in some studies (van de Vijver et al. New Eng. J. Med. 319: 1239 (1988); Walker et al. Br. J. Cancer 60:426 (1989); Tandon et al. J. Clin. Invest. 7:1120 (1989); Wright et al. Cancer Res. 49:2087 (1989); McCann et al. Cancer Res 51:3296 (1991); Paterson et al. Cancer Res. 51:556 (1991); and Winstanley et al. Br. J. Cancer 63:447 (1991)) but not in others (Zhou et al. Oncogene 4:105 (1989); Heintz et al. Arch Path Lab Med 114:160 (1990); Kury et al. Eur. J. Cancer 26:946 (1990); Clark et al. Cancer Res. 51:944 (1991); and Ravdin et al. J. Clin. Oncol. 12:467-74 (1994)).

In an initial evaluation of 103 patients with breast cancer, those having more than three tumor cell positive axillary lymph nodes (node positive) were more likely to overexpress HER2 protein than patients with less than three positive nodes

(Slamon et al. Science 235:177 (1987)). In a subsequent evaluation of 86 node-positive patients with breast cancer, there was a significant correlation among the extent of gene amplification, early relapse, and short survival. HER2 overexpression was determined using Southern and Northern blotting, which correlate with the HER2 oncoprotein expression evaluated by Western blotting and immunohistochemistry (IHC) (Slamon et al. Science 235:177 (1987); Slamon et al. Science 244:707 (1989)). The median period of survival was found to be approximately 5-fold shorter in patients with more than five copies of the her2 gene than in patients without gene amplification. This correlation was present even after correcting for nodal status and other prognostic factors in multivariate analyses. These studies were extended in 187 node-positive patients and indicated that gene amplification, increased amounts of mRNA (determined by Northern blotting), and increased protein expression (determined immunohistochemically) were also correlated with shortened survival time (Slamon et al. Science 244:707 (1989)); (see also U.S. Pat. No. 4,968,603). Nelson et al. have compared her2/neu gene amplification using FISH with immunohistochemically determined overexpression in breast cancer (Nelson et al. Modern Pathology 9 (1) 21A (1996)).

Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing alteration of proteins in a heterogeneous tissue. Immunohistochemistry (IHC) techniques utilize an antibody to probe and visualize cellular antigens in situ, generally by chromagenic or fluorescent methods. This technique excels because it avoids the unwanted effects of disaggregation and allows for evaluation of individual cells in the context of morphology. In addition, the target protein is not altered by the freezing process.

However, in the clinical trial assay (CTA), IHC of formaldehyde-fixed, paraffin embedded tissue samples only demonstrated 50%-80% sensitivity, relative to frozen IHC samples (Press, Cancer Research 54:2771 (1994)). Thus, IHC can lead to false negative results, excluding from treatment patients who might benefit from the treatment.

Fluorescence in situ hybridization (FISH) is a recently developed method for directly assessing the presence of genes in intact cells. FISH is an attractive means of evaluating paraffin-embedded tissue for the presence of malignancy because it provides for cell specificity, yet overcomes the cross-linking problems and other protein-altering effects caused by formalin fixation. FISH has historically been combined with classical staining methodologies in an attempt to correlate genetic abnormalities with cellular morphology (see, e.g., Anastasi et al., Blood 77:2456-2462 (1991); Anastasi et al., Blood 79:1796-1801 (1992); Anastasi et al., Blood 81:1580-1585 (1993); van Lom et al., Blood 82:884-888 (1992); Wolman et al., Diagnostic Molecular Pathology 1(3): 192-199 (1992); Zitzelberger, Journal of Pathology 172:325-335 (1994)).

To date, there has been no correlation of her2 gene amplification with anti-HER2 antibody treatment outcome, only with disease prognosis. The standard assay has been IHC on formalin fixed, paraffin embedded samples. These samples, when scored as 3+ or 2+, identify patients who are likely to benefit from treatment with an anti-HER2 antibody, like Herceptin®. The 3+ and 2+ scores correlate with her2 gene amplification, e.g., as tested by FISH. However, there remains a need for more effective identification of candidates for successful ErbB antagonist therapies, such as Herceptin® treatment. SUMMARY OF THE INVENTION

The invention advantageously provides a method for increasing likelihood of effectiveness of an ErbB antagonist cancer treatment. The method comprises administering a can-

cer treating dose of the ErbB antagonist to a subject wherein an erbB gene in tumor cells in a tissue sample from the subject has been found to be amplified. Preferably the ErbB is HER2. In a specific embodiment, the method further comprises administering a cancer treating dose of a chemotherapeutic, particularly a taxol.

In a specific preferred embodiment, exemplified herein, the invention provides a method for increasing likelihood of effectiveness of an anti-HER2 antibody to treat cancer. This method comprises administering a cancer treating dose of the anti-HER2 antibody to the subject in whom a her2 gene in tumor cells in a tissue sample from the subject have been found to be. amplified.

The unexpected clinical results underlying the invention, in which gene amplification proved to be a more effective indication of antibody-based tumor therapy than protein detection by immunohistochemistry, extends to tumor antigens in general. Thus, any anti-tumor-specific antigen based antibody therapy can have increased likelihood of success in patients who are found to have gene amplification of the gene encoding the tumor antigen.

A particular advantage of the invention is that it permits selection of patients for treatment who, based on immunohistochemical criteria, would be excluded. Thus, in a specific embodiment, the subject has been found to have an antigen level corresponding to a 0 or 1+ score for HER2 by immunohistochemistry on a formaldehyde-fixed tissue sample.

The invention further provides a pharmaceutical package comprising an ErbB antagonist for treating a cancer, and instructions to administer the ErbB antagonist to a subject if an erbB gene in tumor cells in a tissue sample from the subject is amplified. Preferably the ErbB antagonist is an anti-ErbB antibody, such as an anti-HER2 antibody. In a further aspect, the instructions also teach administering a cancer treating dose of a chemotherapeutic, e.g., a taxol. Such pharmaceutical packages, including the instructions for use, can be provided for any antibody-based therapeutic specific for a tumor-specific antigen.

DETAILED DESCRIPTION

The present invention advantageously permits treatment of patients who have a greater likelihood of responding to the treatment by administering therapeutic agents, i. e., anti-tumor antigen therapeutic antibodies or ErbB receptor antagonists, to patients who are found to have an amplified gene encoding such a tumor antigen or ErbB receptor protein. The invention is based, in part, on the unexpected discovery that her2 gene amplification, e.g., as detected by fluorescence in situ hybridization (FISH), although it correlates with HER2 expression as detected by immunohistochemistry (IHC), provides a more accurate basis for selecting patients for treatment because FISH status unexpectedly correlates better with response to treatment. This outcome was surprising in part because FISH status has about the same rate of correlation with a clinical trial assay (CTA) IHC assay as another IHC assay (HercepTest). Based on this observation, FISH would be expected to have a similar correlation with treatment response. This outcome also surprises because direct measurement of protein (by immunoassay) would be expected to provide a more accurate assessment of a cancer therapy targeted to the protein than an indirect measure of expression, like gene amplification.

Evaluation of patient groups and subgroups demonstrates the power of gene amplification analysis for selecting patients more likely to respond to treatment. IHC provides a score for HER2 expression on tumor cells: 0 (no expression) through 3+ (very high levels of expression). Clinical selection criteria exclude patients with 0 and 1+ scores and select patients with 2+ and 3+ scores. The data show that 14% of combined 2+/3+ patients respond to Herceptin®, while 20% of FISH+ (amplified her2 gene) patients respond to Herceptin®. The 3+ subgroup has a 17% response rate, which is very close to the FISH+ subjects' response rate. However, the 2+ subgroup has less than half the response rate of FISH+ subjects. Thus, gene amplification clearly differentiates large sub-populations within the 2+ subgroup, permitting more effective treatment for those who are FISH+, and quickly identifying patients for whom alternative treatment modalities are appropriate and should commence immediately.

Gene amplification analysis also identifies patients who are unnecessarily excluded because of anomalies in the IHC analysis, particularly when the tests are performed on formalyn fixed, paraffin embedded samples (such sample processing can disrupt or destroy antibody epitopes on the HER2 protein, but has much less impact on gene amplification assays). As shown in the examples, a subset of 0 and 1+ subjects are FISH+. These patients are likely to respond to anti-HER2 antibody therapy, e.g., with Herceptin®, although by IHC criteria they would be excluded from receiving this treatment.

Thus, the present invention advantageously permits inclusion of patients who are more likely to benefit from treatment but who, by standard IHC criteria, would be excluded from treatment. At the same time, the invention permits exclusion of patients who should promptly seek an alternative mode of treatment because the anti-tumor antigen therapy (i.e., ErbB antagonist or tumor antigen-specific therapeutic antibody) is not likely to succeed.

In short, the present invention is a powerful adjunct to IHC assays for target protein expression level-based selection of patients. It can also be employed on its own, i.e., without IHC, to provide initial screening and selection of patients. The invention significantly improves screening and selection for subjects to receive a cancer-treating dose of an anti-tumor antigen therapeutic antibody treatment, ErbB receptor antagonist treatment, and other treatment targeted to overexpressed tumor antigens (or tumor-specific antigens), resulting in an increased likelihood of benefit from such treatments.

In another aspect, the invention concerns an article of manufacture or package, comprising a container, a composition within the container comprising an ErbB antagonist, e.g., an anti-ErbB antibody (or other anti-tumor-specific antigen antibody), optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB receptor, and a package insert containing instructions to administer the antagonist to patients who have been found to have an amplified erbB gene.

Definitions

As used herein, an "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3, and ErbB4 receptors, as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid

sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

ErbB receptors are examples of tumor-specific antigens or tumor antigens. The term "tumor antigen" is used herein to refer to a protein that is expressed at a higher level on tumor cells compared to normal cells. Generally, the normal cells for comparison are of the same tissue type, particularly phenotype, as the tumor, or from which the tumor arose. A "tumor specific antigen" refers to an antigen expressed either preferentially or only on tumor cells. Examples of tumor-specific antigens include, in addition to the ErbB receptors, MART1/Melan A, gp-100, and tyrosinase (in melanoma); MAGE-1 and MAGE-3 (in bladder, head and neck, and non-small cell carcinoma); HPV EG and E7 proteins (in cervical cancer); Mucin/MUC-1 (in breast, pancreas, colon, and prostate cancers); prostate specific antigen/PSA (in prostate cancer); and carninoembryonic antigen/CEA (in colon, breast, and gastrointestinal cancers).

By "amplification" is meant the presence of one or more extra gene copies of erbB or other tumor antigen-encoding gene in a chromosome complement. Gene amplification can result in overexpression of protein, e.g., ErbB receptor protein. Gene amplification in cells from a tissue sample can be measured by many techniques, particularly Fluorescence in situ Hybridization (FISH), but also including and not limited to quantitative PCR, quantitative Southern hybridization, and the like.

By "tissue sample" is meant a collection of similar cells obtained from a tissue of a subject or patient, preferably containing nucleated cells with chromosomal material. The four main human tissues are (1) epithelium; (2) the connective tissues, including blood vessels, bone and cartilage; (3) muscle tissue; and (4) nerve tissue. The source of the tissue sample maybe solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like. In one embodiment of the invention, the tissue sample is "non-hematologic tissue" (i.e., not blood or bone marrow tissue).

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, e.g., a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention, provided that it is understood that the present invention comprises a method whereby the same section of tissue sample may be analyzed at both morphological and molecular levels, or may be analyzed with respect to both protein and nucleic acid.

By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis with the performance and/or results of a second analysis. For example, one may use the results of a first analysis in carrying out the second analysis and/or one may use the results of a first analysis to determine whether a second analysis should be performed and/or one may compare the results of a first analysis with the results of a second analysis. In relation to IHC combined with FISH, one may use the results of IHC to determine whether FISH should be performed and/or one may compare the level of protein expression with gene amplification to further characterize a tumor biopsy (e.g. to compare HER2 protein expression with her2 gene amplification). One advantageous feature of the invention is the ability to identify patients likely to benefit from treatment using FISH even if IHC indicates that they are antigen low.

By "nucleic acid" is meant to include any DNA or RNA, for example, chromosomal, mitochondrial, viral and/or bacterial nucleic acid present in tissue sample. The term "nucleic acid" encompasses either or both strands of a double stranded nucleic acid molecule and includes any fragment or portion of an intact nucleic acid molecule.

By "gene" is meant any nucleic acid sequence or portion thereof with a functional role in encoding or transcribing an RNA (rRNA, tRNA, or mRNA, the latter capable of translation as a protein) or regulating other gene expression. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The nucleic acid sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as Epidermal Growth Factor (EGF) (Savage et al., J. Biol. Chem. 247:7612-7621 (1972)); Tansforming Growth Factor alpha (TGF-alpha) (Marquardt et al., Science 223: 1079-1082 (1984)); amphiregulin, also known as schwanoma or keratinocyte autocrine growth factor (Shoyab et al. Science 243:1074-1076 (1989); Kimura et al. Nature 348:257-260 (1990); and Cook et al. Mol. Cell. Biol. 11:2547-2557 (1991)); betacellulin (Shing et al., Science 259:1604-1607 (1993); and Sasada et al. Biochem. Biophys. Res. Commun. 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., Science 251:936-939 (1991)); epiregulin (Toyoda et al., J. Biol. Chem. 270:7495-7500 (1995); and Komurasaki et al. Oncogene 15:2841-2848 (1997)), a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., Nature 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., Proc. Natl. Acad. Sci. 94:9562-9567 (1997)); or cripto (CR-1) (Kannan et al., J. Biol. Chem. 272 (6):3330-3335 (1997)). ErbB ligands that bind EGFR include EGF, TGF-alpha, amphiregulin, betacellulin, HB-EGF and epiregulin. ErbB ligands which bind HER3 include heregulins. ErbB ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide comprising an amino acid sequence encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641,869 or Marchionni et al., Nature, 362:312-318 (1993), and biologically active variants of such polypeptides. Examples of heregulins include heregulin-alpha, heregulin-beta1, heregulin-beta2, and heregulin-beta3 (Holmes et al., Science, 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. Cell 69:205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. Cell 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., Nature, 362:312-318 (1993)); sensory and motor neuron derived factor(SMDF) (Ho et al. J. Biol. Chem. 270:14523-14532 (1995)); gamma-heregulin (Schaefer et al. Oncogene 15:1385-1394 (1997)). An example of a biologically active fragment/amino acid sequence variant of a native sequence HRG polypeptide, is an EGF-like domain fragment (e.g., HRG-beta1, 177-244).

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be

isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., (J. Biol. Chem., 269(20):14661-14665 (1994)), for example. Examples of such ErbB hetero-oligomers include EGFR-HER2, HER2-HER3, and HER3-HER4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more HER2 receptors combined with a different ErbB receptor, such as HER3, HER4, or EGFR. Other proteins, such as a cytokine receptor subunit (e.g., gp130), may be included in the hetero-oligomer.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. (Ann. Rev. Biochem. 56:881-914 (1987)), including variants thereof (e.g., a deletion mutant EGFR as in Humphrey et al., (Proc. Natl. Acad. Sci. U.S.A. 87:4207-4211 (1990)). ErbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), Mab 455 (ATCC CRL HB 8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, PCT Publication No. WO 96/40210).

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to native sequence human HER2 protein described, for example, in Semba et al., (Proc. Natl. Acad. Sci U.S.A. 82:6497-6501 (1985)) and Yamamoto et al. (Nature 319:230-234(1986)) (Genebank accession number X03363), and variants thereof. The term erbB2 refers to the gene encoding human HER2 and neu refers to the gene encoding rat p185neu. Preferred HER2 is native sequence human HER2. Examples of antibodies which bind HER2 include MAbs 4D5 (ATCC CRL 10463), 2C4 (ATCC HB-12697), 7F3 (ATCC HB-12216), and 7C2 (ATCC HB-12215) (see, U.S. Pat. No. 5,772,997; PCT Publication No. WO 98/17797; and U.S. Pat. No. 5,840,525, expressly incorporated herein by reference). Humanized anti-HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, and huMAb4D5-8 (Herceptin®) as described in Table 3 of U.S. Pat. No. 5,821,337, which is expressly incorporated herein by reference; and humanized 520C9 (PCT Publication No. WO 93/21319). Human anti-HER2 antibodies are described in U.S. Pat. No. 5,772,997 and PCT Publication No. WO 97/00271.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968, as well as Kraus et al. (Proc. Natl. Acad. Sci. U.S.A.) 86:9193-9197 (1989)), including variants thereof. Exemplary antibodies that bind HER3 are described in U.S. Pat. No. 5,968,511, e.g., the 8B8 antibody (ATCC HB-12070) or a humanized variant thereof. The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in European Application No. EP 599,274; Plowman et al., (Proc. Natl. Acad. Sci. U.S.A., 90:1746-1750 (1993)); and Plowman et al., (Nature, 366:473-475 (1993)), including variants thereof such as the HER4 isoforms disclosed in PCT Publication No. WO 99/19488.

An "ErbB antagonist" is any molecule that binds to an ErbB receptor and blocks ligand activation of the ErbB receptor. Such antagonists include, but are not limited to, modified ligands, ligand peptides (i.e., ligand fragments), soluble ErbB receptors, and, preferably, anti-ErbB antibodies.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the ErbB antagonist, e.g., anti-ErbB2 antibody, and more generally, any cancer in which administration of an antibody against an over-expressed antigen can treat the cancer. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount elicits antibody-mediated cytotoxicity, activates complement, has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time for disease progression (TTP), survival, tumor size, or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, melanoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small-cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, and various types of head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell surface, such that an anti-ErbB antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90}, and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphorarmide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibi-

otics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; antimetabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; carminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and anti-androgens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

ErbB Receptor Tyrosine Kinases

The ErbB receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes at least four distinct members including Epidermal Growth Factor Receptor (EGFR or ErbB1), HER2 (ErbB2 or p185neu), HER3 (ErbB3), and HER4 (ErbB4 or tyro2).

EGFR, encoded by the ErbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer, as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, Transforming Growth Factor alpha (TGF-alpha), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn Pharmac. Ther. 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-alpha and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn., supra; Masui et al. Cancer Research 44:1002-1007 (1984); and Wu et al. J. Clin. Invest. 95:1897-1905 (1995).

The second member of the ErbB family, p185neu, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., Science, 235:177-182(1987); Slamon et al., Science, 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder.

Antibodies directed against the rat p185neu and human HER2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185neu (see, for example, Drebin et al., Cell 41:695-706 (1985); Myers et al., Meth. Enzym. 198:277-290 (1991); and W094/22478). Drebin et al. (Oncogene 2:273-277(1988)) report that mixtures of antibodies reactive with two distinct regions of p185neu result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice (see also U.S.Pat. No. 5,824,311).

Hudziak et al., (Mol. Cell. Biol. 9(3):1165-1172 (1989)) describe the generation of a panel of anti-HER2 antibodies, which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5, which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-alpha (see, also, U.S. Pat. No. 5,677,171). The anti-HER2 antibodies discussed in Hudziak et al. were further characterized (Fendly et al. Cancer Research 50:1550-1558 (1990); Kotts et al. In Vitro 26(3):

59A (1990); Sarup et al. Growth Regulation 1:72-82 (1991); Shepard et al. J. Clin. Immunol. 11(3):117-127 (1991);Kumar et al. Mol. Cell. Biol. 11(2):979-986 (1991); Lewis et al. CancerImmunol. Immunother. 37:255-263 (1993); Pietras et al. Oncogene 9:1829-1838 (1994); Vitetta et al. Cancer Research 54:5301-5309 (1994); Sliwkowski et al. J. Biol. Chem. 269(20):14661-14665 (1994); Scott et al. J. Biol. Chem. 266:14300-5 (1991); D'souza et al. Proc. Natl. Acad-.Sci. 91:7202-7206 (1994); Lewis et al. Cancer Research 56:1457-1465(1996); and Schaefer et al. Oncogene 15:1385-1394 (1997)).

A recombinant humanized IgG1 version of the murine anti-HER2 antibody 4D5 (rhuMAb HER2 or Herceptin®; commercially available from Genentech, Inc., South San Francisco) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., J. Clin. Oncol. 14:737-744 (1996)). Herceptin®received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. The current treatment protocol employs IHC to determine HER2 protein overexpression.

Other anti-HER2 antibodies with various properties have been described (Tagliabue et al., Int. J. Cancer 47:933-937 (1991); McKenzie et al., Oncogene 4:543-548 (1989); Maier et al., Cancer Res. 51:5361-5369 (1991); Bacus et al., Molecular Carcinogenesis 3:350-362 (1990); Stancovski et al., (Proc. Natl. Acad. Sci. U.S.A.) 88:8691-8695 (1991); Bacus et al., Cancer Research 52:2580-2589 (1992); Xu et al. Int. J. Cancer 53:401-408 (1993); PCT Publication No. WO94/00136; Kasprzyk et al., Cancer Research 52:2771-2776 (1992); Hancock et al., Cancer Res. 51:4575-4580 (1991); Shawver et al., Cancer Res. 54:1367-1373 (1994); Arteaga et al. Cancer Res. 54:3758-3765 (1994); Harwerth et al., J. Biol. Chem. 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; Klapper et al. Oncogene 14:2099-2109 (1997); and PCT Publication No. WO 98/17797).

Homology screening has resulted in the ErbB receptor family members: HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968; Kraus et al., Proc. Natl. Acad. Sci. U.S.A. 86:9193-9197 (1989)) and HER4 (European Patent Application No. EP 599 274; Plowman et al., Proc. Natl. Acad. Sci. U.S.A., 90:1746-1750 (1993); and Plowman et al., Nature, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of ErbB ligands (Earp et al., Breast Cancer Research and Treatment 35:115-132 (1995)). EGFR is bound by six different ligands: Epidermal Growth Factor (EGF), Transforming Growth Factor-alpha (TGF-alpha), amphiregulin, Heparin Binding Epidermal Growth Factor (HB-EGF), betacellulin, and epiregulin (Groenen et al. GrowthFactors 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta, and gamma heregulins (Holmes et al., Science, 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al., Oncogene 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motorneuron derived factor (SMDF) (for a review, see Groenen et al., Growth Factors 11:235-257 (1994); Lemke, G. Molec. & Cell. Neurosci. 7:247-262 (1996) and Lee et al. Pharm. Rev. 47:51-85 (1995)). Recently, two additional ErbB ligands were identified: neuregulin-2 (NRG-2), which is reported to bind either HER3 or HER4 (Chang et al., Nature: 387 509-512 (1997); and Carraway et al Nature 387:512-516 (1997)) and neuregulin-3, which binds HER4 (Zhang et al., (Proc. Natl. Acad. Sci. U.S.A.) 94(18): 9562-7 (1997)). HB-EGF, betacellulin, andepiregulin also bind to HER4.

While EGF and TGF-alpha do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase, (Earp et al., supra.) Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., J. Biol.Chem., 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., Journal of Neuroscience 15:1329-1340 (1995); Morrissey et al., Proc. Natl. Acad. Sci. U.S.A. 92:1431-1435 (1995); and Lewis et al., Cancer Res., 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, Cell 78:5-8(1994)).

Detecting Gene Amplification

The present invention contemplates using any technique to detect gene amplification. (see, Boxer, J. Clin. Pathol. 53:19-21(2000)). These techniques include in situ hybridization (Stoler, Clin. Lab. Med. 12:215-36 (1990), using radioisotope or fluorophore-labeled probes; polymerase chain reaction (PCR); quantitative Southern blotting, and other techniques for quantitating individual genes. Preferably probes or primers selected for gene amplification evaluation are highly specific, to avoid detecting closely related homologous genes.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. A hapten or epitope that is immunospecifically bound by an antibody can also serve as a label.

The term "fluorescently labeled nucleic acid probe" refers to a probe comprising (1) a nucleic acid having a sequence rendering it capable of hybridizing with a target nucleic acid sequence and (2) a fluorescent label. Preferably such hybridization is specific, i. e., it can occur under high stringency conditions.

Sample Preparation

Any tissue sample from a subject may be used. Examples of tissue samples that may be used include, but are not limited to, breast, prostate, ovary, colon, lung, endometrium, stomach, salivary gland or pancreas. The tissue sample can be obtained by a variety of procedures including, but not limited to surgical excision, aspiration, or biopsy. The tissue may be fresh or frozen. In one embodiment, the tissue sample is fixed and embedded in paraffin or the like.

The tissue sample may be fixed (i.e., preserved) by conventional methodology (See e.g., *Manual of Histological Staining Method of the Armed Forces Institute of Pathology, 3rd Edition* Lee G. Luna, HT (ASCP) Editor, The Blakston Division McGraw-Hill Book Company: New York; (1960);

The Armed Forces Institute of Pathology Advanced Laboratory Methods in Histology and Pathology (1994) Ulreka V. Mikel, Editor, Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C.). One of skill in the art will appreciate that the choice of a fixative is determined by the purpose for which the tissue is to be histologically stained or otherwise analyzed. One of skill in the art will also appreciate that the length of fixation depends upon the size of the tissue sample and the fixative used. By way of example, neutral buffered formalin, Bouin's or paraformaldehyde, may be used to fix a tissue sample.

Generally, the tissue sample is first fixed and is then dehydrated through an ascending series of alcohols, infiltrated, and embedded with paraffin or other sectioning media so that the tissue sample may be sectioned. Alternatively, one may section the tissue and fix the sections obtained. By way of example, the tissue sample may be embedded and processed in paraffin by conventional methodology. Examples of paraffin that may be used include, but are not limited to, Paraplast, Broloid, and Tissuemay. Once the tissue sample is embedded, the sample may be sectioned by a. microtome or the like. By way of example for this procedure, sections may range from about three microns to about five microns in thickness. Once sectioned, the sections may be attached to slides by several standard methods. Examples of slide adhesives include, but are not limited to, silane, gelatin, poly-L-lysine, and the like. For example, the paraffin embedded sections may be attached to positively charged slides, slides coated with poly-L-lysine.

If paraffin has been used as the embedding material, the tissue sections are generally deparaffinized and rehydrated to water. The tissue sections may be deparaffinized by several conventional standard methodologies. For example, xylenes and a gradually descending series of alcohols may be used. Alternatively, commercially available deparaffinizing non-organic agents such as Hemo-De7 (CMS, Houston, Tex.) may be used.

Fluorescence In Situ Hybridization (FISH)

In situ hybridization is generally carried out on cells or tissue sections fixed to slides. In situ hybridization may be performed by several conventional methodologies (see, e.g., Leitch et al., *In Situ Hybridization: A Practical Guide*, Oxford BIOS Scientific Publishers, Micropscopy Handbooks v. 27 (1994)). In one in situ procedure, fluorescent dyes (such as fluorescein isothiocyanate (FITC) which fluoresces green when excited by an Argon ion laser) are used to label a nucleic acid sequence probe that is complementary to a target nucleotide sequence in the cell. Each cell containing the target nucleotide sequence will bind the labeled probe producing a fluorescent signal upon exposure, of the cells to a light source of a wavelength appropriate for excitation of the specific fluorochrome used. A "target nucleotide sequence" is a sequence specific for a over-expressed tumor antigen, such as ErbB. FISH analysis can be used in conjunction with other assays, including without limitation morphological staining (of serial sections or the same section; see PCT Publication No. WO 00/20641, specifically incorporated herein by reference).

Various degrees of hybridization stringency can be employed. As the hybridization conditions become more stringent, a greater degree of complementarity is required between the probe and target to form and maintain a stable duplex. Stringency is increased by raising temperature, lowering salt concentration, or raising formamide concentration. Adding dextran sulfate or raising its concentration may also increase the effective concentration of labeled probe to increase the rate of hybridization and ultimate signal intensity. After hybridization, slides are washed in a solution generally containing reagents similar to those found in the hybridization solution with washing time varying from minutes to hours depending on required stringency. Longer or more stringent washes typically lower nonspecific background but run the risk of decreasing overall sensitivity.

Probes used in the FISH analysis may be either RNA or DNA oligonucleotides or polynucleotides and may contain not only naturally occurring nucleotides but their analogs like digoxygenin dCTP, biotin dcTP 7-azaguanosine, azidothymidine, inosine, or uridine. Other useful probes include peptide probes and analogues thereof, branched gene DNA, peptidomimetics, peptide nucleic acid (PNA), and/or antibodies.

Probes should have sufficient complementarity to the target nucleic acid sequence of interest so that stable and specific binding occurs between the target nucleic acid sequence and the probe. The degree of homology required for stable hybridization varies with the stringency of the hybridization medium and/or wash medium. Preferably, completely homologous probes are employed in the present invention, but persons of skill in the art will readily appreciate that probes exhibiting lesser but sufficient homology can be used in the present invention (see e.g., Sambrook, J., et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Press, (1989)).

One of skill in the art will appreciate that the choice of probe depends on the characteristics of the target gene of interest. Examples of amplification include, but are not limited to, her2/neu in breast and ovarian cancer, n-myc in neuroblastoma, c-myc in small cell lung cancer. By way of example for evaluating her2/neu amplification a probe spanning a 140 kb region on the long arm of chromosome 17 containing the her2/neu gene (17q 11.2-17q12) may be used. A probe for the -satellite sequences in the centromeric region of chromosome 17(D1721) may be used to evaluate for aneusomy of chromosome 17 as a source or cause for her2/neu amplification. For example, a cocktailed version of these probes may be obtained from Vysis, Inc. where each probe is directly labeled with easily distinguishable fluorophores, such as SPECTRUM ORANGE® and SPECTRUM GREEN®.

Probes may also be generated and chosen by several means including, but not limited to, mapping by in situ hybridization, somatic cell hybrid panels, or spot blots of sorted chromosomes; chromosomal linkage analysis; or cloned and isolated from sorted chromosome libraries from human cell lines or somatic cell hybrids with human chromosomes, radiation somatic cell hybrids, microdissection of a chromosome region, or from yeast artificial chromosomes (YACs) identified by PCR primers specific for a unique chromosome locus or other suitable means like an adjacent YAC clone. Probes may be genomic DNA, cDNA, or RNA cloned in a plasmid, phage, cosmid, YAC, Bacterial Artificial Chromosomes (BACs), viral vector, or any other suitable vector. Probes may be cloned or synthesized chemically by conventional methods. When cloned, the isolated probe nucleic acid fragments are typically inserted into a vector, such as lambda phage, pBR322, M13, or vectors containing the SP6 or T7 promoter and cloned as a library in a bacterial host (see, e.g., Sambrook, supra).

Probes are preferably labeled with a fluorophor. Examples of fluorophores include, but are not limited to, rare earth chelates (europium chelates), Texas Red, rhodamine, fluorescein, dansyl, Lissamine, umbelliferone, phycocrytherin, phycocyanin, or commercially available fluorophors such SPECTRUM ORANGE® and SPECTRUM GREEN®, and/or

derivatives of any one or more of the above. Multiple probes used in the assay may be labeled with more than one distinguishable fluorescent or pigment color. These color differences provide a means to identify the hybridization positions of specific probes. Moreover, probes that are not separated spatially can be identified by a different color light or pigment resulting from mixing two other colors (e.g., light red+green=yellow), pigment (e.g., blue+yellow=green), or by using a filter set that passes only one color at a time.

Probes can be labeled directly or indirectly with the fluorophor, utilizing conventional methodology. Additional probes and colors may be added to refine and extend this general procedure to include more genetic abnormalities or serve as internal controls. By way of example the her2/neu gene is in chromosome 17, and as an internal control a probe for satellite sequences specific for chromosome 17 (D17Z1) may be used (Vysis, Inc.) to prove diploidy in areas of non-malignant cells and/or to establish the presence or absence of chromosome 17 aneusomy in areas of her2/neu amplification.

After processing for FISH, the slides may be analyzed by standard techniques of fluorescence microscopy (see, e.g., Ploem and Tanke, *Introduction to Fluorescence Microscopy*, Oxford University Press: New York (1987)). Briefly, each slide is observed using a microscope equipped with appropriate excitation filters, dichromic, and barrier filters. Filters are chosen based on the excitation and emission spectra of the fluorochromes used. Photographs of the slides may be taken with the length of time of film exposure depending on the fluorescent label used, the signal intensity and the filter chosen. For FISH analysis the physical loci of the cells of interest determined in the morphological analysis are recalled and visually conformed as being the appropriate area for FISH quantification.

In order to correlate IHC with FISH, one may use a computer-driven, motorized stage which stores location of coordinates. This may be used to evaluate the same area by two different analytical techniques. For example, color images of the morphologically stained areas may be captured and saved using a computer-assisted cooled CCD camera. The same section may be subsequently taken through the FISH procedure, the stored locations recalled, and the designated areas scored for the presence of fluorescent nuclear signals. A similar procedure for IHC followed by FISH is contemplated.

Typically, hundreds of cells are scanned in a tissue sample and quantification of the specific target nucleic acid sequence is determined in the form of fluorescent spots, which are counted relative to the number of cells. Deviation of the number of spots in a cell from a norm (e.g., such as probing for the her2/neu gene in a normal cell will produce two copies, abnormal greater than two) is indicative of a greater likelihood of benefit from a tumor antigen-specific antibody therapy, e.g., an ErbB antagonist therapy. As exemplified infra, her2 gene amplification provides a much more effective indication of the likelihood that an anti-HER2 antibody therapy will be effective.

Pharmaceutical Formulations

Therapeutic formulations of the antagonists, e.g., antibodies, used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2, ErbB3, ErbB4, vascular endothelial factor (VEGF), or an antibody that binds to a different epitope on the target ErbB, in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, a chemotherapeutic, a cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.

The formulations to be used for in vivo administration are preferably, and in the case of humans, must be, sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(—)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mecha-

nism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Treatment with the Anti-ErbB Antagonists

It is contemplated that, according to the present invention, the anti-ErbB antibodies or other antagonists may be used to treat various conditions characterized by overexpression and/or activation of the ErbB receptor in patients who have been found to have an amplified erbB gene. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and any other active agents of the invention are administered to a human patient in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB antibody and a chemotherapeutic agent, e.g., a taxoid. The present invention contemplates administration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells (tumor resection) and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antagonist, e.g. antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Pharmaceutical Packages: Articles of Manufacture

In a related aspect of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, optionally labeled, and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials, such as glass or plastic. The container holds a composition that is effective for treating the condition and preferably has a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-tumor antigen therapeutic antibody or an ErbB antagonist, e.g., an anti-ErbB antibody. A label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. This second buffer can be used to reconstitute the active agent, if that is provided as a lyophilysate or dried powder, or to dilute a concentrated preparation of the active agent. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In addition, the article of manufacture comprises a package insert or inserts with instructions for use in patients who have been found to have erbB gene amplification, e.g., by FISH testing. Such patients may be subjects who, by IHC, would be excluded from treatment with the ErbB antagonist, e.g., patients who score a 0 or 1+ using an anti-HER2 antibody.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., U.S.A. (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLE 1

Concordance Between the Clinical Trials Assay (CTA) and Fluorescence

In Situ Hybridization (FISH) in the Herceptin® Pivotal Trials

Overexpression of HER2 at the 2+ or 3+ level by immunohistochemistry (IHC) was required for enrollment in the

pivotal Herceptin® metastatic breast cancer trials. The Clinical Testing Assay (CTA) involves two separate IHC assays performed with either monoclonal antibodies 4D5 (after protease digestion of the formalin fixed sample) or CB11 (after heat treatment of the formalin fixed sample). Subjects were eligible if either assay was scored at 2+ or 3+. If both were performed, the final score was the higher of the two results.

Concordance between the CTA and another IHC, HercepTest (HT), is 79%. This was the basis for FDA approval of HT to aid in the selection of patients for Herceptin therapy.

This Example describes a similar concordance study, utilizing clinical material submitted for screening for the Herceptin® pivotal trials, that compares the CTA to her2/neu gene amplification measured by the PathVysion FISH assay. In the pivotal trials, 5998 subjects were screened for HER2 expression; 1915 (32%) were positive by the CTA and 4083 (68%) were negative. A random sample of 623 specimens (1:1 ratio of positive:negative) were selected for this analysis, 317 CTA+ and 306 CTA−. Specimens were not freshly cut from blocks. They had been stored between 2 and 4 years as 4-6μ sections on glass slides. Each section was assayed for her2/neu amplification using the protocol specified in the package insert of the PathVysion assay. Amplification was defined as a signal ratio of greater than or equal to 2. The results are shown in Table 1.

TABLE 1

FISH/CTA Concordance

		CTA				
		0	1+	2+	3+	
FISH	−	207	28	67	21	
	+	7	2	21	176	
		4%	7%	24%	89%	529

FISH+ = HER2:CEP17 signal ratio ≧2
Concordance = 82% (79-85%)

For the total 623 specimens tested, a FISH signal result was obtained in 529. Assay failure occurred in 19.9% of CTA− and 10.4% CTA+ samples. Amplification in the 0, 1+, 2, and 3+ groups was 4.2%, 6.7%, 23.9%, and 89.3%, respectively. The sample concordance was 81.3%, similar to the CTA/HT concordance of 79%. Single copy overexpression was 31%, predominantly in the 2+ group. Amplification was rarely (4.6%) noted in the CTA− group. The higher assay failure rate in the CTA− group may be due to non-assay related factors such as tissue fixation. These may have also resulted in false negative results for IHC.

These data were closely interpreted to suggest that her2/neu amplification status may have unexpectedly superior predictive value for identifying patients who are more likely to benefit from Herceptin® treatment as compared to HercepTest. The observation that only 24% of 2+ patients are FISH+ suggest that this sub-group may have less predictable treatment outcomes when selected by IHC only. Identification of FISH+ patients in the 1+ and 0 sub-groups might identify subjects who, though failing the IHC criteria for Herceptin® treatment, would likely benefit from Herceptin® treatment. A direct analysis of Herceptin® benefit based on FISH score compared to IHC score is presented in Example 2.

EXAMPLE 2

FISH/Clinical Outcome Study

This example links the results from three Herceptin® Trials with FISH status. In this study, 805 subjects were selected at random from all three trials. Of these, 167 lacked slides. Another 78 assays (9.7%) failed. Thus, formalin-fixed cut sections stored between 2.5 and 4.5 years from 540 subjects provided the sample pool for this study. There were no imbalances in demographics or prognostic indicators in these samples. Results are reported for different treatment groups.

Correlation of FISH status with response was evaluated for patients who received Herceptin® as a second or third line therapy. These data are reported for 2+and 3+ (by CTA) subjects in Table 2.

TABLE 2

FISH/Response with single agent Herceptin ®, 2nd or 3rd line Therapy, 2+/3+ Combined

	FISH+	FISH−
Response	21	0
No response	84	37
response rate	20%	0%
	(12.5-27.5%)	(0.7%)

N = 142

The 20% response rate of FISH+ subjects unexpectedly exceeds the 15% response rate of 2+ and 3+ patients in this study and 14% response rate observed in patients selected by CTA with a 2+ or 3+ immunohistochemistry score during the pivotal trials. Thus, while FISH correlates well with IHC to about the same degree as another IHC assay, the Hercep Test, as shown in Example 1, it unexpectedly is superior in identifying patients who are more likely to benefit from Herceptin® therapy.

When these data were broken down into the components 3+ and 2+ subjects, the same 20% response rate of FISH+ subjects was seen (Tables 3 and 4).

TABLE 3

FISH/Response with single agent Herceptin ®, 2nd or 3rd line therapy, 3+ subgroup

	FISH+	FISH−
Response	18	0
No response	72	17
response rate	20%	0%
	(12-28%)	(0-14%)

N = 107

TABLE 4

FISH/Response with single agent Herceptin ®, 2nd or 3rd line therapy, 2+ subgroup

	FISH+	FISH−
Response	3	0
No response	12	20
response rate	20%	0%
	(1-40%)	(0-14%)

N = 35

In the 3+ sub-group, the FISH+ response rate (20%) was very close but still exceeded the 17% response rate of 3+ subjects. The 2+ subgroup showed a much greater difference, with only a 9% response rate versus 20% by FISH+ selection. These data show that FISH+ status (her2 gene amplification) greatly increases the likelihood of response to Herceptin®.

Data were also evaluated for patient responses to Herceptin® as a first line therapy (Table 5).

TABLE 5

FISH/Response with single agent Herceptin ® as 1st line therapy, 2+/3+ combined

	FISH+	FISH−
Response	17	1
No response	24	20
response rate	41% (26-56%)	20% (0-14%)

N = 62

The 41% response rate of FISH+ subjects was notably greater than the 27% response rate of 3+, 2+, subjects.

The surprising increase in likelihood of beneficial response based on FISH analysis extended to responses to chemotherapy plus Herceptin®, as shown in Table 6. FISH+ subjects showed a much greater response to chemotherapy and Herceptin® (54%) than FISH−(41%). Tables 7-9 contain more extensive data, broken down by different chemotherapeutic agents (adrinomycin and cyclophosphamide, AC; and Paditaxol, P) and different endpoints (response rate, time to progression, and survival) for Herceptin® in combination with chemotherapy.

TABLE 6

FISH/Response rate to chemotherapy +/− Herceptin ®, 1st line therapy; 2+/3+ combined

	C alone	C + H
FISH−	39% (26-52%)	41% (27-55%)
FISH+	27% (19-35%)	54% (45-63%)

N = 336

TABLE 7

Response rate of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	56*	42	41*	17	50*	32
3+	349	60*	42	49*	17	56*	31
FISH+	240	58*	40	49*	14	54*	27

*p < 0.05

TABLE 8

Time to progression (months) of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	7.8*	6.1	6.9*	2.7	7.4*	4.6
3+	349	8.1*	6.0	7.1*	3.0	7.8*	4.6
FISH+	240	7.8*	6.2	7.0*	3.2	7.3*	4.6

*p < 0.05

TABLE 9

Survival (months) of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	27	21	22	18	25*	20
3+	349	31*	21	25	18	29*	20
FISH+	240	29*	20	25*	14	27*	18

*p < 0.05

These data uniformly confirm that FISH+ analysis, though correlating closely to IHC, provides a much more accurate indicator of likelihood of success with Herceptin® treatment. Across the board, FISH+ selection has about ⅓ (30%) greater response rate than 2+/3+ IHC-selection. When focused on 2+ patients, FISH status provides a much more effective tool for patient selection. FISH states also identifies patients who, because of 0 or 1+ status as determined by IHC, would otherwise be excluded from treatment.

These observations have broad implications for ErbB receptor antagonist-based cancer therapies and anti-tumor antigen cancer therapies in general. Thus erbB antagonists, e.g., anti-erbB receptor antibodies like Herceptin®, can have an increased likelihood of efficacy when administered to patients who are positive for erbB gene amplification, e.g., by a FISH test. This is certainly the case, based on these data, with Herceptin®.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

What is claimed:

1. A method for increasing likelihood of effectiveness of breast cancer treatment with humanized anti-ErbB2 antibody huMAb4D5-8, which method comprises administering a cancer treating dose of said antibody to a human subject diagnosed with breast cancer, wherein an erbB2 gene amplification in breast cancer cells in a tissue sample from the subject has been detected, and wherein the breast cancer cells from the human subject have been found to have a 0 or 1+ score of ErbB2 protein expression by immunohistochemistry.

2. The method according to claim **1**, wherein the breast cancer cells from the human subject have been found to have a 0 or 1+ score of ErbB2 protein expression by immunohistochemistry on a formaldehyde-fixed tissue sample.

3. The method according to claim **1** wherein the erbB2 gene amplification is detected by detecting fluorescence of a fluorescent-labeled nucleic acid probe hybridized to the gene.

4. The method according to claim **1**, which further comprises administering a cancer treating dose of a chemotherapeutic drug.

5. The method according to claim **4**, wherein the chemotherapeutic drug is a taxoid.

6. The method according to claim **1** wherein the likelihood of effectiveness increases by about 30%.

* * * * *

EXHIBIT L

US008076066B2

(12) **United States Patent**
Mass

(10) **Patent No.: US 8,076,066 B2**
(45) **Date of Patent: *Dec. 13, 2011**

(54) **GENE DETECTION ASSAY FOR IMPROVING THE LIKELIHOOD OF AN EFFECTIVE RESPONSE TO A HER2 ANTIBODY CANCER THERAPY**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **11/690,304**

(22) Filed: **Mar. 23, 2007**

(65) **Prior Publication Data**

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(51) **Int. Cl.**
C12Q 1/68 (2006.01)
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(52) **U.S. Cl.** **435/6**; 435/7.23

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

The invention provides a method for more effective treatment of patients susceptible to or diagnosed with tumors overexpressing HER2, as determined by a gene amplification assay, with a HER2 antibody. Such method comprises administering a cancer-treating dose of the HER2 antibody, preferably in addition to chemotherapeutic agents, to a subject in whose tumor cells her2 has been found to be amplified e.g., by fluorescent in situ hybridization.

6 Claims, No Drawings

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GENE DETECTION ASSAY FOR IMPROVING THE LIKELIHOOD OF AN EFFECTIVE RESPONSE TO A HER2 ANTIBODY CANCER THERAPY

This continuation application claims priority to non-provisional application Ser. No. 09/863,101 filed May 18, 2001 now abandoned which claims priority under 35 U.S.C. §119 (e) of provisional application 60/205,754, filed May 19, 2000, which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention concerns the treatment of cancers characterized by the overexpression of a tumor antigen, such as an ErbB receptor, particularly HER2. More specifically, the invention concerns more effective treatment of human patients susceptible to or diagnosed with cancer, in which the tumor cells overexpress ErbB as determined by a gene amplification assay, with an ErbB antagonist, e.g., an anti-ErbB antibody. The invention further provides pharmaceutical packages for such treatment.

BACKGROUND OF THE INVENTION

Advancements in the understanding of genetics and developments in technology and epidemiology have allowed for the correlation of genetic abnormalities with certain malignancies as well as risk assessment of an individual for developing certain malignancies. However, most of the methodologies available for evaluation of tissue for the presence of genes associated with or predisposing an individual to a malignancy have well-known drawbacks. For example, methods that require disaggregation of the tissue, such as Southern, Northern, or Western blot analysis, are rendered less accurate by dilution of the malignant cells by the normal or otherwise non-malignant cells that are present in the same tissue. Furthermore, the resulting loss of tissue architecture precludes the ability to correlate malignant cells with the presence of genetic abnormalities in a context that allows morphological specificity. This issue is particularly problematic in tissue types known to be heterogeneous, such as in human breast carcinoma, where a significant percentage of the cells present in any area may be non-malignant.

The her2/neu gene encodes a protein product, often identified as p185HER2. The native p185HER2 protein is a membrane receptor-like molecule with homology to the epidermal growth factor receptor (EGFR). Amplification and overexpression of HER2 in human breast cancer has been correlated with shorter disease-free interval and shorter overall survival in some studies (van de Vijver et al. New Eng. J. Med. 317: 1239 (1988); Walker et al. Br. J. Cancer 60:426 (1989); Tandon et al. J. Clin. Invest. 7:1120 (1989); Wright et al. Cancer Res. 49:2087 (1989); McCann et al. Cancer Res 51:3296 (1991); Paterson et al. Cancer Res. 51:556 (1991); and Winstanley et al. Br. J. Cancer 63:447 (1991)) but not in others (Zhou et al. Oncogene 4:105 (1989); Heintz et al. Arch Path Lab Med 114:160 (1990); Kury et al. Eur. J. Cancer 26:946 (1990); Clark et al. Cancer Res. 51:944 (1991); and Ravdin et al. J. Clin. Oncol. 12:467-74 (1994)).

In an initial evaluation of 103 patients with breast cancer, those having more than three tumor cell positive axillary lymph nodes (node positive) were more likely to overexpress HER2 protein than patients with less than three positive nodes (Slamon et al. Science 235:177 (1987)). In a subsequent evaluation of 86 node-positive patients with breast cancer, there was a significant correlation among the extent of gene amplification, early relapse, and short survival. HER2 overexpression was determined using Southern and Northern blotting, which correlate with the HER2 oncoprotein expression evaluated by Western blotting and immunohistochemistry (IHC) (Slamon et al. Science 235:177 (1987); Slamon et al. Science 244:707 (1989)). The median period of survival was found to be approximately 5-fold shorter in patients with more than five copies of the her2 gene than in patients without gene amplification. This correlation was present even after correcting for nodal status and other prognostic factors in multivariate analyses. These studies were extended in 187 node-positive patients and indicated that gene amplification, increased amounts of mRNA (determined by Northern blotting), and increased protein expression (determined immunohistochemically) were also correlated with shortened survival time (Slamon et al. Science 244:707 (1989)); (see also U.S. Pat. No. 4,968,603). Nelson et al. have compared her2/neu gene amplification using FISH with immunohistochemically determined overexpression in breast cancer (Nelson et al. Modern Pathology 9 (1) 21A (1996)).

Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing alteration of proteins in a heterogeneous tissue. Immunohistochemistry (IHC) techniques utilize an antibody to probe and visualize cellular antigens in situ, generally by chromagenic or fluorescent methods. This technique excels because it avoids the unwanted effects of disaggregation and allows for evaluation of individual cells in the context of morphology. In addition, the target protein is not altered by the freezing process.

However, in the clinical trial assay (CTA), IHC of formaldehyde-fixed, paraffin embedded tissue samples only demonstrated 50%-80% sensitivity, relative to frozen IHC samples (Press, Cancer Research 54:2771 (1994)). Thus, IHC can lead to false negative results, excluding from treatment patients who might benefit from the treatment.

Fluorescence in situ hybridization (FISH) is a recently developed method for directly assessing the presence of genes in intact cells. FISH is an attractive means of evaluating paraffin-embedded tissue for the presence of malignancy because it provides for cell specificity, yet overcomes the cross-linking problems and other protein-altering effects caused by formalin fixation. FISH has historically been combined with classical staining methodologies in an attempt to correlate genetic abnormalities with cellular morphology (see, e.g., Anastasi et al., Blood 77:2456-2462 (1991); Anastasi et al., Blood 79:1796-1801 (1992); Anastasi et al., Blood 81:1580-1585 (1993); van Lom et al., Blood 82:884-888 (1992); Wolman et al., Diagnostic Molecular Pathology 1(3): 192-199 (1992); Zitzelberger, Journal of Pathology 172:325-335 (1994)).

To date, there has been no correlation of her2 gene amplification with anti-HER2 antibody treatment outcome, only with disease prognosis. The standard assay has been IHC on formalin fixed, paraffin embedded samples. These samples, when scored as 3+ or 2+, identify patients who are likely to benefit from treatment with an anti-HER2 antibody, like HERCEPTIN®. The 3+ and 2+ scores correlate with her2 gene amplification, e.g., as tested by FISH. However, there remains a need for more effective identification of candidates for successful ErbB antagonist therapies, such as HERCEPTIN® treatment.

SUMMARY OF THE INVENTION

The invention advantageously provides a method for increasing likelihood of effectiveness of an ErbB antagonist cancer treatment. The method comprises administering a can-

cer treating dose of the ErbB antagonist to a subject wherein an erbB gene in tumor cells in a tissue sample from the subject has been found to be amplified. Preferably the ErbB is HER2. In a specific embodiment, the method further comprises administering a cancer treating dose of a chemotherapeutic, particularly a taxol.

In a specific preferred embodiment, exemplified herein, the invention provides a method for increasing likelihood of effectiveness of an anti-HER2 antibody to treat cancer. This method comprises administering a cancer treating dose of the anti-HER2 antibody to the subject in whom a her2 gene in tumor cells in a tissue sample from the subject have been found to be amplified.

The unexpected clinical results underlying the invention, in which gene amplification proved to be a more effective indication of antibody-based tumor therapy than protein detection by immunohistochemistry, extends to tumor antigens in general. Thus, any anti-tumor-specific antigen based antibody therapy can have increased likelihood of success in patients who are found to have gene amplification of the gene encoding the tumor antigen.

A particular advantage of the invention is that it permits selection of patients for treatment who, based on immunohistochemical criteria, would be excluded. Thus, in a specific embodiment, the subject has been found to have an antigen level corresponding to a 0 or 1+ score for HER2 by immunohistochemistry on a formaldehyde-fixed tissue sample.

The invention further provides a pharmaceutical package comprising an ErbB antagonist for treating a cancer, and instructions to administer the ErbB antagonist to a subject if an erbB gene in tumor cells in a tissue sample from the subject is amplified. Preferably the ErbB antagonist is an anti-ErbB antibody, such as an anti-HER2 antibody. In a further aspect, the instructions also teach administering a cancer treating dose of a chemotherapeutic, e.g., a taxol. Such pharmaceutical packages, including the instructions for use, can be provided for any antibody-based therapeutic specific for a tumor-specific antigen.

DETAILED DESCRIPTION

The present invention advantageously permits treatment of patients who have a greater likelihood of responding to the treatment by administering therapeutic agents, i.e., anti-tumor antigen therapeutic antibodies or ErbB receptor antagonists, to patients who are found to have an amplified gene encoding such a tumor antigen or ErbB receptor protein. The invention is based, in part, on the unexpected discovery that her2 gene amplification, e.g., as detected by fluorescence in situ hybridization (FISH), although it correlates with HER2 expression as detected by immunohistochemistry (IHC), provides a more accurate basis for selecting patients for treatment because FISH status unexpectedly correlates better with response to treatment. This outcome was surprising in part because FISH status has about the same rate of correlation with a clinical trial assay (CTA) IHC assay as another IHC assay (HERCEPTEST®). Based on this observation, FISH would be expected to have a similar correlation with treatment response. This outcome also surprises because direct measurement of protein (by immunoassay) would be expected to provide a more accurate assessment of a cancer therapy targeted to the protein than an indirect measure of expression, like gene amplification.

Evaluation of patient groups and subgroups demonstrates the power of gene amplification analysis for selecting patients more likely to respond to treatment. IHC provides a score for HER2 expression on tumor cells: 0 (no expression) through 3+ (very high levels of expression). Clinical selection criteria exclude patients with 0 and 1+ scores and select patients with 2+ and 3+ scores. The data show that 14% of combined 2+/3+ patients respond to HERCEPTIN®, while 20% of FISH+ (amplified her2 gene) patients respond to HERCEPTIN®. The 3+ subgroup has a 17% response rate, which is very close to the FISH+ subjects' response rate. However, the 2+ subgroup has less than half the response rate of FISH+ subjects. Thus, gene amplification clearly differentiates large subpopulations within the 2+ subgroup, permitting more effective treatment for those who are FISH+, and quickly identifying patients for whom alternative treatment modalities are appropriate and should commence immediately.

Gene amplification analysis also identifies patients who are unnecessarily excluded because of anomalies in the IHC analysis, particularly when the tests are performed on formalyn fixed, paraffin embedded samples (such sample processing can disrupt or destroy antibody epitopes on the HER2 protein, but has much less impact on gene amplification assays). As shown in the examples, a subset of 0 and 1+ subjects are FISH+. These patients are likely to respond to anti-HER2 antibody therapy, e.g., with HERCEPTIN®, although by IHC criteria they would be excluded from receiving this treatment.

Thus, the present invention advantageously permits inclusion of patients who are more likely to benefit from treatment but who, by standard IHC criteria, would be excluded from treatment. At the same time, the invention permits exclusion of patients who should promptly seek an alternative mode of treatment because the anti-tumor antigen therapy (i.e., ErbB antagonist or tumor antigen-specific therapeutic antibody) is not likely to succeed.

In short, the present invention is a powerful adjunct to IHC assays for target protein expression level-based selection of patients. It can also be employed on its own, i.e., without IHC, to provide initial screening and selection of patients. The invention significantly improves screening and selection for subjects to receive a cancer-treating dose of an anti-tumor antigen therapeutic antibody treatment, ErbB receptor antagonist treatment, and other treatment targeted to overexpressed tumor antigens (or tumor-specific antigens), resulting in an increased likelihood of benefit from such treatments.

In another aspect, the invention concerns an article of manufacture or package, comprising a container, a composition within the container comprising an ErbB antagonist, e.g., an anti-ErbB antibody (or other anti-tumor-specific antigen antibody), optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB receptor, and a package insert containing instructions to administer the antagonist to patients who have been found to have an amplified erbB gene.

Definitions

As used herein, an "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3, and ErbB4 receptors, as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid

sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

ErbB receptors are examples of tumor-specific antigens or tumor antigens. The term "tumor antigen" is used herein to refer to a protein that is expressed at a higher level on tumor cells compared to normal cells. Generally, the normal cells for comparison are of the same tissue type, particularly phenotype, as the tumor, or from which the tumor arose. A "tumor specific antigen" refers to an antigen expressed either preferentially or only on tumor cells. Examples of tumor-specific antigens include, in addition to the ErbB receptors, MART1/Melan A, gp-100, and tyrosinase (in melanoma); MAGE-1 and MAGE-3 (in bladder, head and neck, and non-small cell carcinoma); HPV EG and E7 proteins (in cervical cancer); Mucin/MUC-1 (in breast, pancreas, colon, and prostate cancers); prostate specific antigen/PSA (in prostate cancer); and carninoembryonic antigen/CEA (in colon, breast, and gastrointestinal cancers).

By "amplification" is meant the presence of one or more extra gene copies of erbB or other tumor antigen-encoding gene in a chromosome complement. Gene amplification can result in overexpression of protein, e.g., ErbB receptor protein. Gene amplification in cells from a tissue sample can be measured by many techniques, particularly Fluorescence in situ Hybridization (FISH), but also including and not limited to quantitative PCR, quantitative Southern hybridization, and the like.

By "tissue sample" is meant a collection of similar cells obtained from a tissue of a subject or patient, preferably containing nucleated cells with chromosomal material. The four main human tissues are (1) epithelium; (2) the connective tissues, including blood vessels, bone and cartilage; (3) muscle tissue; and (4) nerve tissue. The source of the tissue sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like. In one embodiment of the invention, the tissue sample is "non-hematologic tissue" (i.e., not blood or bone marrow tissue).

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, e.g., a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention, provided that it is understood that the present invention comprises a method whereby the same section of tissue sample may be analyzed at both morphological and molecular levels, or may be analyzed with respect to both protein and nucleic acid.

By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis with the performance and/or results of a second analysis. For example, one may use the results of a first analysis in carrying out the second analysis and/or one may use the results of a first analysis to determine whether a second analysis should be performed and/or one may compare the results of a first analysis with the results of a second analysis. In relation to IHC combined with FISH, one may use the results of IHC to determine whether FISH should be performed and/or one may compare the level of protein expression with gene amplification to further characterize a tumor biopsy (e.g. to compare HER2 protein expression with her2 gene amplification). One advantageous feature of the invention is the ability to identify patients likely to benefit from treatment using FISH even if IHC indicates that they are antigen low.

By "nucleic acid" is meant to include any DNA or RNA, for example, chromosomal, mitochondrial, viral and/or bacterial nucleic acid present in tissue sample. The term "nucleic acid" encompasses either or both strands of a double stranded nucleic acid molecule and includes any fragment or portion of an intact nucleic acid molecule.

By "gene" is meant any nucleic acid sequence or portion thereof with a functional role in encoding or transcribing an RNA (rRNA, tRNA, or mRNA, the latter capable of translation as a protein) or regulating other gene expression. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The nucleic acid sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as Epidermal Growth Factor (EGF) (Savage et al., J. Biol. Chem. 247:7612-7621 (1972)); Tansforming Growth Factor alpha (TGF-alpha) (Marquardt et al., Science 223: 1079-1082 (1984)); amphiregulin, also known as schwanoma or keratinocyte autocrine growth factor (Shoyab et al. Science 243:1074-1076 (1989); Kimura et al. Nature 348:257-260 (1990); and Cook et al. Mol. Cell. Biol. 11:2547-2557 (1991)); betacellulin (Shing et al., Science 259:1604-1607 (1993); and Sasada et al. Biochem. Biophys. Res. Commun. 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., Science 251:936-939 (1991)); epiregulin (Toyoda et al, J. Biol. Chem. 270:7495-7500 (1995); and Komurasaki et al. Oncogene 15:2841-2848 (1997)), a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., Nature 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., Proc. Natl. Acad. Sci. 94:9562-9567 (1997)); or cripto (CR-1) (Kannan et al., J. Biol. Chem. 272 (6):3330-3335 (1997)). ErbB ligands that bind EGFR include EGF, TGF-alpha, amphiregulin, betacellulin, HB-EGF andepiregulin. ErbB ligands which bind HER3 include heregulins. ErbB ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide comprising an amino acid sequence encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641,869 or Marchionni et al., Nature, 362:312-318 (1993), and biologically active variants of such polypeptides. Examples of heregulins include heregulin-alpha, heregulin-beta1, heregulin-beta2, and heregulin-beta3 (Holmes et al., Science, 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. Cell 69: 205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. Cell 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., Nature, 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. J. Biol. Chem. 270:14523-14532 (1995)); gamma-heregulin (Schaefer et al. Oncogene 15:1385-1394 (1997)). An example of a biologically active fragment/amino acid sequence variant of a native sequence HRG polypeptide, is an EGF-like domain fragment (e.g., HRG-beta1, 177-244).

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be

isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., (J. Biol. Chem., 269(20): 14661-14665 (1994)), for example. Examples of such ErbB hetero-oligomers include EGFR-HER2, HER2-HER3, and HER3-HER4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more HER2 receptors combined with a different ErbB receptor, such as HER3, HER4, or EGFR. Other proteins, such as a cytokine receptor subunit (e.g., gp130), may be included in the hetero-oligomer.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. (Ann. Rev. Biochem. 56:881-914 (1987)), including variants thereof (e.g., a deletion mutant EGFR as in Humphrey et al., (Proc. Natl. Acad. Sci. USA 87:4207-4211 (1990)). ErbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), Mab 455 (ATCC CRL HB 8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, PCT Publication No. WO 96/40210).

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to native sequence human HER2 protein described, for example, in Semba et al., (Proc. Natl. Acad. Sci USA 82:6497-6501 (1985)) and Yamamoto et al. (Nature 319:230-234 (1986)) (Genebank accession number X03363), and variants thereof. The term erbB2 refers to the gene encoding human HER2 and neu refers to the gene encoding rat p185neu. Preferred HER2 is native sequence human HER2. Examples of antibodies which bind HER2 include MAbs 4D5 (ATCC CRL 10463), 2C4 (ATCC HB-12697), 7F3 (ATCC HB-12216), and 7C2 (ATCC HB-12215) (see, U.S. Pat. No. 5,772,997; PCT Publication No. WO 98/17797; and U.S. Pat. No. 5,840,525, expressly incorporated herein by reference). Humanized anti-HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, and huMAb4D5-8 (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337, which is expressly incorporated herein by reference; and humanized 520C9 (PCT Publication No. WO 93/21319). Human anti-HER2 antibodies are described in U.S. Pat. No. 5,772,997 and PCT Publication No. WO 97/00271.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968, as well as Kraus et al. (Proc. Natl. Acad. Sci. USA) 86:9193-9197 (1989)), including variants thereof. Exemplary antibodies that bind HER3 are described in U.S. Pat. No. 5,968,511, e.g., the 8B8 antibody (ATCC HB-12070) or a humanized variant thereof. The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in European Application No. EP 599,274; Plowman et al., (Proc. Natl. Acad. Sci. USA, 90:1746-1750 (1993)); and Plowman et al., (Nature, 366:473-475 (1993)), including variants thereof such as the HER4 isoforms disclosed in PCT Publication No. WO 99/19488.

An "ErbB antagonist" is any molecule that binds to an ErbB receptor and blocks ligand activation of the ErbB receptor. Such antagonists include, but are not limited to, modified ligands, ligand peptides (i.e., ligand fragments), soluble ErbB receptors, and, preferably, anti-ErbB antibodies.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the ErbB antagonist, e.g., anti-ErbB2 antibody, and more generally, any cancer in which administration of an antibody against an over-expressed antigen can treat the cancer. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount elicits antibody-mediated cytotoxicity, activates complement, has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time for disease progression (TTP), survival, tumor size, or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, melanoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small-cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, and various types of head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell surface, such that an anti-ErbB antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90}, and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibi-

otics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; antimetabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; carminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and anti-androgens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W B Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

ErbB Receptor Tyrosine Kinases

The ErbB receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes at least four distinct members including Epidermal Growth Factor Receptor (EGFR or ErbB1), HER2 (ErbB2 or p 185neu), HER3 (ErbB3), and HER4 (ErbB4 or tyro2).

EGFR, encoded by the ErbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer, as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, Transforming Growth Factor alpha (TGF-alpha), by the same tumor cells resulting inreceptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn Pharmac. Ther. 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-alpha and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn., supra; Masui et al. Cancer Research 44: 1002-1007 (1984); and Wu et al. J. Clin. Invest. 95:1897-1905 (1995).

The second member of the ErbB family, p185neu, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., Science, 235:177-182(1987); Slamon et al., Science, 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder.

Antibodies directed against the rat p185neu and human HER2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185neu (see, for example, Drebin et al., Cell 41:695-706 (1985); Myers et al., Meth. Enzym. 198:277-290 (1991); and WO94/22478). Drebin et al. (Oncogene 2:273-277(1988)) report that mixtures of antibodies reactive with two distinct regions of p185neu result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice (see also U.S. Pat. No. 5,824,311).

Hudziak et al., (Mol. Cell. Biol. 9(3):1165-1172 (1989)) describe the generation of a panel of anti-HER2 antibodies, which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5, which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-alpha (see, also, U.S. Pat. No. 5,677,171). The anti-HER2 antibodies discussed in Hudziak et al. were further characterized (Fendly et al. Cancer Research 50:1550-1558 (1990); Kotts et al. In Vitro 26(3):

59A (1990); Sarup et al. Growth Regulation 1:72-82 (1991); Shepard et al. J. Clin. Immunol. 11(3):117-127 (1991); Kumar et al. Mol. Cell. Biol. 11(2):979-986 (1991); Lewis et al. CancerImmunol. Immunother. 37:255-263 (1993); Pietras et al. Oncogene 9: 1829-1838 (1994); Vitetta et al. Cancer Research 54:5301-5309 (1994); Sliwkowski et al. J. Biol. Chem. 269(20):14661-14665 (1994); Scott et al. J. Biol. Chem. 266:14300-5 (1991); D'souza et al. Proc. Natl. Acad. Sci. 91:7202-7206 (1994); Lewis et al. Cancer Research 56:1457-1465(1996); and Schaefer et al. Oncogene 15:1385-1394 (1997)).

A recombinant humanized IgG1 version of the murine anti-HER2 antibody 4D5 (rhuMAb HER2 or HERCEPTIN®; commercially available from Genentech, Inc., South San Francisco) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., J. Clin. Oncol. 14:737-744 (1996)). HERCEPTIN® received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. The current treatment protocol employs IHC to determine HER2 protein overexpression.

Other anti-HER2 antibodies with various properties have been described (Tagliabue et al., Int. J. Cancer 47:933-937 (1991); McKenzie et al., Oncogene 4:543-548 (1989); Maier et al., Cancer Res. 51:5361-5369 (1991); Bacus et al., Molecular Carcinogenesis 3:350-362 (1990); Stancovski et al., (Proc. Natl. Acad. Sci. USA) 88:8691-8695 (1991); Bacus et al., Cancer Research 52:2580-2589 (1992); Xu et al. Int. J. Cancer 53:401-408 (1993); PCT Publication No. WO94/00136; Kasprzyk et al., Cancer Research 52:2771-2776 (1992); Hancock et al., Cancer Res. 51:4575-4580 (1991); Shawver et al., Cancer Res. 54:1367-1373 (1994); Arteaga et al. Cancer Res. 54:3758-3765 (1994); Harwerth et al., J. Biol. Chem. 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; Klapper et al. Oncogene 14:2099-2109 (1997); and PCT Publication No. WO 98/17797).

Homology screening has resulted in the ErbB receptor family members: HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968; Kraus et al., Proc. Natl. Acad. Sci. USA 86:9193-9197 (1989)) and HER4 (European Patent Application No. EP 599 274; Plowman et al., Proc. Natl. Acad. Sci. USA, 90:1746-1750 (1993); and Plowman et al., Nature, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of ErbB ligands (Earp et al., Breast Cancer Research and Treatment 35: 115-132 (1995)). EGFR is bound by six different ligands: Epidermal Growth Factor (EGF), Transforming Growth Factor-alpha (TGF-alpha), amphiregulin, Heparin Binding Epidermal Growth Factor (HB-EGF), betacellulin, and epiregulin (Groenen et al. GrowthFactors 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta, and gamma heregulins (Holmes et al., Science, 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al., Oncogene 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motorneuron derived factor (SMDF) (for a review, see Groenen et al., Growth Factors 11:235-257 (1994); Lemke, G. Molec. & Cell. Neurosci. 7:247-262 (1996) and Lee et al. Pharm. Rev. 47:51-85 (1995)). Recently, two additional ErbB ligands were identified: neuregulin-2 (NRG-2), which is reported to bind either HER3 or HER4 (Chang et al., Nature: 387 509-512 (1997); and Carraway et al Nature 387:512-516 (1997)) and neuregulin-3, which binds HER4 (Zhang et al., (Proc. Natl. Acad. Sci. USA) 94(18): 9562-7 (1997)). HB-EGF, betacellulin, and epiregulin also bind to HER4.

While EGF and TGF-alpha do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase, (Earp et al., supra.) Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., J. Biol. Chem., 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., Journal of Neuroscience 15: 1329-1340 (1995); Morrissey et al., Proc. Natl. Acad. Sci. USA 92:1431-1435 (1995); and Lewis et al., Cancer Res., 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, Cell 78:5-8(1994)).

Detecting Gene Amplification

The present invention contemplates using any technique to detect gene amplification. (see, Boxer, J. Clin. Pathol. 53: 19-21(2000)). These techniques include in situ hybridization (Stoler, Clin. Lab. Med. 12:215-36 (1990), using radioisotope or fluorophore-labeled probes; polymerase chain reaction (PCR); quantitative Southern blotting, and other techniques for quantitating individual genes. Preferably probes or primers selected for gene amplification evaluation are highly specific, to avoid detecting closely related homologous genes.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. A hapten or epitope that is immunospecifically bound by an antibody can also serve as a label.

The term "fluorescently labeled nucleic acid probe" refers to a probe comprising (1) a nucleic acid having a sequence rendering it capable of hybridizing with a target nucleic acid sequence and (2) a fluorescent label. Preferably such hybridization is specific, i.e., it can occur under high stringency conditions.

Sample Preparation

Any tissue sample from a subject may be used. Examples of tissue samples that may be used include, but are not limited to, breast, prostate, ovary, colon, lung, endometrium, stomach, salivary gland or pancreas. The tissue sample can be obtained by a variety of procedures including, but not limited to surgical excision, aspiration, or biopsy. The tissue may be fresh or frozen. In one embodiment, the tissue sample is fixed and embedded in paraffin or the like.

The tissue sample may be fixed (i.e., preserved) by conventional methodology (See e.g., *Manual of Histological Staining Method of the Armed Forces Institute of Pathology,* 3rd Edition Lee G. Luna, HT (ASCP) Editor, The Blakston Division McGraw-Hill Book Company: New York; (1960); *The Armed Forces Institute of Pathology Advanced Laboratory Methods in Histology and Pathology* (1994) Ulreka V. Mikel, Editor, Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C.). One of skill in the

art will appreciate that the choice of a fixative is determined by the purpose for which the tissue is to be histologically stained or otherwise analyzed. One of skill in the art will also appreciate that the length of fixation depends upon the size of the tissue sample and the fixative used. By way of example, neutral buffered formalin, Bouin's or paraformaldehyde, may be used to fix a tissue sample.

Generally, the tissue sample is first fixed and is then dehydrated through an ascending series of alcohols, infiltrated, and embedded with paraffin or other sectioning media so that the tissue sample may be sectioned. Alternatively, one may section the tissue and fix the sections obtained. By way of example, the tissue sample may be embedded and processed in paraffin by conventional methodology. Examples of paraffin that may be used include, but are not limited to, Paraplast, Broloid, and Tissuemay. Once the tissue sample is embedded, the sample may be sectioned by a microtome or the like. By way of example for this procedure, sections may range from about three microns to about five microns in thickness. Once sectioned, the sections may be attached to slides by several standard methods. Examples of slide adhesives include, but are not limited to, silane, gelatin, poly-L-lysine, and the like. For example, the paraffin embedded sections may be attached to positively charged slides, slides coated with poly-L-lysine.

If paraffin has been used as the embedding material, the tissue sections are generally deparaffinized and rehydrated to water. The tissue sections may be deparaffinized by several conventional standard methodologies. For example, xylenes and a gradually descending series of alcohols may be used. Alternatively, commercially available deparaffinizing non-organic agents such as Hemo-De7 (CMS, Houston, Tex.) may be used.

Fluorescence In Situ Hybridization (FISH)

In situ hybridization is generally carried out on cells or tissue sections fixed to slides. In situ hybridization may be performed by several conventional methodologies (see, e.g., Leitch et al., *In Situ Hybridization: A Practical Guide*, Oxford BIOS Scientific Publishers, Micropscopy Handbooks v. 27 (1994)). In one in situ procedure, fluorescent dyes (such as fluorescein isothiocyanate (FITC) which fluoresces green when excited by an Argon ion laser) are used to label a nucleic acid sequence probe that is complementary to a target nucleotide sequence in the cell. Each cell containing the target nucleotide sequence will bind the labeled probe producing a fluorescent signal upon exposure, of the cells to a light source of a wavelength appropriate for excitation of the specific fluorochrome used. A "target nucleotide sequence" is a sequence specific for a over-expressed tumor antigen, such as ErbB. FISH analysis can be used in conjunction with other assays, including without limitation morphological staining (of serial sections or the same section; see PCT Publication No. WO 00/20641, specifically incorporated herein by reference).

Various degrees of hybridization stringency can be employed. As the hybridization conditions become more stringent, a greater degree of complementarity is required between the probe and target to form and maintain a stable duplex. Stringency is increased by raising temperature, lowering salt concentration, or raising formamide concentration. Adding dextran sulfate or raising its concentration may also increase the effective concentration of labeled probe to increase the rate of hybridization and ultimate signal intensity. After hybridization, slides are washed in a solution generally containing reagents similar to those found in the hybridization solution with washing time varying from minutes to hours depending on required stringency. Longer or more stringent washes typically lower nonspecific background but run the risk of decreasing overall sensitivity.

Probes used in the FISH analysis may be either RNA or DNA oligonucleotides or polynucleotides and may contain not only naturally occurring nucleotides but their analogs like digoxygenin dCTP, biotin dcTP 7-azaguanosine, azidothymidine, inosine, or uridine. Other useful probes include peptide probes and analogues thereof, branched gene DNA, peptidomimetics, peptide nucleic acid (PNA), and/or antibodies.

Probes should have sufficient complementarity to the target nucleic acid sequence of interest so that stable and specific binding occurs between the target nucleic acid sequence and the probe. The degree of homology required for stable hybridization varies with the stringency of the hybridization medium and/or wash medium. Preferably, completely homologous probes are employed in the present invention, but persons of skill in the art will readily appreciate that probes exhibiting lesser but sufficient homology can be used in the present invention (see e.g., Sambrook, J., et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Press, (1989)).

One of skill in the art will appreciate that the choice of probe depends on the characteristics of the target gene of interest. Examples of amplification include, but are not limited to, her2/neu in breast and ovarian cancer, n-myc in neuroblastoma, c-myc in small cell lung cancer. By way of example for evaluating her2/neu amplification a probe spanning a 140 kb region on the long arm of chromosome 17 containing the her2/neu gene (17q 11.2-17q12) may be used. A probe for the -satellite sequences in the centromeric region of chromosome 17(D1721) may be used to evaluate for aneusomy of chromosome 17 as a source or cause for her2/neu amplification. For example, a cocktailed version of these probes may be obtained from Vysis, Inc. where each probe is directly labeled with easily distinguishable fluorophores, such as SPECTRUM ORANGE® and SPECTRUM GREEN®.

Probes may also be generated and chosen by several means including, but not limited to, mapping by in situ hybridization, somatic cell hybrid panels, or spot blots of sorted chromosomes; chromosomal linkage analysis; or cloned and isolated from sorted chromosome libraries from human cell lines or somatic cell hybrids with human chromosomes, radiation somatic cell hybrids, microdissection of a chromosome region, or from yeast artificial chromosomes (YACs) identified by PCR primers specific for a unique chromosome locus or other suitable means like an adjacent YAC clone. Probes may be genomic DNA, cDNA, or RNA cloned in a plasmid, phage, cosmid, YAC, Bacterial Artificial Chromosomes (BACs), viral vector, or any other suitable vector. Probes may be cloned or synthesized chemically by conventional methods. When cloned, the isolated probe nucleic acid fragments are typically inserted into a vector, such as lambda phage, pBR322, M13, or vectors containing the SP6 or T7 promoter and cloned as a library in a bacterial host (see, e.g., Sambrook, supra).

Probes are preferably labeled with a fluorophor. Examples of fluorophores include, but are not limited to, rare earth chelates (europium chelates), Texas Red, rhodamine, fluorescein, dansyl, Lissamine, umbelliferone, phycocrytherin, phycocyanin, or commercially available fluorophors such SPECTRUM ORANGE® and SPECTRUM GREEN®, and/or derivatives of any one or more of the above. Multiple probes used in the assay may be labeled with more than one distinguishable fluorescent or pigment color. These color differences provide a means to identify the hybridization positions of specific probes. Moreover, probes that are not separated spatially can be identified by a different color light or pigment resulting from mixing two other colors (e.g., light red+green=yellow), pigment (e.g., blue+yellow=green), or by using a filter set that passes only one color at a time.

Probes can be labeled directly or indirectly with the fluorophor, utilizing conventional methodology. Additional probes and colors may be added to refine and extend this general procedure to include more genetic abnormalities or serve as internal controls. By way of example the her2/neu gene is in chromosome 17, and as an internal control a probe for satellite sequences specific for chromosome 17 (D17Z1) may be used (Vysis, Inc.) to prove diploidy in areas of non-malignant cells and/or to establish the presence or absence of chromosome 17 aneusomy in areas of her2/neu amplification.

After processing for FISH, the slides may be analyzed by standard techniques of fluorescence microscopy (see, e.g., Ploem and Tanke, *Introduction to Fluorescence Microscopy*, Oxford University Press: New York (1987)). Briefly, each slide is observed using a microscope equipped with appropriate excitation filters, dichromic, and barrier filters. Filters are chosen based on the excitation and emission spectra of the fluorochromes used. Photographs of the slides may be taken with the length of time of film exposure depending on the fluorescent label used, the signal intensity and the filter chosen. For FISH analysis the physical loci of the cells of interest determined in the morphological analysis are recalled and visually conformed as being the appropriate area for FISH quantification.

In order to correlate IHC with FISH, one may use a computer-driven, motorized stage which stores location of coordinates. This may be used to evaluate the same area by two different analytical techniques. For example, color images of the morphologically stained areas may be captured and saved using a computer-assisted cooled CCD camera. The same section may be subsequently taken through the FISH procedure, the stored locations recalled, and the designated areas scored for the presence of fluorescent nuclear signals. A similar procedure for IHC followed by FISH is contemplated.

Typically, hundreds of cells are scanned in a tissue sample and quantification of the specific target nucleic acid sequence is determined in the form of fluorescent spots, which are counted relative to the number of cells. Deviation of the number of spots in a cell from a norm (e.g., such as probing for the her2/neu gene in a normal cell will produce two copies, abnormal greater than two) is indicative of a greater likelihood of benefit from a tumor antigen-specific antibody therapy, e.g., an ErbB antagonist therapy. As exemplified infra, her2 gene amplification provides a much more effective indication of the likelihood that an anti-HER2 antibody therapy will be effective.

Pharmaceutical Formulations

Therapeutic formulations of the antagonists, e.g., antibodies, used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2, ErbB3, ErbB4, vascular endothelial factor (VEGF), or an antibody that binds to a different epitope on the target ErbB, in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, a chemotherapeutic, a cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.

The formulations to be used for in vivo administration are preferably, and in the case of humans, must be, sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Treatment with the Anti-ErbB Antagonists

It is contemplated that, according to the present invention, the anti-ErbB antibodies or other antagonists may be used to treat various conditions characterized by overexpression and/or activation of the ErbB receptor in patients who have been found to have an amplified erbB gene. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal,

liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and any other active agents of the invention are administered to a human patient in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB antibody and a chemotherapeutic agent, e.g., a taxoid. The present invention contemplates administration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells (tumor resection) and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antagonist, e.g., antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Pharmaceutical Packages: Articles of Manufacture

In a related aspect of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, optionally labeled, and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials, such as glass or plastic. The container holds a composition that is effective for treating the condition and preferably has a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-tumor antigen therapeutic antibody or an ErbB antagonist, e.g., an anti-ErbB antibody. A label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. This second buffer can be used to reconstitute the active agent, if that is provided as a lyophilysate or dried powder, or to dilute a concentrated preparation of the active agent. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In addition, the article of manufacture comprises a package insert or inserts with instructions for use in patients who have been found to have erbB gene amplification, e.g., by FISH testing. Such patients may be subjects who, by IHC, would be excluded from treatment with the ErbB antagonist, e.g., patients who score a 0 or 1+ using an anti-HER2 antibody.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLE 1

Concordance Between the Clinical Trials Assay (CTA) and Fluorescence In Situ Hybridization (FISH) in the HERCEPTIN® Pivotal Trials

Overexpression of HER2 at the 2+ or 3+ level by immunohistochemistry (IHC) was required for enrollment in the pivotal HERCEPTIN® metastatic breast cancer trials. The Clinical Testing Assay (CTA) involves two separate IHC assays performed with either monoclonal antibodies 4D5 (after protease digestion of the formalin fixed sample) or CB11 (after heat treatment of the formalin fixed sample). Subjects were eligible if either assay was scored at 2+ or 3+. If both were performed, the final score was the higher of the two results.

Concordance between the CTA and another IHC, HERCEPTEST® (HT), is 79%. This was the basis for FDA approval of HT to aid in the selection of patients for HERCEPTIN® therapy.

This Example describes a similar concordance study, utilizing clinical material submitted for screening for the HERCEPTIN® pivotal trials, that compares the CTA to her2/neu gene amplification measured by the PathVysion FISH assay. In the pivotal trials, 5998 subjects were screened for HER2 expression; 1915 (32%) were positive by the CTA and 4083 (68%) were negative. A random sample of 623 specimens (1:1 ratio of positive:negative) were selected for this analysis, 317 CTA+ and 306 CTA−. Specimens were not freshly cut

from blocks. They had been stored between 2 and 4 years as 4-6μ sections on glass slides. Each section was assayed for her2/neu amplification using the protocol specified in the package insert of the PathVysion assay. Amplification was defined as a signal ratio of greater than or equal to 2. The results are shown in Table 1.

TABLE 1

FISH/CTA Concordance

		CTA				
		0	1+	2+	3+	
FISH	–	207	28	67	21	
	+	7	2	21	176	
		4%	7%	24%	89%	529

FISH+ = HER2:CEP17 signal ratio ≧ 2

Concordance = 82% (79-85%)

For the total 623 specimens tested, a FISH signal result was obtained in 529. Assay failure occurred in 19.9% of CTA– and 10.4% CTA+ samples. Amplification in the 0, 1+, 2, and 3+ groups was 4.2%, 6.7%, 23.9%, and 89.3%, respectively. The sample concordance was 81.3%, similar to the CTA/HT concordance of 79%. Single copy overexpression was 31%, predominantly in the 2+ group. Amplification was rarely (4.6%) noted in the CTA– group. The higher assay failure rate in the CTA– group may be due to non-assay related factors such as tissue fixation. These may have also resulted in false negative results for IHC.

These data were closely interpreted to suggest that her2/neu amplification status may have unexpectedly superior predictive value for identifying patients who are more likely to benefit from HERCEPTIN® treatment as compared to HERCEPTEST®. The observation that only 24% of 2+ patients are FISH+ suggest that this sub-group may have less predictable treatment outcomes when selected by IHC only. Identification of FISH+ patients in the 1+ and 0 sub-groups might identify subjects who, though failing the IHC criteria for HERCEPTIN® treatment, would likely benefit from HERCEPTIN® treatment. A direct analysis of HERCEPTIN® benefit based on FISH score compared to IHC score is presented in Example 2.

EXAMPLE 2

FISH/Clinical Outcome Study

This example links the results from three HERCEPTIN® Trials with FISH status. In this study, 805 subjects were selected at random from all three trials. Of these, 167 lacked slides. Another 78 assays (9.7%) failed. Thus, formalin-fixed cut sections stored between 2.5 and 4.5 years from 540 subjects provided the sample pool for this study. There were no imbalances in demographics or prognostic indicators in these samples. Results are reported for different treatment groups.

Correlation of FISH status with response was evaluated for patients who received HERCEPTIN® as a second or third line therapy. These data are reported for 2+ and 3+ (by CTA) subjects in Table 2.

TABLE 2

FISH/Response with single agent HERCEPTIN ®, 2nd or 3rd line Therapy, 2+/3+ Combined

	FISH+	FISH–
Response	21	0
No response	84	37
response rate	20%	0%
	(12.5-27.5%)	(0.7%)

N = 142

The 20% response rate of FISH+ subjects unexpectedly exceeds the 15% response rate of 2+ and 3+ patients in this study and 14% response rate observed in patients selected by CTA with a 2+ or 3+ immunohistochemistry score during the pivotal trials. Thus, while FISH correlates well with IHC to about the same degree as another IHC assay, the Hercep Test, as shown in Example 1, it unexpectedly is superior in identifying patients who are more likely to benefit from HERCEPTIN® therapy.

When these data were broken down into the components 3+ and 2+ subjects, the same 20% response rate of FISH+ subjects was seen (Tables 3 and 4).

TABLE 3

FISH/Response with single agent HERCEPTIN ®, 2nd or 3rd line therapy, 3+ subgroup

	FISH+	FISH–
Response	18	0
No response	72	17
response rate	20%	0%
	(12-28%)	(0-14%)

N = 107

TABLE 4

FISH/Response with single agent HERCEPTIN ®, 2nd or 3rd line therapy, 2+ subgroup

	FISH+	FISH–
Response	3	0
No response	12	20
response rate	20%	0%
	(1-40%)	(0-14%)

N = 35

In the 3+ sub-group, the FISH+ response rate (20%) was very close but still exceeded the 17% response rate of 3+ subjects. The 2+ subgroup showed a much greater difference, with only a 9% response rate versus 20% by FISH+ selection. These data show that FISH+ status (her2 gene amplification) greatly increases the likelihood of response to HERCEPTIN®.

Data were also evaluated for patient responses to HERCEPTIN® as a first line therapy (Table 5).

TABLE 5

FISH/Response with single agent HERCEPTIN ® as 1st line therapy, 2+/3+ combined

	FISH+	FISH–
Response	17	1
No response	24	20
response rate	41%	20%
	(26-56%)	(0-14%)

N = 62

The 41% response rate of FISH+ subjects was notably greater than the 27% response rate of 3+, 2+ subjects.

The surprising increase in likelihood of beneficial response based on FISH analysis extended to responses to chemotherapy plus HERCEPTIN®, as shown in Table 6. FISH+ subjects showed a much greater response to chemotherapy and HERCEPTIN® (54%) than FISH-(41%). Tables 7-9 contain more extensive data, broken down by different chemotherapeutic agents (adrinomycin and cyclophosphamide, AC; and Paditaxol, P) and different endpoints (response rate, time to progression, and survival) for HERCEPTIN® in combination with chemotherapy.

TABLE 6

FISH/Response rate to chemotherapy +/− HERCEPTIN ®, 1st line therapy; 2+/3+ combined

	C alone	C + H
FISH−	39% (26-52%)	41% (27-55%)
FISH+	27% (19-35%)	54% (45-63%)

N = 336

TABLE 7

Response rate of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	56*	42	41*	17	50*	32
3+	349	60*	42	49*	17	56*	31
FISH+	240	58*	40	49*	14	54*	27

*p < 0.05

TABLE 8

Time to progression (months) of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	7.8*	6.1	6.9*	2.7	7.4*	4.6
3+	349	8.1*	6.0	7.1*	3.0	7.8*	4.6
FISH+	240	7.8*	6.2	7.0*	3.2	7.3*	4.6

*p < 0.05

TABLE 9

Survival (months) of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	27	21	22	18	25*	20
3+	349	31*	21	25	18	29*	20
FISH+	240	29*	20	25*	14	27*	18

*p < 0.05

These data uniformly confirm that FISH+ analysis, though correlating closely to IHC, provides a much more accurate indicator of likelihood of success with HERCEPTIN® treatment. Across the board, FISH+ selection has about ⅓ (30%) greater response rate than 2+/3+ IHC-selection. When focused on 2+ patients, FISH status provides a much more effective tool for patient selection. FISH states also identifies patients who, because of 0 or 1+ status as determined by IHC, would otherwise be excluded from treatment.

These observations have broad implications for ErbB receptor antagonist-based cancer therapies and anti-tumor antigen cancer therapies in general. Thus erbB antagonists, e.g., anti-erbB receptor antibodies like HERCEPTIN®, can have an increased likelihood of efficacy when administered to patients who are positive for erbB gene amplification, e.g., by a FISH test. This is certainly the case, based on these data, with HERCEPTIN®.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

What is claimed:

1. A method of identifying and treating a breast cancer patient disposed to respond favorably to a HER2 antibody, huMAb4D5-8, which method comprises detecting her2 gene amplification in cancer cells in a breast tissue sample from the patient and treating the patient with her2 gene amplification with the HER2 antibody in an amount effective to treat the breast cancer, wherein the patient's cancer cells express HER2 at a 0 or 1+ level by immunohistochemistry.

2. The method of claim **1** wherein her2 gene amplification is detected by detecting fluorescence of a fluorescent-labeled nucleic acid probe hybridized to the gene.

3. The method of claim **1** wherein a formaldehyde-fixed tissue sample containing the patient's breast cancer cells has been subjected to immunohistochemistry assay and found to express HER2 at a 0 or 1+ level.

4. The method of claim **3** wherein the immunohistochemistry assay is performed prior to detecting her2 gene amplification.

5. The method of claim **1** which further comprises administering a cancer treating dose of a chemotherapeutic drug.

6. The method of claim **5** wherein the chemotherapeutic drug is a taxoid.

* * * * *

EXHIBIT M

US008440402B2

(12) **United States Patent**
Mass

(10) **Patent No.: US 8,440,402 B2**
(45) **Date of Patent: *May 14, 2013**

(54) **GENE DETECTION ASSAY FOR IMPROVING THE LIKELIHOOD OF AN EFFECTIVE RESPONSE TO A HER2 ANTIBODY CANCER THERAPY**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/323,322**

(22) Filed: **Dec. 12, 2011**

(65) **Prior Publication Data**

US 2012/0093838 A1 Apr. 19, 2012

Related U.S. Application Data

(63) Continuation of application No. 11/690,304, filed on Mar. 23, 2007, now Pat. No. 8,076,066, which is a continuation of application No. 09/863,101, filed on May 18, 2001, now abandoned.

(60) Provisional application No. 60/205,754, filed on May 19, 2000.

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(52) **U.S. Cl.**
USPC **435/6.1**; 435/7.23

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

The invention provides a method for more effective treatment of patients susceptible to or diagnosed with tumors overexpressing HER2, as determined by a gene amplification assay, with a HER2 antibody. Such method comprises administering a cancer-treating dose of the HER2 antibody, preferably in addition to chemotherapeutic agents, to a subject in whose tumor cells her2 has been found to be amplified e.g., by fluorescent in situ hybridization.

6 Claims, No Drawings

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GENE DETECTION ASSAY FOR IMPROVING THE LIKELIHOOD OF AN EFFECTIVE RESPONSE TO A HER2 ANTIBODY CANCER THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 11/690,304, filed Mar. 23, 2007 now U.S. Pat. No. 8,076,066, which is a continuation application of and claims priority to U.S. patent application Ser. No. 09/863,101 filed May 18, 2001 (abandoned) which claims priority under 35 U.S.C. §119(e) of provisional application 60/205,754, filed May 19, 2000, all of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

The present invention concerns the treatment of cancers characterized by the overexpression of a tumor antigen, such as an ErbB receptor, particularly HER2. More specifically, the invention concerns more effective treatment of human patients susceptible to or diagnosed with cancer, in which the tumor cells overexpress ErbB as determined by a gene amplification assay, with an ErbB antagonist, e.g., an anti-ErbB antibody. The invention further provides pharmaceutical packages for such treatment.

BACKGROUND OF THE INVENTION

Advancements in the understanding of genetics and developments in technology and epidemiology have allowed for the correlation of genetic abnormalities with certain malignancies as well as risk assessment of an individual for developing certain malignancies. However, most of the methodologies available for evaluation of tissue for the presence of genes associated with or predisposing an individual to a malignancy have well-known drawbacks. For example, methods that require disaggregation of the tissue, such as Southern, Northern, or Western blot analysis, are rendered less accurate by dilution of the malignant cells by the normal or otherwise non-malignant cells that are present in the same tissue. Furthermore, the resulting loss of tissue architecture precludes the ability to correlate malignant cells with the presence of genetic abnormalities in a context that allows morphological specificity. This issue is particularly problematic in tissue types known to be heterogeneous, such as in human breast carcinoma, where a significant percentage of the cells present in any area may be non-malignant.

The her2/neu gene encodes a protein product, often identified as p185HER2. The native p185HER2 protein is a membrane receptor-like molecule with homology to the epidermal growth factor receptor (EGFR). Amplification and overexpression of HER2 in human breast cancer has been correlated with shorter disease-free interval and shorter overall survival in some studies (van de Vijver et al. New Eng. J. Med. 317: 1239 (1988); Walker et al. Br. J. Cancer 60:426 (1989); Tandon et al. J. Clin. Invest. 7:1120 (1989); Wright et al. Cancer Res. 49:2087 (1989); McCann et al. Cancer Res 51:3296 (1991); Paterson et al. Cancer Res. 51:556 (1991); and Winstanley et al. Br. J. Cancer 63:447 (1991)) but not in others (Zhou et al. Oncogene 4:105 (1989); Heintz et al. Arch Path Lab Med 114:160 (1990); Kury et al. Eur. J. Cancer 26:946 (1990); Clark et al. Cancer Res. 51:944 (1991); and Ravdin et al. J. Clin. Oncol. 12:467-74 (1994)).

In an initial evaluation of 103 patients with breast cancer, those having more than three tumor cell positive axillary lymph nodes (node positive) were more likely to overexpress HER2 protein than patients with less than three positive nodes (Slamon et al. Science 235:177 (1987)). In a subsequent evaluation of 86 node-positive patients with breast cancer, there was a significant correlation among the extent of gene amplification, early relapse, and short survival. HER2 overexpression was determined using Southern and Northern blotting, which correlate with the HER2 oncoprotein expression evaluated by Western blotting and immunohistochemistry (IHC) (Slamon et al. Science 235:177 (1987); Slamon et al. Science 244:707 (1989)). The median period of survival was found to be approximately 5-fold shorter in patients with more than five copies of the her2 gene than in patients without gene amplification. This correlation was present even after correcting for nodal status and other prognostic factors in multivariate analyses. These studies were extended in 187 node-positive patients and indicated that gene amplification, increased amounts of mRNA (determined by Northern blotting), and increased protein expression (determined immunohistochemically) were also correlated with shortened survival time (Slamon et al. Science 244:707 (1989)); (see also U.S. Pat. No. 4,968,603). Nelson et al. have compared her2/neu gene amplification using FISH with immunohistochemically determined overexpression in breast cancer (Nelson et al. Modern Pathology 9 (1) 21A (1996)).

Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing alteration of proteins in a heterogeneous tissue. Immunohistochemistry (IHC) techniques utilize an antibody to probe and visualize cellular antigens in situ, generally by chromagenic or fluorescent methods. This technique excels because it avoids the unwanted effects of disaggregation and allows for evaluation of individual cells in the context of morphology. In addition, the target protein is not altered by the freezing process.

However, in the clinical trial assay (CTA), IHC of formaldehyde-fixed, paraffin embedded tissue samples only demonstrated 50%-80% sensitivity, relative to frozen IHC samples (Press, Cancer Research 54:2771 (1994)). Thus, IHC can lead to false negative results, excluding from treatment patients who might benefit from the treatment.

Fluorescence in situ hybridization (FISH) is a recently developed method for directly assessing the presence of genes in intact cells. FISH is an attractive means of evaluating paraffin-embedded tissue for the presence of malignancy because it provides for cell specificity, yet overcomes the cross-linking problems and other protein-altering effects caused by formalin fixation. FISH has historically been combined with classical staining methodologies in an attempt to correlate genetic abnormalities with cellular morphology (see, e.g., Anastasi et al., Blood 77:2456-2462 (1991); Anastasi et al., Blood 79:1796-1801 (1992); Anastasi et al., Blood 81:1580-1585 (1993); van Lom et al., Blood 82:884-888 (1992); Wolman et al., Diagnostic Molecular Pathology 1(3): 192-199 (1992); Zitzelberger, Journal of Pathology 172:325-335 (1994)).

To date, there has been no correlation of her2 gene amplification with anti-HER2 antibody treatment outcome, only with disease prognosis. The standard assay has been IHC on formalin fixed, paraffin embedded samples. These samples, when scored as 3+ or 2+, identify patients who are likely to benefit from treatment with an anti-HER2 antibody, like HERCEPTIN®. The 3+ and 2+ scores correlate with her2 gene amplification, e.g., as tested by FISH. However, there

remains a need for more effective identification of candidates for successful ErbB antagonist therapies, such as HERCEPTIN® treatment.

SUMMARY OF THE INVENTION

The invention advantageously provides a method for increasing likelihood of effectiveness of an ErbB antagonist cancer treatment. The method comprises administering a cancer treating dose of the ErbB antagonist to a subject wherein an erbB gene in tumor cells in a tissue sample from the subject has been found to be amplified. Preferably the ErbB is HER2. In a specific embodiment, the method further comprises administering a cancer treating dose of a chemotherapeutic, particularly a taxol.

In a specific preferred embodiment, exemplified herein, the invention provides a method for increasing likelihood of effectiveness of an anti-HER2 antibody to treat cancer. This method comprises administering a cancer treating dose of the anti-HER2 antibody to the subject in whom a her2 gene in tumor cells in a tissue sample from the subject have been found to be amplified.

The unexpected clinical results underlying the invention, in which gene amplification proved to be a more effective indication of antibody-based tumor therapy than protein detection by immunohistochemistry, extends to tumor antigens in general. Thus, any anti-tumor-specific antigen based antibody therapy can have increased likelihood of success in patients who are found to have gene amplification of the gene encoding the tumor antigen.

A particular advantage of the invention is that it permits selection of patients for treatment who, based on immunohistochemical criteria, would be excluded. Thus, in a specific embodiment, the subject has been found to have an antigen level corresponding to a 0 or 1+ score for HER2 by immunohistochemistry on a formaldehyde-fixed tissue sample.

The invention further provides a pharmaceutical package comprising an ErbB antagonist for treating a cancer, and instructions to administer the ErbB antagonist to a subject if an erbB gene in tumor cells in a tissue sample from the subject is amplified. Preferably the ErbB antagonist is an anti-ErbB antibody, such as an anti-HER2 antibody. In a further aspect, the instructions also teach administering a cancer treating dose of a chemotherapeutic, e.g., a taxol. Such pharmaceutical packages, including the instructions for use, can be provided for any antibody-based therapeutic specific for a tumor-specific antigen.

DETAILED DESCRIPTION

The present invention advantageously peimits treatment of patients who have a greater likelihood of responding to the treatment by administering therapeutic agents, i.e., anti-tumor antigen therapeutic antibodies or ErbB receptor antagonists, to patients who are found to have an amplified gene encoding such a tumor antigen or ErbB receptor protein. The invention is based, in part, on the unexpected discovery that her2 gene amplification, e.g., as detected by fluorescence in situ hybridization (FISH), although it correlates with HER2 expression as detected by immunohistochemistry (NC), provides a more accurate basis for selecting patients for treatment because FISH status unexpectedly correlates better with response to treatment. This outcome was surprising in part because FISH status has about the same rate of correlation with a clinical trial assay (CTA) NC assay as another NC assay (HERCEPTEST®). Based on this observation, FISH would be expected to have a similar correlation with treatment response. This outcome also surprises because direct measurement of protein (by immunoassay) would be expected to provide a more accurate assessment of a cancer therapy targeted to the protein than an indirect measure of expression, like gene amplification.

Evaluation of patient groups and subgroups demonstrates the power of gene amplification analysis for selecting patients more likely to respond to treatment. NC provides a score for HER2 expression on tumor cells: 0 (no expression) through 3+ (very high levels of expression). Clinical selection criteria exclude patients with 0 and 1+ scores and select patients with 2+ and 3+ scores. The data show that 14% of combined 2+/3+ patients respond to HERCEPTIN®, while 20% of FISH+ (amplified her2 gene) patients respond to HERCEPTIN®. The 3+ subgroup has a 17% response rate, which is very close to the FISH+ subjects' response rate. However, the 2+ subgroup has less than half the response rate of FISH+ subjects. Thus, gene amplification clearly differentiates large subpopulations within the 2+ subgroup, permitting more effective treatment for those who are FISH+, and quickly identifying patients for whom alternative treatment modalities are appropriate and should commence immediately.

Gene amplification analysis also identifies patients who are unnecessarily excluded because of anomalies in the IHC analysis, particularly when the tests are performed on formalyn fixed, paraffin embedded samples (such sample processing can disrupt or destroy antibody epitopes on the HER2 protein, but has much less impact on gene amplification assays). As shown in the examples, a subset of 0 and 1+ subjects are FISH+. These patients are likely to respond to anti-HER2 antibody therapy, e.g., with HERCEPTIN®, although by IHC criteria they would be excluded from receiving this treatment.

Thus, the present invention advantageously permits inclusion of patients who are more likely to benefit from treatment but who, by standard IHC criteria, would be excluded from treatment. At the same time, the invention permits exclusion of patients who should promptly seek an alternative mode of treatment because the anti-tumor antigen therapy (i.e., ErbB antagonist or tumor antigen-specific therapeutic antibody) is not likely to succeed.

In short, the present invention is a powerful adjunct to INC assays for target protein expression level-based selection of patients. It can also be employed on its own, i.e., without IHC, to provide initial screening and selection of patients. The invention significantly improves screening and selection for subjects to receive a cancer-treating dose of an anti-tumor antigen therapeutic antibody treatment, ErbB receptor antagonist treatment, and other treatment targeted to overexpressed tumor antigens (or tumor-specific antigens), resulting in an increased likelihood of benefit from such treatments.

In another aspect, the invention concerns an article of manufacture or package, comprising a container, a composition within the container comprising an ErbB antagonist, e.g., an anti-ErbB antibody (or other anti-tumor-specific antigen antibody), optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB receptor, and a package insert containing instructions to administer the antagonist to patients who have been found to have an amplified erbB gene.

DEFINITIONS

As used herein, an "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3, and ErbB4 receptors, as

well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

ErbB receptors are examples of tumor-specific antigens or tumor antigens. The term "tumor antigen" is used herein to refer to a protein that is expressed at a higher level on tumor cells compared to normal cells. Generally, the normal cells for comparison are of the same tissue type, particularly phenotype, as the tumor, or from which the tumor arose. A "tumor specific antigen" refers to an antigen expressed either preferentially or only on tumor cells. Examples of tumor-specific antigens include, in addition to the ErbB receptors, MART1/Melan A, gp-100, and tyrosinase (in melanoma); MAGE-1 and MAGE-3 (in bladder, head and neck, and non-small cell carcinoma); HPV EG and E7 proteins (in cervical cancer); Mucin/MUC-1 (in breast, pancreas, colon, and prostate cancers); prostate specific antigen PSA (in prostate cancer); and caminoembryonic antigen/CEA (in colon, breast, and gastrointestinal cancers).

By "amplification" is meant the presence of one or more extra gene copies of erbB or other tumor antigen-encoding gene in a chromosome complement. Gene amplification can result in overexpression of protein, e.g., ErbB receptor protein. Gene amplification in cells from a tissue sample can be measured by many techniques, particularly Fluorescence in situ Hybridization (FISH), but also including and not limited to quantitative PCR, quantitative Southern hybridization, and the like.

By "tissue sample" is meant a collection of similar cells obtained from a tissue of a subject or patient, preferably containing nucleated cells with chromosomal material. The four main human tissues are (1) epithelium; (2) the connective tissues, including blood vessels, bone and cartilage; (3) muscle tissue; and (4) nerve tissue. The source of the tissue sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like. In one embodiment of the invention, the tissue sample is "non-hematologic tissue" (i.e., not blood or bone marrow tissue).

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, e.g., a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention, provided that it is understood that the present invention comprises a method whereby the same section of tissue sample may be analyzed at both morphological and molecular levels, or may be analyzed with respect to both protein and nucleic acid.

By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis with the performance and/or results of a second analysis. For example, one may use the results of a first analysis in carrying out the second analysis and/or one may use the results of a first analysis to determine whether a second analysis should be performed and/or one may compare the results of a first analysis with the results of a second analysis. In relation to IHC combined with FISH, one may use the results of INC to determine whether FISH should be performed and/or one may compare the level of protein expression with gene amplification to further characterize a tumor biopsy (e.g. to compare HER2 protein expression with her2 gene amplification). One advantageous feature of the invention is the ability to identify patients likely to benefit from treatment using FISH even if IHC indicates that they are antigen low.

By "nucleic acid" is meant to include any DNA or RNA, for example, chromosomal, mitochondrial, viral and/or bacterial nucleic acid present in tissue sample. The term "nucleic acid" encompasses either or both strands of a double stranded nucleic acid molecule and includes any fragment or portion of an intact nucleic acid molecule.

By "gene" is meant any nucleic acid sequence or portion thereof with a functional role in encoding or transcribing an RNA (rRNA, tRNA, or mRNA, the latter capable of translation as a protein) or regulating other gene expression. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The nucleic acid sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as Epidermal Growth Factor (EGF) (Savage et al., J. Biol. Chem. 247:7612-7621 (1972)); Tansforming Growth Factor alpha (TGF-alpha) (Marquardt et al., Science 223: 1079-1082 (1984)); amphiregulin, also known as schwanoma or keratinocyte autocrine growth factor (Shoyab et al. Science 243:1074-1076 (1989); Kimura et al. Nature 348:257-260 (1990); and Cook et al. Mol. Cell. Biol. 11:2547-2557 (1991)); betacellulin (Shing et al., Science 259:1604-1607 (1993); and Sasada et al. Biochem. Biophys. Res. Commun. 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., Science 251:936-939 (1991)); epiregulin (Toyoda et al., J. Biol. Chem. 270:7495-7500 (1995); and Komurasaki et al. Oncogene 15:2841-2848 (1997)), a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., Nature 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., Proc. Natl. Acad. Sci. 94:9562-9567 (1997)); or cripto (CR-1) (Kannan et al., J. Biol. Chem. 272 (6):3330-3335 (1997)). ErbB ligands that bind EGFR include EGF, TGF-alpha, amphiregulin, betacellulin, HB-EGF andepiregulin. ErbB ligands which bind HER3 include heregulins. ErbB ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide comprising an amino acid sequence encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641, 869 or Marchionni et al., Nature, 362:312-318 (1993), and biologically active variants of such polypeptides. Examples of heregulins include heregulin-alpha, heregulin-beta1, heregulin-beta2, and heregulin-beta3 (Holmes et al., Science, 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. Cell 69: 205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. Ce1172:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., Nature, 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. J. Biol. Chem. 270:14523-14532 (1995)); gamma-heregulin

(Schaefer et al. Oncogene 15:1385-1394 (1997)). An example of a biologically active fragment/amino acid sequence variant of a native sequence HRG polypeptide, is an EGF-like domain fragment (e.g., HRG-beta1, 177-244).

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., (J. Biol. Chem., 269(20):14661-14665 (1994)), for example. Examples of such ErbB hetero-oligomers include EGFR-HER2, HER2-HER3, and HER3-HER4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more HER2 receptors combined with a different ErbB receptor, such as HER3, HER4, or EGFR. Other proteins, such as a cytokine receptor subunit (e.g., gp130), may be included in the hetero-oligomer.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. (Ann. Rev. Biochem. 56:881-914 (1987)), including variants thereof (e.g., a deletion mutant EGFR as in Humphrey et al., (Proc. Natl. Acad. Sci. USA 87:4207-4211 (1990)). ErbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), Mab 455 (ATCC CRL HB 8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, PCT Publication No. WO 96/40210).

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to native sequence human HER2 protein described, for example, in Semba et al., (Proc. Natl. Acad. Sci. USA 82:6497-6501 (1985)) and Yamamoto et al. (Nature319:230-234 (1986)) (Genebank accession number X03363), and variants thereof. The term erbB2 refers to the gene encoding human HER2 and neu refers to the gene encoding rat p185neu. Preferred HER2 is native sequence human HER2. Examples of antibodies which bind HER2 include MAbs 4D5 (ATCC CRL 10463), 2C4 (ATCC HB-12697), 7F3 (ATCC HB-12216), and 7C2 (ATCC HB-12215) (see, U.S. Pat. No. 5,772,997; PCT Publication No. WO 98/17797; and U.S. Pat. No. 5,840,525, expressly incorporated herein by reference). Humanized anti-HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, and huMAb4D5-8 (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337, which is expressly incorporated herein by reference; and humanized 520C9 (PCT Publication No. WO 93/21319). Human anti-HER2 antibodies are described in U.S. Pat. No. 5,772,997 and PCT Publication No. WO 97/00271.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968, as well as Kraus et al. (Proc. Natl. Acad. Sci. USA) 86:9193-9197 (1989)), including variants thereof. Exemplary antibodies that bind HER3 are described in U.S. Pat. No. 5,968,511, e.g., the 8B8 antibody (ATCC BIB-12070) or a humanized variant thereof. The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in European Application No. EP 599,274; Plowman et al., (Proc. Natl. Acad. Sci. USA, 90:1746-1750 (1993)); and Plowman et al., (Nature, 366:473-475 (1993)), including variants thereof such as the HER4 isoforms disclosed in PCT Publication No. WO 99/19488.

An "ErbB antagonist" is any molecule that binds to an ErbB receptor and blocks ligand activation of the ErbB receptor. Such antagonists include, but are not limited to, modified ligands, ligand peptides (i.e., ligand fragments), soluble ErbB receptors, and, preferably, anti-ErbB antibodies.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the ErbB antagonist, e.g., anti-ErbB2 antibody, and more generally, any cancer in which administration of an antibody against an over-expressed antigen can treat the cancer. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount elicits antibody-mediated cytotoxicity, activates complement, has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time for disease progression (TTP), survival, tumor size, or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, melanoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small-cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, and various types of head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell surface, such that an anti-ErbB antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90}, and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide,

triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; antimetabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (Taxotere, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; caminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and anti-androgens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

ErbB Receptor Tyrosine Kinases

The ErbB receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes at least four distinct members including Epidermal Growth Factor Receptor (EGFR or ErbB1), HER2 (ErbB2 or p185neu), HER3 (ErbB3), and HER4 (ErbB4 or tyro2).

EGFR, encoded by the ErbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer, as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, Transforming Growth Factor alpha (TGF-alpha), by the same tumor cells resulting inreceptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn Pharmac. Ther. 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-alpha and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn., supra; Masui et al. Cancer Research44:1002-1007 (1984); and Wu et al. J. Clin. Invest. 95:1897-1905 (1995).

The second member of the ErbB family, p185neu, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., Science, 235:177-182 (1987); Slamon et al., Science, 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder.

Antibodies directed against the rat p185neu and human HER2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185neu (see, for example, Drebin et al., Cell 41:695-706 (1985); Myers et al., Meth. Enzym. 198:277-290 (1991); and WO94/22478). Drebin et al. (Oncogene 2:273-277 (1988)) report that mixtures of antibodies reactive with two distinct regions of p185neu result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice (see also U.S. Pat. No. 5,824,311).

Hudziak et al., (Mol. Cell. Biol. 9(3):1165-1172 (1989)) describe the generation of a panel of anti-HER2 antibodies, which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5, which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to

a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-alpha (see, also, U.S. Pat. No. 5,677,171). The anti-HER2 antibodies discussed in Hudziak et al. were further characterized (Fendly et al. Cancer Research 50:1550-1558 (1990); Kotts et al. In Vitro 26(3): 59A (1990); Sarup et al. Growth Regulation 1:72-82 (1991); Shepard et al. J. Clin. Immunol. 11(3):117-127 (1991); Kumar et al. Mol. Cell. Biol. 11(2):979-986 (1991); Lewis et al. CancerImmunol. Immunother. 37:255-263 (1993); Pietras et al. Oncogene 9:1829-1838 (1994); Vitetta et al. Cancer Research 54:5301-5309 (1994); Sliwkowski et al. J. Biol. Chem. 269(20):14661-14665 (1994); Scott et al. J. Biol. Chem. 266:14300-5 (1991); D'souza et al. Proc. Natl. Acad. Sci. 91:7202-7206 (1994); Lewis et al. Cancer Research 56:1457-1465 (1996); and Schaefer et al. Oncogene 15:1385-1394 (1997)).

A recombinant humanized IgG1 version of the murine anti-HER2 antibody 4D5 (rhuMAb HER2 or HERCEPTIN®; commercially available from Genentech, Inc., South San Francisco) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., J. Clin. Oncol. 14:737-744 (1996)). HERCEPTIN® received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. The current treatment protocol employs IHC to determine HER2 protein overexpression.

Other anti-HER2 antibodies with various properties have been described (Tagliabue et al., Int. J. Cancer 47:933-937 (1991); McKenzie et al., Oncogene 4:543-548 (1989); Maier et al., Cancer Res. 51:5361-5369 (1991); Bacus et al., Molecular Carcinogenesis 3:350-362 (1990); Stancovski et al., (Proc. Natl. Acad. Sci. USA) 88:8691-8695 (1991); Bacus et al., Cancer Research 52:2580-2589 (1992); Xu et al. Int. J. Cancer 53:401-408 (1993); PCT Publication No. WO94/00136; Kasprzyk et al., Cancer Research 52:2771-2776 (1992); Hancock et al., Cancer Res. 51:4575-4580 (1991); Shawver et al., Cancer Res. 54:1367-1373 (1994); Arteaga et al. Cancer Res. 54:3758-3765 (1994); Harwerth et al., J. Biol. Chem. 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; Klapper et al. Oncogene 14:2099-2109 (1997); and PCT Publication No. WO 98/17797).

Homology screening has resulted in the ErbB receptor family members: HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968; Kraus et al., Proc. Natl. Acad. Sci. USA 86:9193-9197 (1989)) and HER4 (European Patent Application No. EP 599 274; Plowman et al., Proc. Natl. Acad. Sci. USA, 90:1746-1750 (1993); and Plowman et al., Nature, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of ErbB ligands (Earp et al., Breast Cancer Research and Treatment 35: 115-132 (1995)). EGFR is bound by six different ligands: Epidermal Growth Factor (EGF), Transforming Growth Factor-alpha (TGF-alpha), amphiregulin, Heparin Binding Epidermal Growth Factor (HB-EGF), betacellulin, and epiregulin (Groenen et al. GrowthFactors 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta, and gamma heregulins (Holmes et al., Science, 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al., Oncogene 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motorneuron derived factor (SMDF) (for a review, see Groenen et al., Growth Factors 11:235-257 (1994); Lemke, G. Molec. & Cell. Neurosci. 7:247-262 (1996) and Lee et al. Pharm. Rev. 47:51-85 (1995)). Recently, two additional ErbB ligands were identified: neuregulin-2 (NRG-2), which is reported to bind either HER3 or HER4 (Chang et al., Nature: 387 509-512 (1997); and Carraway et al Nature 387:512-516 (1997)) and neuregulin-3, which binds HER4 (Zhang et al., (Proc. Natl. Acad. Sci. USA) 94(18): 9562-7 (1997)). HB-EGF, betacellulin, and epiregulin also bind to HER4.

While EGF and TGF-alpha do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase, (Earp et al., supra.) Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., J. Biol. Chem., 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., Journal of Neuroscience15: 1329-1340 (1995); Morrissey et al., Proc. Natl. Acad. Sci. USA 92:1431-1435 (1995); and Lewis et al., Cancer Res., 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, Cell 78:5-8 (1994)).

Detecting Gene Amplification

The present invention contemplates using any technique to detect gene amplification. (see, Boxer, J. Clin. Pathol. 53: 19-21 (2000)). These techniques include in situ hybridization (Stoler, Clin. Lab. Med. 12:215-36 (1990), using radioisotope or fluorophore-labeled probes; polymerase chain reaction (PCR); quantitative Southern blotting, and other techniques for quantitating individual genes. Preferably probes or primers selected for gene amplification evaluation are highly specific, to avoid detecting closely related homologous genes.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. A hapten or epitope that is immunospecifically bound by an antibody can also serve as a label.

The term "fluorescently labeled nucleic acid probe" refers to a probe comprising (1) a nucleic acid having a sequence rendering it capable of hybridizing with a target nucleic acid sequence and (2) a fluorescent label. Preferably such hybridization is specific, i.e., it can occur under high stringency conditions.

Sample Preparation

Any tissue sample from a subject may be used. Examples of tissue samples that may be used include, but are not limited to, breast, prostate, ovary, colon, lung, endometrium, stomach, salivary gland or pancreas. The tissue sample can be obtained by a variety of procedures including, but not limited to surgical excision, aspiration, or biopsy. The tissue may be

fresh or frozen. In one embodiment, the tissue sample is fixed and embedded in paraffin or the like.

The tissue sample may be fixed (i.e., preserved) by conventional methodology (See e.g., *Manual of Histological Staining Method of the Armed Forces Institute of Pathology, 3rd Edition* Lee G. Luna, H T (ASCP) Editor, The Blakston Division McGraw-Hill Book Company: New York; (1960); *The Armed Forces Institute of Pathology Advanced Laboratory Methods in Histology and Pathology* (1994) Ulreka V. Mikel, Editor, Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C.). One of skill in the art will appreciate that the choice of a fixative is determined by the purpose for which the tissue is to be histologically stained or otherwise analyzed. One of skill in the art will also appreciate that the length of fixation depends upon the size of the tissue sample and the fixative used. By way of example, neutral buffered formalin, Bouin's or paraformaldehyde, may be used to fix a tissue sample.

Generally, the tissue sample is first fixed and is then dehydrated through an ascending series of alcohols, infiltrated, and embedded with paraffin or other sectioning media so that the tissue sample may be sectioned. Alternatively, one may section the tissue and fix the sections obtained. By way of example, the tissue sample may be embedded and processed in paraffin by conventional methodology. Examples of paraffin that may be used include, but are not limited to, Paraplast, Broloid, and Tissuemay. Once the tissue sample is embedded, the sample may be sectioned by a microtome or the like. By way of example for this procedure, sections may range from about three microns to about five microns in thickness. Once sectioned, the sections may be attached to slides by several standard methods. Examples of slide adhesives include, but are not limited to, silane, gelatin, poly-L-lysine, and the like. For example, the paraffin embedded sections may be attached to positively charged slides, slides coated with poly-L-lysine.

If paraffin has been used as the embedding material, the tissue sections are generally deparaffinized and rehydrated to water. The tissue sections may be deparaffinized by several conventional standard methodologies. For example, xylenes and a gradually descending series of alcohols may be used. Alternatively, commercially available deparaffinizing non-organic agents such as Hemo-De7 (CMS, Houston, Tex.) may be used.

Fluorescence In Situ Hybridization (FISH)

In situ hybridization is generally carried out on cells or tissue sections fixed to slides. In situ hybridization may be performed by several conventional methodologies (see, e.g., Leitch et al., *In Situ Hybridization: A Practical Guide*, Oxford BIOS Scientific Publishers, Micropscopy Handbooks v. 27 (1994)). In one in situ procedure, fluorescent dyes (such as fluorescein isothiocyanate (FITC) which fluoresces green when excited by an Argon ion laser) are used to label a nucleic acid sequence probe that is complementary to a target nucleotide sequence in the cell. Each cell containing the target nucleotide sequence will bind the labeled probe producing a fluorescent signal upon exposure, of the cells to a light source of a wavelength appropriate for excitation of the specific fluorochrome used. A "target nucleotide sequence" is a sequence specific for a over-expressed tumor antigen, such as ErbB. FISH analysis can be used in conjunction with other assays, including without limitation morphological staining (of serial sections or the same section; see PCT Publication No. WO 00/20641, specifically incorporated herein by reference).

Various degrees of hybridization stringency can be employed. As the hybridization conditions become more stringent, a greater degree of complementarity is required between the probe and target to form and maintain a stable duplex. Stringency is increased by raising temperature, lowering salt concentration, or raising formamide concentration. Adding dextran sulfate or raising its concentration may also increase the effective concentration of labeled probe to increase the rate of hybridization and ultimate signal intensity. After hybridization, slides are washed in a solution generally containing reagents similar to those found in the hybridization solution with washing time varying from minutes to hours depending on required stringency. Longer or more stringent washes typically lower nonspecific background but run the risk of decreasing overall sensitivity.

Probes used in the FISH analysis may be either RNA or DNA oligonucleotides or polynucleotides and may contain not only naturally occurring nucleotides but their analogs like digoxygenin dCTP, biotin dcTP 7-azaguanosine, azidothymidine, inosine, or uridine. Other useful probes include peptide probes and analogues thereof, branched gene DNA, peptidometics, peptide nucleic acid (PNA), and/or antibodies.

Probes should have sufficient complementarity to the target nucleic acid sequence of interest so that stable and specific binding occurs between the target nucleic acid sequence and the probe. The degree of homology required for stable hybridization varies with the stringency of the hybridization medium and/or wash medium. Preferably, completely homologous probes are employed in the present invention, but persons of skill in the art will readily appreciate that probes exhibiting lesser but sufficient homology can be used in the present invention (see e.g., Sambrook, J., et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Press, (1989)).

One of skill in the art will appreciate that the choice of probe depends on the characteristics of the target gene of interest. Examples of amplification include, but are not limited to, her2/neu in breast and ovarian cancer, n-myc in neuroblastoma, c-myc in small cell lung cancer. By way of example for evaluating her2/neu amplification a probe spanning a 140 kb region on the long arm of chromosome 17 containing the her2/neu gene (17q 11.2-17q12) may be used. A probe for the -satellite sequences in the centromeric region of chromosome 17(D1721) may be used to evaluate for aneusomy of chromosome 17 as a source or cause for her2/neu amplification. For example, a cocktailed version of these probes may be obtained from Vysis, Inc. where each probe is directly labeled with easily distinguishable fluorophores, such as SPECTRUM ORANGE® and SPECTRUM GREEN®.

Probes may also be generated and chosen by several means including, but not limited to, mapping by in situ hybridization, somatic cell hybrid panels, or spot blots of sorted chromosomes; chromosomal linkage analysis; or cloned and isolated from sorted chromosome libraries from human cell lines or somatic cell hybrids with human chromosomes, radiation somatic cell hybrids, microdissection of a chromosome region, or from yeast artificial chromosomes (YACs) identified by PCR primers specific for a unique chromosome locus or other suitable means like an adjacent YAC clone. Probes may be genomic DNA, cDNA, or RNA cloned in a plasmid, phage, cosmid, YAC, Bacterial Artificial Chromosomes (BACs), viral vector, or any other suitable vector. Probes may be cloned or synthesized chemically by conventional methods. When cloned, the isolated probe nucleic acid fragments are typically inserted into a vector, such as lambda phage,

pBR322, M13, or vectors containing the SP6 or T7 promoter and cloned as a library in a bacterial host (see, e.g., Sambrook, supra).

Probes are preferably labeled with a fluorophor. Examples of fluorophores include, but are not limited to, rare earth chelates (europium chelates), Texas Red, rhodamine, fluorescein, dansyl, Lissamine, umbelliferone, phycocrytherin, phycocyanin, or commercially available fluorophors such SPECTRUM ORANGE® and SPECTRUM GREEN®, and/or derivatives of any one or more of the above. Multiple probes used in the assay may be labeled with more than one distinguishable fluorescent or pigment color. These color differences provide a means to identify the hybridization positions of specific probes. Moreover, probes that are not separated spatially can be identified by a different color light or pigment resulting from mixing two other colors (e.g., light red+green=yellow), pigment (e.g., blue+yellow=green), or by using a filter set that passes only one color at a time.

Probes can be labeled directly or indirectly with the fluorophor, utilizing conventional methodology. Additional probes and colors may be added to refine and extend this general procedure to include more genetic abnormalities or serve as internal controls. By way of example the her2/neu gene is in chromosome 17, and as an internal control a probe for satellite sequences specific for chromosome 17 (D17Z1) may be used (Vysis, Inc.) to prove diploidy in areas of non-malignant cells and/or to establish the presence or absence of chromosome 17 aneusomy in areas of her2/neu amplification.

After processing for FISH, the slides may be analyzed by standard techniques of fluorescence microscopy (see, e.g., Ploem and Tanke, Introduction to Fluorescence Microscopy, Oxford University Press: New York (1987)). Briefly, each slide is observed using a microscope equipped with appropriate excitation filters, dichromic, and barrier filters. Filters are chosen based on the excitation and emission spectra of the fluorochromes used. Photographs of the slides may be taken with the length of time of film exposure depending on the fluorescent label used, the signal intensity and the filter chosen. For FISH analysis the physical loci of the cells of interest determined in the morphological analysis are recalled and visually conformed as being the appropriate area for FISH quantification.

In order to correlate IHC with FISH, one may use a computer-driven, motorized stage which stores location of coordinates. This may be used to evaluate the same area by two different analytical techniques. For example, color images of the morphologically stained areas may be captured and saved using a computer-assisted cooled CCD camera. The same section may be subsequently taken through the FISH procedure, the stored locations recalled, and the designated areas scored for the presence of fluorescent nuclear signals. A similar procedure for IHC followed by FISH is contemplated.

Typically, hundreds of cells are scanned in a tissue sample and quantification of the specific target nucleic acid sequence is determined in the form of fluorescent spots, which are counted relative to the number of cells. Deviation of the number of spots in a cell from a norm (e.g., such as probing for the her2/neu gene in a normal cell will produce two copies, abnormal greater than two) is indicative of a greater likelihood of benefit from a tumor antigen-specific antibody therapy, e.g., an ErbB antagonist therapy. As exemplified infra, her2 gene amplification provides a much more effective indication of the likelihood that an anti-HER2 antibody therapy will be effective.

Pharmaceutical Formulations

Therapeutic formulations of the antagonists, e.g., antibodies, used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2, ErbB3, ErbB4, vascular endothelial factor (VEGF), or an antibody that binds to a different epitope on the target ErbB, in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, a chemotherapeutic, a cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.

The formulations to be used for in vivo administration are preferably, and in the case of humans, must be, sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over

100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Treatment with the Anti-ErbB Antagonists

It is contemplated that, according to the present invention, the anti-ErbB antibodies or other antagonists may be used to treat various conditions characterized by overexpression and/or activation of the ErbB receptor in patients who have been found to have an amplified erbB gene. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and any other active agents of the invention are administered to a human patient in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB antibody and a chemotherapeutic agent, e.g., a taxoid. The present invention contemplates administration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells (tumor resection) and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antagonist, e.g., antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Pharmaceutical Packages

Articles of Manufacture

In a related aspect of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, optionally labeled, and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials, such as glass or plastic. The container holds a composition that is effective for treating the condition and preferably has a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-tumor antigen therapeutic antibody or an ErbB antagonist, e.g., an anti-ErbB antibody. A label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. This second buffer can be used to reconstitute the active agent, if that is provided as a lyophilysate or dried powder, or to dilute a concentrated preparation of the active agent. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In addition, the article of manufacture comprises a package insert or inserts with instructions for use in patients who have been found to have erbB gene amplification, e.g., by FISH testing. Such patients may be subjects who, by IHC, would be excluded from treatment with the ErbB antagonist, e.g., patients who score a 0 or 1+ using an anti-HER2 antibody.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Examples.

Example 1

Concordance Between the Clinical Trials Assay (CTA) and Fluorescence In Situ Hybridization (FISH) in the HERCEPTIN® Pivotal Trials

Overexpression of HER2 at the 2+ or 3+ level by immunohistochemistry (IHC) was required for enrollment in the pivotal HERCEPTIN® metastatic breast cancer trials. The Clinical Testing Assay (CTA) involves two separate IHC assays performed with either monoclonal antibodies 4D5 (after protease digestion of the formalin fixed sample) or CB11 (after heat treatment of the formalin fixed sample). Subjects were eligible if either assay was scored at 2+ or 3+. If both were performed, the final score was the higher of the two results.

Concordance between the CTA and another IHC, HERCEPTEST® (HT), is 79%. This was the basis for FDA approval of HT to aid in the selection of patients for HERCEPTIN® therapy.

This Example describes a similar concordance study, utilizing clinical material submitted for screening for the HERCEPTIN® pivotal trials, that compares the CTA to her2/neu gene amplification measured by the PathVysion FISH assay. In the pivotal trials, 5998 subjects were screened for HER2 expression; 1915 (32%) were positive by the CTA and 4083 (68%) were negative. A random sample of 623 specimens (1:1 ratio of positive:negative) were selected for this analysis, 317 CTA+ and 306 CTA−. Specimens were not freshly cut from blocks. They had been stored between 2 and 4 years as 4-6μ sections on glass slides. Each section was assayed for her2/neu amplification using the protocol specified in the package insert of the PathVysion assay. Amplification was defined as a signal ratio of greater than or equal to 2. The results are shown in Table 1.

TABLE 1

FISH/CTA Concordance

		CTA				
		0	1+	2+	3+	
FISH	−	207	28	67	21	
	+	7	2	21	176	
		4%	7%	24%	89%	529

FISH+ = HER2:CEP17 signal ratio ≧2
Concordance = 82% (79-85%)

For the total 623 specimens tested, a FISH signal result was obtained in 529. Assay failure occurred in 19.9% of CTA− and 10.4% CTA+ samples. Amplification in the 0, 1+, 2, and 3+ groups was 4.2%, 6.7%, 23.9%, and 89.3%, respectively. The sample concordance was 81.3%, similar to the CTA/HT concordance of 79%. Single copy overexpression was 31%, predominantly in the 2+ group. Amplification was rarely (4.6%) noted in the CTA− group. The higher assay failure rate in the CTA− group may be due to non-assay related factors such as tissue fixation. These may have also resulted in false negative results for IHC.

These data were closely interpreted to suggest that her2/neu amplification status may have unexpectedly superior predictive value for identifying patients who are more likely to benefit from HERCEPTIN® treatment as compared to HERCEPTEST®. The observation that only 24% of 2+ patients are FISH+ suggest that this sub-group may have less predictable treatment outcomes when selected by IHC only. Identification of FISH+ patients in the 1+ and 0 sub-groups might identify subjects who, though failing the IHC criteria for HERCEPTIN® treatment, would likely benefit from HERCEPTIN® treatment. A direct analysis of HERCEPTIN® benefit based on FISH score compared to IHC score is presented in Example 2.

Example 2

FISH/Clinical Outcome Study

This example links the results from three HERCEPTIN® Trials with FISH status. In this study, 805 subjects were selected at random from all three trials. Of these, 167 lacked slides. Another 78 assays (9.7%) failed. Thus, formalin-fixed cut sections stored between 2.5 and 4.5 years from 540 subjects provided the sample pool for this study. There were no imbalances in demographics or prognostic indicators in these samples. Results are reported for different treatment groups.

Correlation of FISH status with response was evaluated for patients who received HERCEPTIN® as a second or third line therapy. These data are reported for 2+ and 3+ (by CTA) subjects in Table 2.

TABLE 2

FISH/Response with single agent HERCEPTIN ®, 2nd or 3rd line therapy, 2+/3+ Combined

	FISH+	FISH−
Response	21	0
No response	84	37
response rate	20%	0%
	(12.5-27.5%)	(0.7%)

N = 142

The 20% response rate of FISH+ subjects unexpectedly exceeds the 15% response rate of 2+ and 3+ patients in this study and 14% response rate observed in patients selected by CTA with a 2+ or 3+ immunohistochemistry score during the pivotal trials. Thus, while FISH correlates well with NC to about the same degree as another NC assay, the Hercep Test, as shown in Example 1, it unexpectedly is superior in identifying patients who are more likely to benefit from HERCEPTIN® therapy.

When these data were broken down into the components 3+ and 2+ subjects, the same 20% response rate of FISH+ subjects was seen (Tables 3 and 4).

TABLE 3

FISH/Response with single agent HERCEPTIN ®, 2nd or 3rd line therapy, 3+ subgroup

	FISH+	FISH−
Response	18	0
No response	72	17
response rate	20%	0%
	(12-28%)	(0-14%)

N = 107

TABLE 4

FISH/Response with single agent HERCEPTIN ®, 2nd or 3rd line therapy, 2+ subgroup

	FISH+	FISH−
Response	3	0
No response	12	20
response rate	20% (1-40%)	0% (0-14%)

N = 35

In the 3+ sub-group, the FISH+ response rate (20%) was very close but still exceeded the 17% response rate of 3+ subjects. The 2+ subgroup showed a much greater difference, with only a 9% response rate versus 20% by FISH+ selection. These data show that FISH+ status (her2 gene amplification) greatly increases the likelihood of response to HERCEPTIN®.

Data were also evaluated for patient responses to HERCEPTIN® as a first line therapy (Table 5).

TABLE 5

FISH/Response with single agent HERCEPTIN ® as 1st line therapy, 2+/3+ combined

	FISH+	FISH−
Response	17	1
No response	24	20
response rate	41% (26-56%)	20% (0-14%)

N = 62

The 41% response rate of FISH+ subjects was notably greater than the 27% response rate of 3+, 2+ subjects.

The surprising increase in likelihood of beneficial response based on FISH analysis extended to responses to chemotherapy plus HERCEPTIN®, as shown in Table 6. FISH+ subjects showed a much greater response to chemotherapy and HERCEPTIN® (54%) than FISH−(41%). Tables 7-9 contain more extensive data, broken down by different chemotherapeutic agents (adrinomycin and cyclophosphamide, AC; and Paditaxol, P) and different endpoints (response rate, time to progression, and survival) for HERCEPTIN® in combination with chemotherapy.

TABLE 6

FISH/Response rate to chemotherapy +/− HERCEPTIN ®, 1st line therapy; 2+/3+ combined

	C alone	C + H
FISH−	39% (26-52%)	41% (27-55%)
FISH+	27% (19-35%)	54% (45-63%)

N = 336

TABLE 7

Response rate of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	56*	42	41*	17	50*	32
3+	349	60*	42	49*	17	56*	31
FISH+	240	58*	40	49*	14	54*	27

*p < 0.05

TABLE 8

Time to progression (months) of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	7.8*	6.1	6.9*	2.7	7.4*	4.6
3+	349	8.1*	6.0	7.1*	3.0	7.8*	4.6
FISH+	240	7.8*	6.2	7.0*	3.2	7.3*	4.6

*p < 0.05

TABLE 9

Survival (months) of newly defined populations

		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	27	21	22	18	25*	20
3+	349	31*	21	25	18	29*	20
FISH+	240	29*	20	25*	14	27*	18

*p < 0.05

These data uniformly confirm that FISH+ analysis, though correlating closely to IHC, provides a much more accurate indicator of likelihood of success with HERCEPTIN® treatment. Across the board, FISH+ selection has about ⅓ (30%) greater response rate than 2+/3+ IHC-selection. When focused on 2+ patients, FISH status provides a much more effective tool for patient selection. FISH states also identifies patients who, because of 0 or 1+ status as determined by IHC, would otherwise be excluded from treatment.

These observations have broad implications for ErbB receptor antagonist-based cancer therapies and anti-tumor antigen cancer therapies in general. Thus erbB antagonists, e.g., anti-erbB receptor antibodies like HERCEPTIN®, can have an increased likelihood of efficacy when administered to patients who are positive for erbB gene amplification, e.g., by a FISH test. This is certainly the case, based on these data, with HERCEPTIN®.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

What is claimed:

1. A method for identifying and treating a patient disposed to respond favorably to a HER2 antibody, huMAb4D5-8, for treating breast cancer, which method comprises detecting her2 gene amplification in tumor cells in a tissue sample from the patient and treating the patient with her2 gene amplification with the HER2 antibody in an amount effective to treat the breast cancer, wherein the patient's tumor cells express HER2 at a 0 or 1+ level by immunohistochemistry.

2. The method of claim **1** wherein her2 gene amplification is detected by detecting fluorescence of a fluorescent-labeled nucleic acid probe hybridized to the gene.

3. The method of claim 1 wherein a formaldehyde-fixed sample of the patient's tumor cells has been subjected to an immunohistochemistry assay and found to express HER2 at a 0 or 1+ level.

4. The method of claim 3 wherein the immunohistochemistry assay is performed prior to detecting her2 gene amplification.

5. The method of claim 1 which further comprises administering a cancer treating dose of a chemotherapeutic drug.

6. The method of claim 5 wherein the chemotherapeutic drug is a taxoid.

* * * * *

EXHIBIT N

US006121428A

United States Patent [19]

Blank et al.

[11] **Patent Number: 6,121,428**

[45] **Date of Patent: Sep. 19, 2000**

[54] **PROTEIN RECOVERY**

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[73] Assignee: **Genentech, Inc.**, South San Francisco, Calif.

[21] Appl. No.: **09/097,309**

[22] Filed: **Jun. 12, 1998**

Related U.S. Application Data

[60] Provisional application No. 60/050,951, Jun. 13, 1997.

[51] **Int. Cl.**[7] **C07K 1/22**; C07K 1/34

[52] **U.S. Cl.** **530/413**; 435/269; 435/272; 530/344; 530/412; 530/414; 530/417

[58] **Field of Search** 435/68.1, 803, 435/269, 272; 530/387.1, 413, 412, 344, 390.5, 414, 417; 436/518, 824

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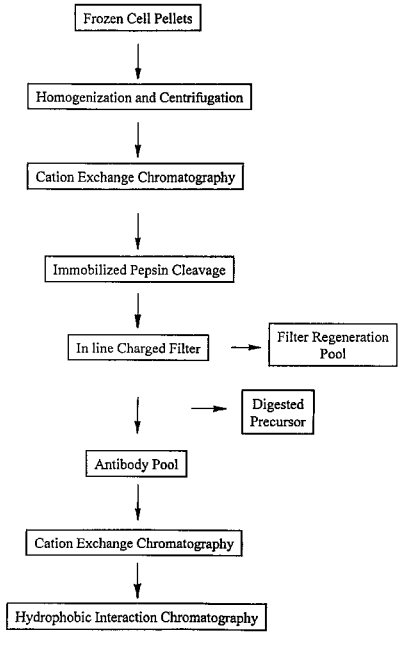
Primary Examiner—David Saunders

Attorney, Agent, or Firm—Lee K. Tan; Genentech, Inc.

[57] **ABSTRACT**

The invention herein provides a method for recovering a polypeptide comprising exposing a composition comprising a polypeptide to a reagent which binds to, or modifies, the polypeptide, wherein the reagent is immobilized on a solid phase; and then passing the composition through a filter bearing a charge which is opposite to the charge of the reagent in the composition, so as to remove leached reagent from the composition.

16 Claims, 8 Drawing Sheets



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EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAGINPKNG
GTSHNQRFMDRFTISVDKSTSTAYMQMNSLRAEDTAVYYCARWRGLNYGFDVRYFD
VWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPELLGGRMKQLEDKVEELLSKNYHLENEVARLKKLVGER

FIG. 1A

DIQMTQSPSSLSASVGDRVTITCRASQDINNYLNWYQQKPGKAPKLLIYYTSTLHSGVP
SRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 1B

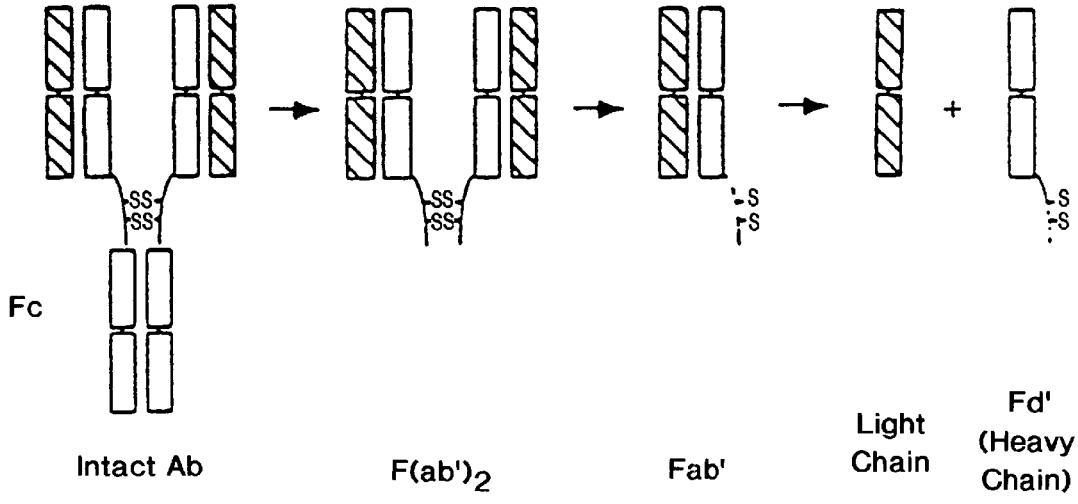
Fc
Intact Ab
F(ab')2
Fab'
Light Chain
Fd' (Heavy Chain)

FIG. 2A

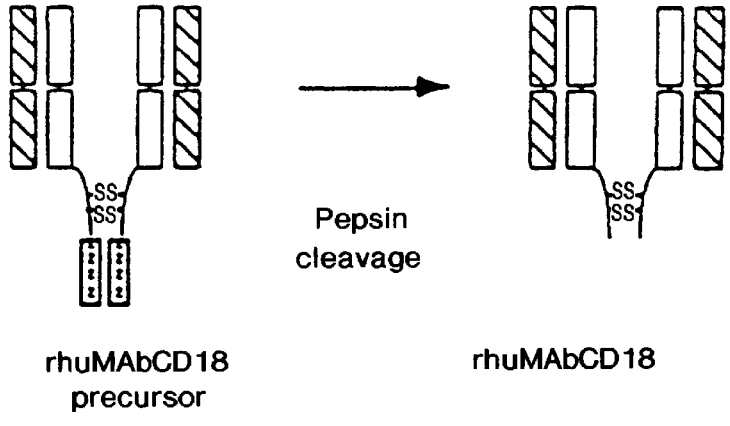
Pepsin cleavage
rhuMAbCD18 precursor
rhuMAbCD18

FIG. 2B

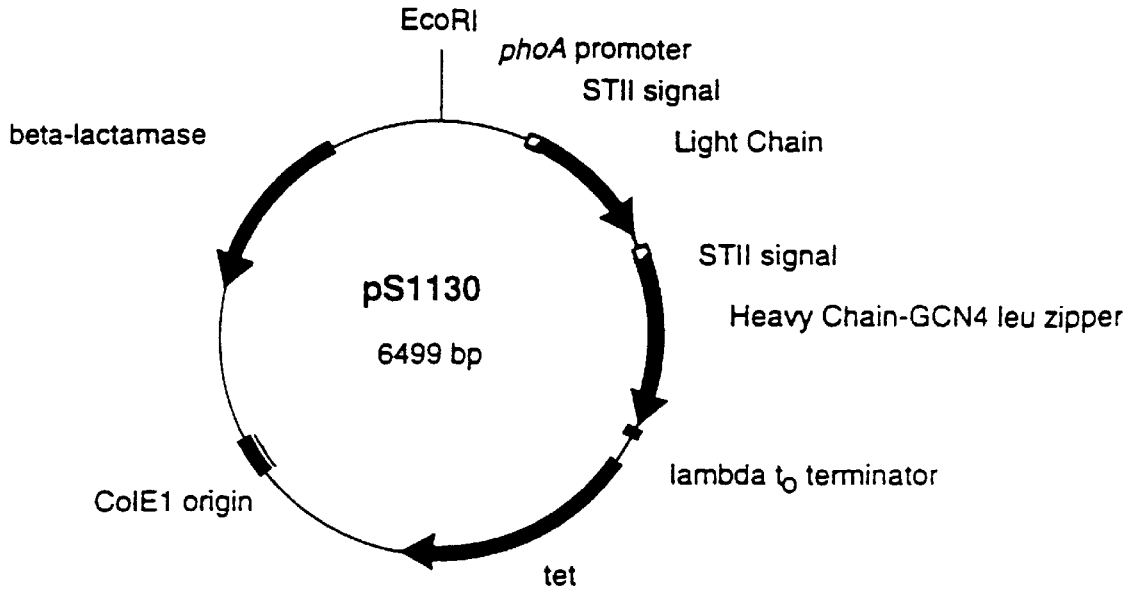
EcoRI
phoA promoter
STII signal
Light Chain
STII signal
Heavy Chain-GCN4 leu zipper
lambda t_o terminator
tet
ColE1 origin
beta-lactamase
pS1130
6499 bp

FIG. 3

```
   1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA

  61 GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC TTCGCAATAT GGCGCAAAAT

 121 GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC

 181 AGCATTCCTG ACGACGATAC GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT

 241 CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT

 301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGCCGGGG

 361 ATCCTCTAGA GGTTGAGGTG ATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT
 -23                           M   K   K   N   I   A   F   L   L

 413 GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAC GCT GAT ATC
 -14 A   S   M   F   V   F   S   I   A   T   N   A   Y   A   D   I

 461 CAG ATG ACC CAG TCC CCG AGC TCC CTG TCC GCC TCT GTG GGC GAT AGG
   3 Q   M   T   Q   S   P   S   S   L   S   A   S   V   G   D   R

 509 GTC ACC ATC ACC TGT CGT GCC AGT CAG GAC ATC AAC AAT TAT CTG AAC
  19 V   T   I   T   C   R   A   S   Q   D   I   N   N   Y   L   N

 557 TGG TAT CAA CAG AAA CCA GGA AAA GCT CCG AAA CTA CTG ATT TAC TAT
  35 W   Y   Q   Q   K   P   G   K   A   P   K   L   L   I   Y   Y

 605 ACC TCC ACC CTC CAC TCT GGA GTC CCT TCT CGC TTC TCT GGT TCT GGT
  51 T   S   T   L   H   S   G   V   P   S   R   F   S   G   S   G

 653 TCT GGG ACG GAT TAC ACT CTG ACC ATC AGC AGT CTG CAA CCG GAG GAC
  67 S   G   T   D   Y   T   L   T   I   S   S   L   Q   P   E   D

 701 TTC GCA ACT TAT TAC TGT CAG CAA GGT AAT ACT CTG CCG CCG ACG TTC
  83 F   A   T   Y   Y   C   Q   Q   G   N   T   L   P   P   T   F

 749 GGA CAG GGC ACG AAG GTG GAG ATC AAA CGA ACT GTG GCT GCA CCA TCT
  99 G   Q   G   T   K   V   E   I   K   R   T   V   A   A   P   S

 797 GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC
 115 V   F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A

 845 TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA
 131 S   V   V   C   L   L   N   N   F   Y   P   R   E   A   K   V

 893 CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT
 147 Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q   E   S

 941 GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC
 163 V   T   E   Q   D   S   K   D   S   T   Y   S   L   S   S   T

 989 CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC
 179 L   T   L   S   K   A   D   Y   E   K   H   K   V   Y   A   C

1037 GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC
 195 E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N

1085 AGG GGA GAG TGT TAA  G CTGATCCTCT ACGCCGGACG CATCGTGGCG
 211 R   G   E   C
```

FIG. 4A

```
1131 CTAGTACGCA AGTTCACGTA AAAACGGTAT CTAGAGGTTG AGGTGATTTT  ATG AAA
 -23                                                        M   K

1187 AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT
 -21 K   N   I   A   F   L   L   A   S   M   F   V   F   S   I   A

1235 ACA AAC GCG TAC GCT GAG GTT CAG CTG GTG GAG TCT GGC GGT GGC CTG
  -5 T   N   A   Y   A   E   V   Q   L   V   E   S   G   G   G   L

1283 GTG CAG CCA GGG GGC TCA CTC CGT TTG TCC TGT GCA ACT TCT GGC TAC
  12 V   Q   P   G   G   S   L   R   L   S   C   A   T   S   G   Y

1331 ACC TTT ACC GAA TAC ACT ATG CAC TGG ATG CGT CAG GCC CCG GGT AAG
  28 T   F   T   E   Y   T   M   H   W   M   R   Q   A   P   G   K

1379 GGC CTG GAA TGG GTT GCA GGG ATT AAT CCT AAA AAC GGT GGT ACC AGC
  44 G   L   E   W   V   A   G   I   N   P   K   N   G   G   T   S

1427 CAC AAC CAG AGG TTC ATG GAC CGT TTC ACT ATA AGC GTA GAT AAA TCC
  60 H   N   Q   R   F   M   D   R   F   T   I   S   V   D   K   S

1475 ACC AGT ACA GCC TAC ATG CAA ATG AAC AGC CTG CGT GCT GAG GAC ACT
  76 T   S   T   A   Y   M   Q   M   N   S   L   R   A   E   D   T

1523 GCC GTC TAT TAT TGT GCT AGA TGG CGA GGC CTG AAC TAC GGC TTT GAC
  92 A   V   Y   Y   C   A   R   W   R   G   L   N   Y   G   F   D

1571 GTC CGT TAT TTT GAC GTC TGG GGT CAA GGA ACC CTG GTC ACC GTC TCC
 108 V   R   Y   F   D   V   W   G   Q   G   T   L   V   T   V   S

1619 TCG GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC
 124 S   A   S   T   K   G   P   S   V   F   P   L   A   P   S   S

1667 AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC
 140 K   S   T   S   G   G   T   A   A   L   G   C   L   V   K   D

1715 TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC
 156 Y   F   P   E   P   V   T   V   S   W   N   S   G   A   L   T

1763 AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA CTC TAC
 172 S   G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y

1811 TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG
 188 S   L   S   S   V   V   T   V   P   S   S   S   L   G   T   Q

1859 ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTC GAC
 204 T   Y   I   C   N   V   N   H   K   P   S   N   T   K   V   D

1907 AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCG CCG
 220 K   K   V   E   P   K   S   C   D   K   T   H   T   C   P   P

1955 TGC CCA GCA CCA GAA CTG CTG GGC GGC CGC ATG AAA CAG CTA GAG GAC
 236 C   P   A   P   E   L   L   G   G   R   M   K   Q   L   E   D

2003 AAG GTC GAA GAG CTA CTC TCC AAG AAC TAC CAC CTA GAG AAT GAA GTG
 252 K   V   E   E   L   L   S   K   N   Y   H   L   E   N   E   V

2051 GCA AGA CTC AAA AAG CTT GTC GGG GAG CGC TAA  GCATGCG ACGGCCCTAG
 268 A   R   L   K   K   L   V   G   E   R

2101 AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA
```

FIG. 4B

Strain	Genotype
W3110	K-12 F⁻ lambda⁻ IN*rmD-rmE1*
↓	
1A2	W3110 Δ*fhuA*
↓	
7C1	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*)
↓	
16C9	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC*
↓	
23E3	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP*
↓	
33B6	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP ilvG*
↓	
49B2	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP ilvG* Δ*fucP*
↓	
49A5	W3110 ΔfhuA ΔphoA Δ(*argF-lac*) *deoC* ΔdegP *ilvG* ΔfucP ΔmalE

FIG. 5

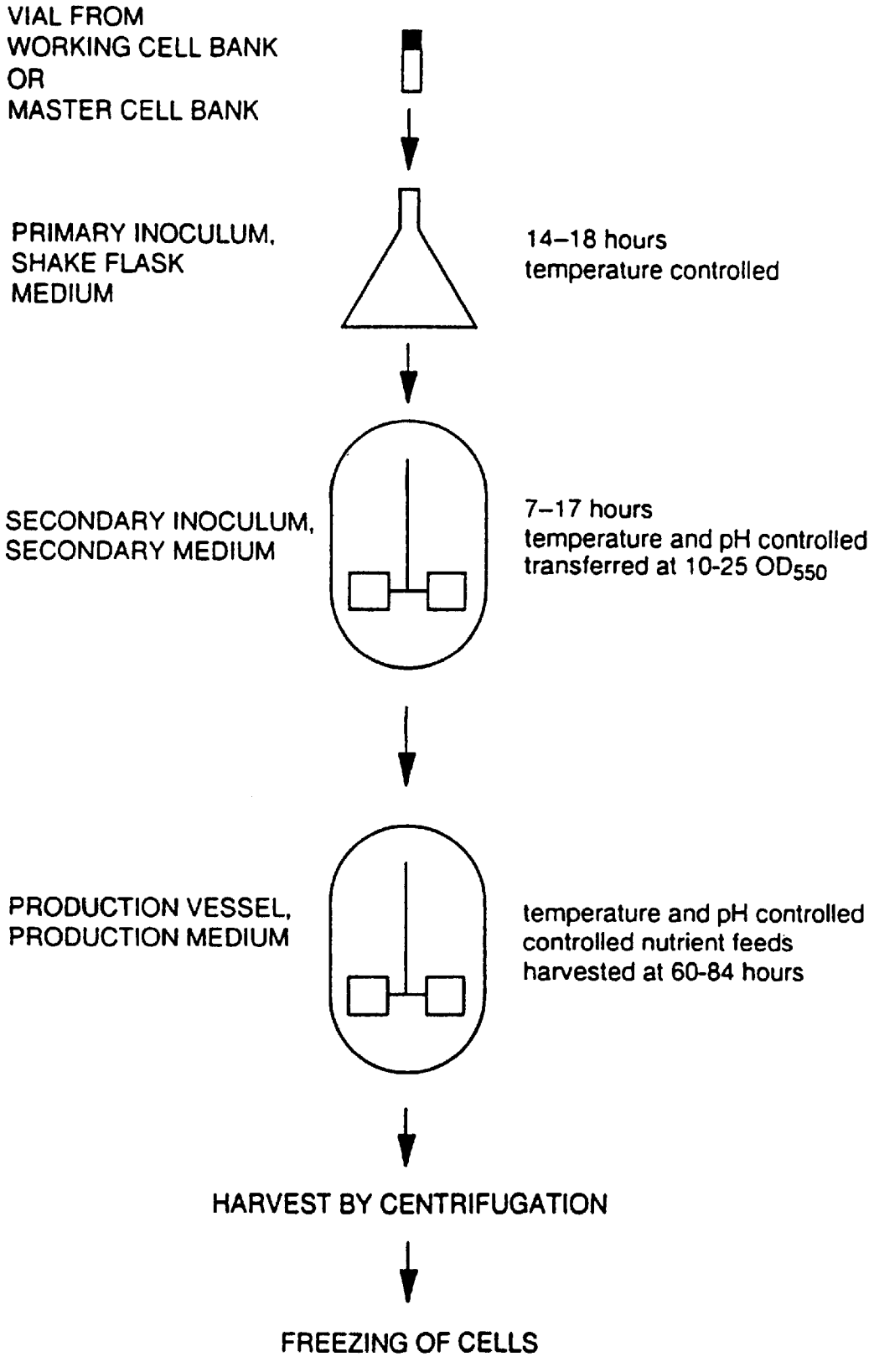
VIAL FROM
WORKING CELL BANK
OR
MASTER CELL BANK
PRIMARY INOCULUM,
SHAKE FLASK
MEDIUM
14–18 hours
temperature controlled
SECONDARY INOCULUM,
SECONDARY MEDIUM
7–17 hours
temperature and pH controlled
transferred at 10-25 OD550
PRODUCTION VESSEL,
PRODUCTION MEDIUM
temperature and pH controlled
controlled nutrient feeds
harvested at 60-84 hours
HARVEST BY CENTRIFUGATION
FREEZING OF CELLS

FIG. 6

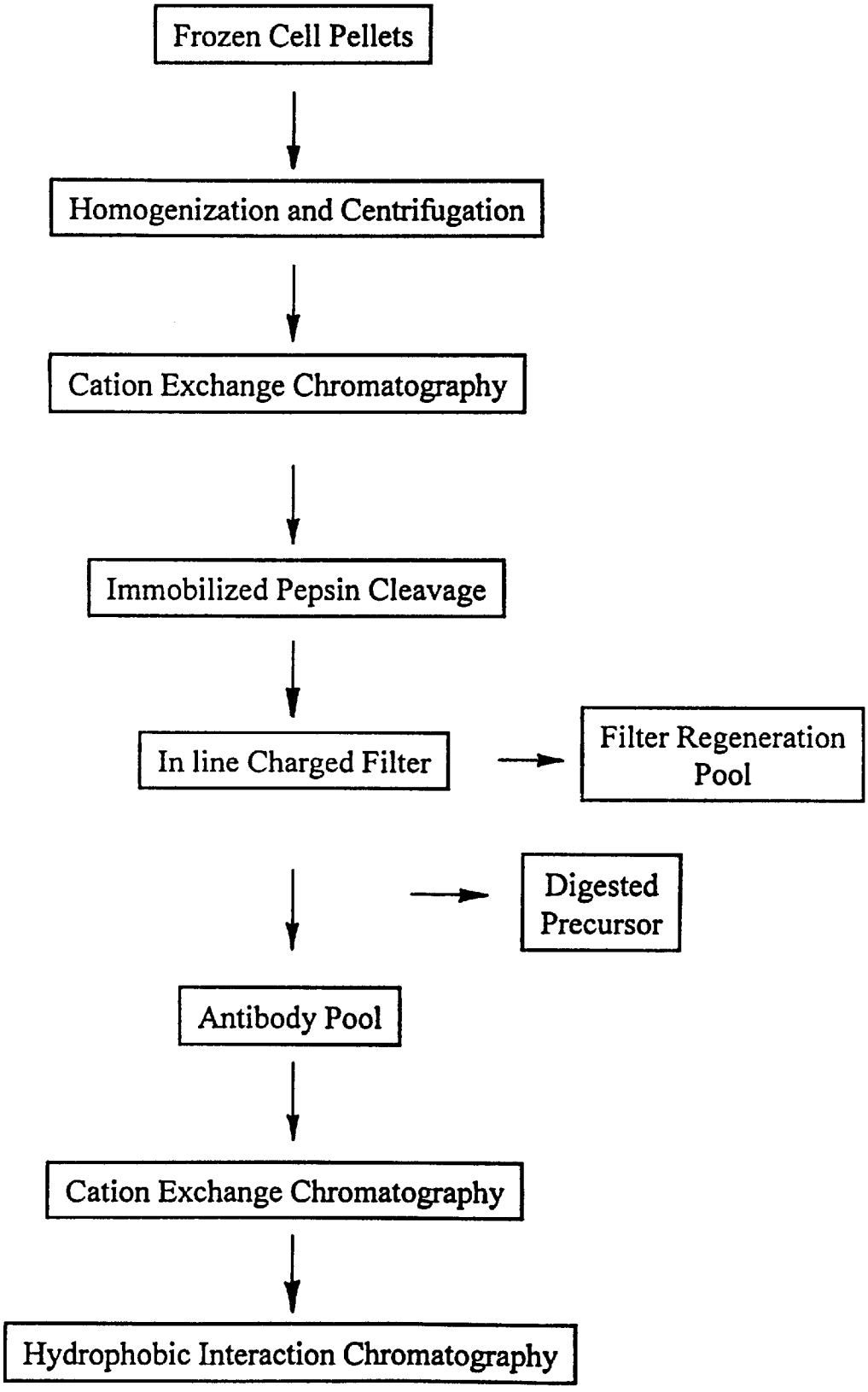
Frozen Cell Pellets
Homogenization and Centrifugation
Cation Exchange Chromatography
Immobilized Pepsin Cleavage
In line Charged Filter
Filter Regeneration Pool
Digested Precursor
Antibody Pool
Cation Exchange Chromatography
Hydrophobic Interaction Chromatography

FIG. 7

PROTEIN RECOVERY

RELATED APPLICATION

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60/050,951 filed Jun. 13, 1997, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to protein recovery. In particular, it pertains to recovery of a polypeptide, wherein the polypeptide is exposed to an immobilized reagent which binds to, or modifies, the polypeptide.

2. Description of Related Art

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through."

As part of the overall recovery process for the protein, the protein may be exposed to an immobilized reagent which

binds to or modifies the protein. For example, the protein may be subjected to affinity chromatography wherein an immobilized reagent which binds specifically to the protein, such as an antibody, captures the antibody and impurities pass through the affinity chromatography column. The protein can be subsequently eluted from the column by changing the conditions such that the protein no longer binds to the immobilized reagent. The immobilized reagent may also be an enzyme which modifies the protein. Sahni et al., *Anal. Biochem.* 193:178–185 (1991) and Voyksner et al., *Anal. Biochem.* 188:72–81 (1990) describe immobilized proteases.

Another type of purification process is filtration. Filtration of fine particle size contaminants from fluids has been accomplished by the use of various porous filter media through which a contaminated composition is passed such that the filter retains the contaminant. Retention of the contaminant may occur by mechanical straining or electrokinetic particle capture and adsorption. In mechanical straining, a particle is retained by physical entrapment when it attempts to pass through a pore smaller than itself. In the case of electrokinetic capture mechanisms, the particle collides with a surface within the porous filter and is retained on the surface by short range attractive forces. To achieve electrokinetic capture, charge modifying systems can be used to alter the surface charge characteristics of a filter (see, e.g., W090/11814). For example, where the contaminant to be removed is anionic, a cationic charge modifier can be used to alter the charge characteristics of the filter such that the contaminant is retained by the filter.

There is a need in the art for improved methods for recovering polypeptides, especially those polypeptides produced by recombinant techniques.

SUMMARY OF THE INVENTION

Accordingly, the invention provides a method for recovering a polypeptide comprising: (a) exposing a composition comprising a polypeptide to a reagent which binds to, or modifies, the polypeptide, wherein the reagent is immobilized on a solid phase; and then (b) passing the composition through a filter bearing a charge which is opposite to the charge of the reagent in the composition, so as to remove leached reagent from the composition. Preferably the charge characteristics of the polypeptide in the composition in step (b) are such that the polypeptide passes through the filter and preferably the filter is placed in line with the composition exposed to the reagent as in step (a). In one embodiment of the invention, the polypeptide to be treated in step (a) is a precursor polypeptide and the immobilized reagent is a protease (e.g. pepsin) which removes a precursor domain (e.g. a leucine zipper dimerization domain) from the polypeptide.

The invention also provides a method for recovering a polypeptide comprising removing a leached reagent from a composition comprising the polypeptide and the leached reagent by passing the composition through a filter bearing a charge opposite to that of the leached reagent, wherein the leached reagent was previously immobilized on a solid phase.

In yet a further embodiment, the invention provides a method for modifying a precursor antibody comprising a leucine zipper dimerization domain, comprising exposing the precursor antibody to a protease immobilized on a solid phase such that the protease removes the leucine zipper from the precursor antibody. This method optionally further comprises passing the antibody free of the leucine zipper through

a positively charged filter placed in line with antibody which has been exposed to the immobilized protease.

The anti-CD18 purification process is an example of a process in which an immobilized reagent is required to remove a leucine zipper dimerization domain from the anti-CD18 antibody precursor. The antibody precursor is initially purified using ABX cation exchange chromatography before the leucine zipper domain is removed by digestion with pepsin. The amount of pepsin necessary to completely remove the leucine zipper from the antibody precursor is considerable. A ratio of 1 mg of pepsin per 20 mg of antibody is necessary to carry out the digestion over a reasonable period of time. Treatment like this will leave a large amount of pepsin to be removed in the remaining steps of the anti-CD18 purification process (FIG. **7**). Quick removal of pepsin was found to be beneficial, since excessive exposure to pepsin resulted in overdigestion of the anti-CD18 antibody, with significant loses of intact product. In order to effectively control the amount of pepsin added to the anti-CD18 precursor antibody, and effectively eliminate any traces of pepsin that can persist through the purification process, two methods were implemented into the anti-CD18 antibody purification process. First, to considerably reduce the amount of pepsin added to the ABX purified antibody precursor pool, pepsin was immobilized on a solid phase (i.e. coupled to control pore glass beads (CPG) and packed into a column). The digestion reaction was then carried out by flowing the antibody precursor pool through the pepsin-CPG column. This procedure limited the amount of pepsin added into the antibody precursor pool. Nevertheless, a further problem was identified in that pepsin was found to leach from the solid phase. A small amount of pepsin leaching from the solid phase was found to be sufficient to cause overdigestion of the anti-CD18 antibody, resulting in a reduction in product yields. To overcome this problem of pepsin leaching from the solid phase, a positively charged filter was placed in line with the effluent from the pepsin-CPG column. The filter was found to remove all pepsin leaching from the solid phase, thereby preventing overdigestion of the antibody precursor. Pepsin is an acidic protein with a low pI. Therefore at pH 4, the pH of the digestion step, pepsin remained negatively charged and bound strongly to the positively charged filter. The use of a charged filter instead of a resin to remove leachables was found to be advantageous, since filters are compact and capable of very high flow rates with minimal backpressure. A filter can be implemented in line without the need to perform a separate recovery step, therefore reducing process complexity and time.

It is envisaged that negatively and positively charged filters can be used to solve problems associated with leaching of formerly immobilized reagents in other recovery processes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and **1**B depict the amino acid sequence of rhuMAb CD18 heavy chain (FIG. **1**A; SEQ ID NO:1) and light chain (FIG. **1**B; SEQ ID NO:2). The sequence in italics in FIG. **1**A (SEQ ID NO:3) is that of the leucine zipper.

FIGS. **2**A and **2**B depict intact antibody (Ab) and a variety of antibody fragments ($F(ab')_2$, Fab', light chain and Fd'). Heavy chains are depicted in white and light chains are hatched. The two disulfide bonds that form between two heavy chains are shown as -ss-. FIG. **2**B shows pepsin cleavage of the rhuMAb CD18 precursor to yield rhuMAb CD18, free of the leucine zipper.

FIG. **3** depicts the structure of plasmid pS1130 used to produce rhuMAb CD18 of the example below.

FIGS. **4**A and **4**B depict the full sequence of the pS1130 expression cassette (SEQ ID NO:5).

FIG. **5** shows derivation of the 49A5 production cell line.

FIG. **6** is a schematic of the fermentation process for rhuMAb CD18.

FIG. **7** is a flow diagram depicting the purification steps for rhuMAb CD18.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1–3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more

preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch et al., *Proc. Natl. Acad. Sci. USA* 80:1382–1386 (1983), version of the algorithm described by Needleman et al., *J. Mol. Biol.* 48:443–453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide are prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

In preferred embodiments of the invention, the polypeptide is a recombinant polypeptide. A "recombinant polypeptide" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the polypeptide, or produces the polypeptide as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in in vitro cell culture as well a cell within a host animal. Methods for recombinant production of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

A "precursor polypeptide" herein is a polypeptide to which is fused one or more precursor domains, e.g. where the precursor domain is part of a polypeptide chain of the polypeptide or is covalently attached to the polypeptide by a chemical linker, for example. The "precursor domain" may be an amino acid residue or polypeptide. For example, the precursor domain may be a dimerization domain such as a leucine zipper, an amino acid sequence such as polyglutamic acid which bears a negative charge and another amino acid sequence such as polylysine which bears a positive charge, or a peptide helix bundle comprising a helix, a turn and another helix; an epitope tag useful, e.g., in purification of the polypeptide of interest; an amino acid residue or peptide at the amino or carboxy terminus of the polypeptide which is desired to be removed to generate a homogenous polypeptide preparation; a N-terminal methionine, an artifact of production of the polypeptide in recombinant cell culture; a pre, pro or prepro domain of a mature polypeptide (e.g. the pro domain of prothrombin, wherein removal of the pro domain generates the biologically active mature thrombin molecule); a polylysine polypeptide; an enzyme such as glutathione transferase; or the Fc region of an intact antibody which is removed to generate an $F(ab')_2$.

An "epitope tag" polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the polypeptide to which it is fused. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable epitope tag polypeptides generally have at least 6 amino acid residues and usually between about 8–50 amino acid residues (preferably between about 914 30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field et al. *Mol. Cell. Biol.* 8:2159–2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., *Mol. Cell. Biol.* 5(12):3610–3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky et al., *Protein Engineering* 3(6):547–553 (1990)).

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

The antibody herein is directed against an "antigen" of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA4, ICAM-1, VCAM and $\alpha v/\beta 3$ integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from

antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552–554 (1990). Clackson et al, *Nature,* 352:624–628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al, *Nuc. Acids. Res.,* 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255–258 (1993); Bruggemann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24–34 (L1), 50–56 (L2) and 89–97 (L3) in the light chain variable domain and (H1), 50–65 (H2) and 95–102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Polypeptides of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26–32 (L1), 50–52 (L2) and 91–96 (L3) in the light chain variable domain and 26–32 (H1), 53–55 (H2) and 96–101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901–917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the H52 antibody of the example below are identified in Eigenbrot et al. *Polypeptides: Structure, Function and Genetics* 18:49–62 (1994).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

In a preferred embodiment of the invention, the antibody is an antibody fragment which is preferably human or humanized (see above discussion concerning humanized antibodies).

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et

al., *Journal of Biochemical and Biophysical Methods* 24:107–117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163–167 (1992)). In another embodiment as described in the Example below, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, N.Y., pp. 269–315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H–V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Holliger et al., *Proc. Natl. Acad. Sci. USA* 90:6444–6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Polypeptide Eng.* 8(10):1057–1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

"Multispecific antibodies" have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, -anti-CD3/MoV18, anti-neural cell adhesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, FcγRII or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-$p185^{HER2}$/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD3/, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al., *Nature,* 305:537–539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93108829, and in Traunecker et al., *EMBO J.,* 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule

provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO92/20372, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al, *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547–1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Holliger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444–6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et aL, *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. ImmunoL* 147: 60 (1991).

By "recovering a polypeptide" is meant obtaining a polypeptide preparation from a "pre-recovery preparation" by purifying the pre-recovery preparation (see below) or by modifying a precursor polypeptide to generate a form of the polypeptide which is free of the precursor domain.

By "purifying" a composition comprising an polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in an "essentially pure" composition, which is used herein to refer to a composition comprising at least about 90% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" herein refers to a composition comprising at least about 99% by weight of polypeptide of interest, based on total weight of the composition.

The "reagent" of interest herein is a compound or composition (preferably a polypeptide) which is able to bind to and/or modify a polypeptide of interest. A "leached" reagent is one which has come free from the solid phase. The reagent may, for example, bind to the polypeptide as is the case for "capture reagents" used in affinity purification methods. Examples of such "capture reagents" include protein A or protein G for capturing polypeptides such as antibodies and immunoadhesins; antibodies which can be used for affinity purification of polypeptides; a ligand binding domain of a receptor for capturing a ligand thereto; a receptor binding domain for capturing a receptor or a fragment thereof binding protein (e.g. IGFBPs such as IGFBP-3 and growth hormone binding proteins (GHBPs)); and immunoadhesins. Alternatively, or in addition, the reagent may modify the polypeptide of interest. For example, the reagent may chemically or physically alter the polypeptide. By "chemical alteration" is meant modification of the polypeptide by, e.g., bond formation or cleavage resulting in a new chemical entity. By "physical alteration" is meant changes in the higher order structure of the polypeptide. Enzymes are examples of reagents which can chemically and/or physically modify the polypeptide. The preferred enzyme is a protease (e.g. for removing one or more precursor domains from a precursor polypeptide). A "proteasel" is an enzyme which can hydrolyze a polypeptide. Examples of proteases include pepsin, cathepsin, trypsin, papain, elastase, carboxypeptidases, aminopeptidases, subtilisin, chymotrypsin, thermolysin, V_8 protease, prolinase and other endo- or exopeptidases.

By "solid phase" is meant a non-aqueous matrix to which a reagent can adhere. The solid phase may be a purification column, a discontinuous phase of discrete particles, a membrane or filter. Examples of materials for forming the solid phase include polysaccharides (such as agarose and

cellulose); and other mechanically stable matrices such as silica (e.g. controlled pore glass), poly(styrenedivinyl) benzene, polyacrylamide, ceramic particles and derivatives of any of the above. In preferred embodiments, the solid phase comprises controlled pore glass beads retained in a column. In certain embodiments, the solid phase is coated with a reagent (such as glycerol) which is intended to prevent nonspecific adherence of contaminants to the solid phase.

The reagent discussed above may be "immobilized" on or in the solid phase by forming a covalent bond between a functional group of the reagent and a reactive group on the surface of the solid phase. In other embodiments, the reagent is "immobilized" on the solid phase by adsorption and ionic binding or may be entrapped in the solid phase, e.g., within cells or lattice type polymers or microcapsules (See Holenberg and Roberts in *Enzymes as Drugs* John Wiley & Sons NY (1981), pages 396–411). The reagent should essentially retain its ability to bind to and/or modify the polypeptide of interest once immobilized to the solid phase. Reagent immobilization may be achieved by matrix activation. Briefly, this generally involves first activating the solid phase by a specific chemical reaction depending on the surface chemistry and then immobilizing the reagent by combining it with the activated solid phase. Activation of the solid phase can involve activation of hydroxyl groups (e.g. cyanogen bromide activation of the solid phase); carboxyl groups (e.g. using N-hydroxybenzotriazole in the presence of a water-soluble carbodiimide); acyl hydrazide (using, e.g., glutaraldehyde to generate aldehyde groups); amines (using, e.g., nitrous acid, phosgene and thiosphosgene, or cyanogen bromide); or acrylonitrile. In another embodiment, the reagent may be immobilized using a cross-linking agent (i.e. the reagent is immobilized indirectly to the solid phase) such as zero-length cross-linkers (e.g. carbodiimide, Woodward's reagent K, chloroformates and carbonyidiimidazole); homobifunctional cross-linkers (e.g. glutaraldehyde, chloroformates and carbonyidiimidazole, heterocyclic halides, divinylsulfone, quinones and transition metal ions); heterobifunctional cross-linkers including, for example, monohalogenacetyl halide, epichlorohydrin as well as amino and thiol group-directed reagents. In yet a further embodiment, the reagent is cross-linked to the solid phase through a carbohydrate chain. To achieve this, the sugar moieties may be first oxidized to aldheydes which form Schiff bases with either ethylenediamine or glycyltyrosine. Sodium borohydride may be used to stabilize the bonds. The derivatized glycoprotein is immobilized to the solid phase. For a review of immobilization techniques, see Wong, S. *Chemistry of Protein Conjugation and Cross-Linking* CRC Press Inc., Boston (1991).

A "leucine zipper" is a peptide (often about 20–40 amino acid residues long) having several repeating amino acids, in which every seventh amino acid is a leucine residue. Such leucine zipper sequences form amphipathic α-helices, with the leucine residues lined up on the hydrophobic side for dimer formation. Leucine zippers may have the general structural formula known as the heptad repeat (Leucine-X_1-X_2-X_3,-X_4-X_5-X_6;SEQ ID NO:4)$_n$, where X may be any of the conventional 20 amino acids, but is most likely to be amino acids with tight α-helix forming potential, for example, alanine, valine, aspartic acid, glutamic acid and lysine, and n may be three or greater, although typically n is 4 or 5. Examples of leucine zippers herein include the Fos-Jun leucine zipper (O'Shea et al. *Science* 245:646 (1989)) which may be used for forming heterodimers (e.g. bispecific antibodies); the GCN4 leucine zipper from yeast (Landschulz et al. *Science* 240:1759–1764 (1988)) which may be used for forming homodimers (e.g. monospecific antibodies, as in the example below); and leucine zippers found in other DNA-binding proteins, such as C/EBP and c-myc, as well as variants of any of these.

The term "filter" when used herein refers to a porous filter media through which an aqueous phase can pass but which retains one or more contaminants. The filter can be formed from a variety of materials such as cellulose fibers, including, e.g. cellulose acetate (SARTOBIND™ membrane adsorbers by Sartorius); silica based particulate; fibrous and particulate filter elements; nylon membranes or any combination of these. The filter of interest herein is a "charged filter" (i.e. positively or negatively charged) which means that it bears an overall net positive charge or an overall net negative charge. This may be achieved, for example, by attaching "charge modifying groups" to the filter. Anionic charge modifiers include water soluble polymers having anionic functional groups such as carboxyl, phosphorous, phosphonic, sulfonic groups (U.S. Pat. No. 4,604,208). Cationic charge modifiers include melamine formaldehyde cationic colloid (U.S. Pat. No. 4,007,113), inorganic cationic colloidal silica (U.S. Pat. No. 4,305,782), polyamido-polyamine epichlorohydrin cationic resin, polyamine epichlorohydrin. The filter is preferably one which allows high flow rates, without sacrificing binding capacity (as opposed to bead based columns, for example). Various configurations of the filter are contemplated, such as multilayer modules and spiral wound arrangements.

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. An "equilibration buffer" is that used to prepare a solid phase for loading the polypeptide of interest. The "loading buffer" is that which is used to load the composition comprising the polypeptide and contaminants onto the solid phase. Often, the equilibration and loading buffers are the same. The "elution buffer" is used to elute the polypeptide from the solid phase.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the "binding domain" of a heterologous "adhesin" polypeptide (e.g. a receptor, ligand or enzyme) with the effector functions of an immunoglobulin constant domain. Structurally, the immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (i.e. is "heterologous") and an immunoglobulin constant domain sequence. The immunoglobulin constant domain sequence in the immunoadhesin is preferably derived from γ1, γ2, or γ4 heavy chains since immunoadhesins comprising these regions can be purified by protein A chromatography (Lindmark et al., *J Immunol. Meth.* 62:1–13 (1983)).

The term "ligand binding domain" as used herein refers to any native cell-surface receptor or any. region or derivative thereof retaining at least a qualitative ligand binding of a corresponding native receptor. In a specific embodiment, the receptor is from a cell-surface polypeptide having an extracellular domain which is homologous to a member of the immunoglobulin supergenefamily. Other receptors, which are not members of the immunoglobulin supergenefamily but are nonetheless specifically covered by this definition, are receptors for cytokines, and in particular receptors with tyrosine kinase activity (receptor tyrosine kinases), members of the hematopoietin and nerve growth factor receptor superfamilies, and cell adhesion molecules, e.g. (E-, L- and P-) selectins.

The term "receptor binding domain" is used to designate any native ligand for a receptor, including cell adhesion molecules, or any region or derivative of such native ligand retaining at least a qualitative receptor binding ability of a corresponding native ligand. This definition, among others, specifically includes binding sequences from ligands for the above-mentioned receptors.

Modes for Carrying Out the Invention

The invention herein provides a method for modifying a polypeptide and/or purifying a polypeptide from a composition comprising the polypeptide and one or more contaminants. The composition is generally one resulting from the recombinant production of the polypeptide, but may be that resulting from production of the polypeptide by peptide synthesis (or other synthetic means) or the polypeptide may be purified from a native source of the polypeptide. Preferably the polypeptide is an antibody, e.g. one which binds the CD18 antigen.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., *E. coli*, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., *Salmonella typhimurium*, Serratia, e.g., *Serratia marcescans*, and Shigella, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as *P. aeruginosa*, and Streptomyces. One preferred *E. coli* cloning host is *E. coil* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coil* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; Kluyveromyces hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K bulgaricus* (ATCC 16,045), *K wickeramii* (ATCC 24,178), *K waltii* (ATCC 56,500), *K drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; yarrowia (EP 402,226); *Pichia pastoris* (EP 183,070); Candida; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; Schwanniomyces such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243–251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y Acad. Sci.* 383:44–68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The polypeptide is then subjected to one or more purification steps. Examples of purification procedures include fractionation on an ion-exchange column, hydrophobic interaction chromatography (e.g. on phenyl sepharose), ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin Sepharosem™, anion exchange chromatography, cation exchange chromatography (e.g. on a Bakerbond ABX column or SP-Sepharose HP column), chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (e.g. using protein A, protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

In one embodiment of the invention, the recovery step involves exposing a composition comprising the polypeptide (and optionally one or more contaminants) to a solid phase to which is immobilized a reagent which binds to, or modifies, the polypeptide. This step may be at the start or end or anywhere in a sequence of recovery steps for the polypeptide. In one embodiment, the solid phase is packed in a column and the immobilized reagent captures the polypeptide. In another embodiment, the reagent chemically and/or physically modifies the polypeptide and is immobilized on the solid phase which is, e.g., packed in a column, and the composition is passed through the column. For example, the polypeptide may comprise a precursor domain which the immobilized regent removes as part of the recovery process. In the example below, the precursor polypeptide was an antibody with a leucine zipper dimerization domain which was removed by immobilized pepsin in the recovery process. Following this step, the solid phase (e.g. chromatography column) may be regenerated using techniques applicable for regenerating such a solid phase.

It has been discovered herein that leaching of the immobilized reagent from the solid phase can occur and this can result in decreased yields and/or contamination of the polypeptide preparation following this step. In particular, in the example below, it was found that the pepsin could leach from a column to which it was immobilized and result in digestion of the antibody following removal of the leucine zipper, thereby reducing yields of functional antibody.

In order to obviate this problem, the invention provides a step following exposure of the composition to the immobilized reagent as discussed above. This involves passing the composition comprising the polypeptide and leached reagent (and optionally one or more further contaminants) through a filter bearing a charge which is opposite to the charge of the reagent at the pH of the composition, so as to remove leached reagent from the composition. The filter may be positively charged to remove contaminants that are negatively charged at the pH of the composition, such as acidic proteases, protein A, protein G or other reagents that can leach from affinity columns. Alternatively, the filter may be negatively charged to remove contaminants that are positively charged at the pH of the composition, such as basic proteases. Preferably, the charge characteristics of the polypeptide of interest in the composition passed through the filter are such that the polypeptide is not significantly retained by the filter and passes therethrough. The ability of the leached reagent to bind to the filter and the polypeptide to pass through it varies depending on the pH of the composition passing though the filter. To determine which filter to use (i.e. positively or negatively charged filter), one may investigate the pI of the leached reagent and, optionally, the pI of the polypeptide exposed to the immobilized reagent as discussed above. In one embodiment (e.g. as in the example below), the pH of the composition will be such that the leached reagent and polypeptide already have opposite net charges. In another embodiment, it may be beneficial to adjust the pH of the composition to be passed through the charged filter such that the leached reagent and polypeptide have opposite charges. Such alteration of the pH of the composition may serve to increase binding of oppositely charged contaminants to the filter and/or decrease binding of the polypeptide of interest to the filter. Other modifications of the composition to achieve the same effect are envisaged herein. Following any optional modifications of the composition, a filter may be selected which has a charge opposite to that of the leached reagent to be removed from the composition.

In a preferred embodiment of the invention, the filter is placed "in line" with the effluent treated as in the previous step (i.e. the effluent flows directly though the filter). This can be achieved by connecting the filter directly to the column effluent port, before the effluent is collected into a pool tank. The filter may be regenerated using techniques applicable to the type of filter used.

The polypeptide preparation may be subjected to additional purification, if necessary. Exemplary further purification steps have been discussed above. The polypeptide thus recovered may be formulated in a pharmaceutically acceptable carrier and is used for various diagnostic, therapeutic or other uses known for such molecules.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of ail citations in the specification are expressly incorporated herein by reference.

EXAMPLE

This example concerns an antibody (rhuMAb CD18) produced as a precursor polypeptide with a leucine zipper domain which is removed during the purification process of the instant invention. Recombinant humanized anti-CD18 antibody (rhuMAb CD18) having the amino acid sequence shown in FIG. **1**A (heavy chain; SEQ ID NO:1) and FIG. **1**B (light chain; SEQ ID NO:2) was created by humanization of the murine monoclonal antibody muMAb H52 (Hildreth et al. *J. Immunology* 134:3272–3280 (1985)).

Recombinant production of rhuMAb CD18: Plasmid pS1130 was constructed to direct production of the rhuMAb CD18 precursor molecule in *E. coli*. The precursor is cleaved during the purification process by the protease pepsin to yield rhuMAb CD18. rhuMAb CD18 is an $F(ab')_2$ molecule composed of 2 different peptides (light and heavy chains) linked by disulfide bonds. The Fc region of intact antibodies normally holds the 2 Fab arms together (FIG. **2**A), so when Fab' is produced in *E. coli* very little $F(ab')_2$ is formed. Fusion of a yeast GCN4 leucine zipper dimerization domain to the C-terminus of an Fab' substitutes for the Fc region and allows for efficient $F(ab')_2$ production in *E. coli*. The GCN4 leucine zipper domains interact to form

stable dimeric structures (parallel coiled coils) that hold the hinge region cysteine residues of two heavy chains together so that the two native interchain disulfide bonds can form. This results in formation of $F(ab')_2$ complexes that are covalently linked by disulfide bonds. The leucine zipper domains are later removed from the rhuMAb CD18 precursor during the purification process using the protease pepsin, which cleaves uniformly between the 2 leucine residues of the hinge. This results in the formation of the rhuMAb CD18 $F(ab')_2$ molecule (FIG. **2**B).

Plasmid pS1130 (FIG. **3**) is based on the well characterized plasmid pBR322 with a 2143 bp expression cassette (FIG. **4**) inserted into the EcoRI restriction site. Plasmid pS1130 is resistant to both tetracycline and β-lactam antibiotics. The expression cassette contains a single copy of each gene linked in tandem. Transcription of each gene into a single dicistronic mRNA is directed by the *E. coli* phoA promoter (Chang et al. *Gene* 44:121–125 (1986)) and ends at the phage lamda t_0 terminator (Scholtissek and Grosse *Nucleic Acids Research* 15:3185 (1987)). Translation initiation signals for each chain are provided by *E. coli* STII (heat stable enterotoxin) (Picken et al. *Infection and Immunity* 42:269–275 (1983)) Shine-Dalgarno sequences. Translation of each chain begins with a 23 residue STII signal peptide that directs translocation of the peptides across the cytoplasmic membrane into the periplasmic space (SEQ ID NOs: 6 and 7). The STII signal peptide is then removed by the *E. coli* leader peptidase. The light and heavy chains fold into their native conformations after secretion into the periplasm and associate into the rhuMAb CD18 precursor, a covalently linked $F(ab')_2$ (FIG. **2**B). The leucine zipper domain is cleaved from the precursor during the purification process (see below) to yield rhuMAb CD18 (FIG. **2**B). The cell line used in the production of rhuMAb CD18 is 49A5, derived from *E. coli* cell line W3110 (ATCC 27,325) as shown in FIG. **5**. The fermentation procedure takes place as shown in FIG. **6**. Production of rhuMAb CD18 precursor occurs when the medium becomes depleted in phosphate, typically 30–60 hours after inoculation.

Purification of rhuMAb CD18 precursor from the *E. coli* cell paste was as follows.

Homogenization and Centrifugation: Frozen cell pellets containing anti-CD18 precursor antibody, were dissolved in about 3 volumes of extraction buffer (120 mM MES, 5mM EDTA buffer, pH 6) heated to 30–40° C. This resulted in a suspension with a pH between about 5.4 and 6.5. This suspension was passed twice through a Gaulin homogenizer at 5500 to 6500 psi and kept below 20° C. with a heat exchanger. 5% polyethyleineimine (PEI) (w/v), pH 6 was added to the homogenate to a final concentration of 0.2% PEI. The mixture was incubated for about one hour at 2–8° C. About one volume of extraction buffer (120 mM MES, 5 mM EDTA, pH 6) was added before the solids were removed by centrifugation at 15,280 g. The clear supernatant was conditioned to a conductivity of less than 3 mohms by the addition of cold water.

Ion Exchange Chromatography: The conditioned supernatant was loaded onto a cation exchange column (ABX column; Mallinckrodt Baker, Inc., NJ, USA) equilibrated in 50 mM MES, pH 6.0. The column was washed with the equilibration buffer and the anti-CD18 precursor was eluted with a linear gradient from 50 mM MES, pH 6.0 to 50 mM MES, 100 mM sodium citrate, pH 6.0. The column was monitored by absorbance at 280 nm, and the eluate was collected in fractions. The appropriate fractions were pooled based on analytical cation exchange hydrophobic liquid chromatography (HPLC). After use, the cation exchange column was regenerated using 3.0 M guanidine HCl, 20 mM HEPES buffer, pH 7.4, followed by 1% acetic acid, 120 mM phosphoric acid. The column was stored in 1% acetic acid, 120 mM phosphoric acid.

Precursor digestion: Pepsin (Sigma, MO, USA) was chemically coupled to controlled pore glass (CPG) by Bioprocess Ltd., UK. The CPG was activated with $NaIO_4$ followed by reduction of schiff base formation between CPG and pepsin using $NaBH_3CN$.

The cation exchange anti-CD18 precursor antibody pool of the previous step was diluted with 50 mM MES, 36 mM sodium citrate, pH 4.0 to a concentration of approximately 2 g/L. The pool was then adjusted to pH 4 by addition of 2 M citric acid and flowed through a column containing immobilized pepsin (pepsin-CPG) previously equilibrated with 50 mM MES, 36 mM sodium citrate pH 4.0. This procedure removed the zippers from the hinge region while leaving intact $F(ab')_2$. After use, the pepsin column was regenerated with 0.12% aqueous HCl, pH 1.5 and stored in 100 mM sodium acetate, 150 mM sodium chloride, 0.01% Thimerosal, 50% glycerol, pH 4.5.

Anion exchange filtration: The effluent from the pepsin-CPG column was passed directly in line through an anion exchange Sartobind Q membrane (Sartorius, Goettingen, West Germany). The generated anti-CD18 $F(ab')_2$ antibody flows through the membrane while pepsin and other negatively charge impurities bind strongly to the membrane. The membrane was regenerated using 50 mM MES, 36 mM sodium citrate, 1 M sodium chloride, pH 4.0 and was stored in 0.1 N sodium hydroxide.

Analysis of the digestion reaction: Digestion of the anti-CD18 precursor antibody was analyzed by HPLC cation-exchange chromatography on a BAKERBOND™ carboxysulfon (CSX) 50×4.6 mm column (J. T. Baker Phillipsburg, N.J.) maintained at 55° C. The polypeptides were eluted using an increasing linear gradient from pH 6.0 to pH 8.0 at a flow rate of 4 ml/min using a detection wavelength of 280 nm. Buffer A contained 16 mM of each HEPES/PIPES/MES, pH 6.0 and Buffer B contained 16 mM of each HEPES/PIPES/MES, pH 8.0. For the separation of digested and undigested anti-CD18 precursor antibody, a linear gradient was run for 10 min from 40% B to 100% B.

Pepsin analysis: The amount of pepsin leached from the pepsin-CPG column was determined by reverse phase HPLC analysis and by pepsin ELISA analysis.

For HPLC analysis, a TosoHass TSK-Phenyl (7.5×75 mm) column was monitored with 90% solvent A (0.1% TFA in water) and 10% solvent B (0.1% TFA in acetonitrile). Upon 75 μg sample injection, a 30 minute gradient from 10% to 25% solvent B was initiated; the flow rate was 1 ml/min, and the temperature was maintained at 55° C. throughout.

For the ELISA, a sandwich ELISA was performed. Polyclonal goat anti-pepsin antibodies were used to coat a 96-well microtiter plate. Pepsin containing samples and standards were incubated in the coated wells. The sandwich was completed with biotinylated-goat-anti-pepsin. Prior to biotinylation, the second antibodies were affinity purified using CPG-pepsin. The immunological complexes were detected in the plates using streptavidin-alkaline phosphatase and p-nitrophenyl phosphate substrate. Absorbance at 405 nm was measured in a microtiter plate reader. Standards cover the range of 33.3 μg/ml down to 0.5 μg/ml in 2-fold dilutions. Dilutions were made for the samples (pure sample or diluted 1:2, 1:4, and 1:8). Samples were also spiked at the level of 10 μg/ml with pepsin and assayed as

samples. The detection limit of the assay was 1 μg/ml. A 4-parameter logistic curve fit to the data produced an acceptable standard curve.

Cation exchange chromatography: The pool was diluted to give a conductivity of approx. 7 mohms by the addition of water. The pool was applied to a cation exchange column (SP Sepharose High Performance; SPHP) equilibrated in 25 mM MES, 60 mM acetic acid, pH 4.0. The SP Sepharose column was washed with 25 mM MES, 75 mM sodium acetate pH 5.6 and eluted in a linear gradient of 75–110 mM sodium acetate in 25 mM MES pH 5.6. The column eluate was monitored at 280 nm and the eluate fractions were pooled based on analytical ion exchange HPLC. The SP Sepharose column was regenerated in 25 mM MES, 4 mM sodium acetate pH 5.6 followed by a wash with 0.5% sodium hydroxide. The column was stored in 0.1% NaOH.

Hydrophobic Interaction Chromatography (HIC): The pooled fraction from the SP sepharose column was diluted with the addition of 3.0M ammonium sulphate, 25 mM MES pH 6.0 at a ratio of 0.26 liters per liter of pool. This was then passed through a HIC column (phenyl sepharose FF—low substitution) previously equilibrated in 0.625 M ammonium sulphate, 25 mM MES pH 6.0. After loading, the column was washed with the same buffer used in the equilibration and the rhuMAb CD18 eluted in 0.375M ammonium sulphate, 25 mM MES pH 6.0. The eluate was monitored at 280 nm and the fractions are collected based on analytical reversed phase HPLC. The HIC column was regenerated in 25 mM MES, pH 6.0, followed by a wash in 0.5% NaOH. The column was stored in 0.1% NaOH.

Results

Two separate large scale purification runs were performed (see FIG. **7**). The purification process started with *E. coli* cell paste containing anti-CD18 precursor antibody, and completed with the anti-CD18 $F(ab')_2$ lacking the leucine zipper dimerization domain. During both purification runs, digestion of the antibody precursor molecule was performed by passing partially purified anti-CD18 precursor antibody through a pepsin-CPG column. Digestion was monitored by SDS PAGE and analytical cation exchange HPLC. The total amount of pepsin leached from the pepsin-CPG column was determined by measuring pepsin in the digested precursor antibody pool after the CPG-pepsin digestion and filtration step and in the anion exchange membrane regeneration pool. Regeneration of the membrane was performed by eluting pepsin and contaminants attached to the membrane using 50 mM MES, 36 mM sodium citrate, 1 M sodium chloride, buffer pH 4.0 (see FIG. **7**). The effective removal of pepsin throughout the purification steps was monitored by Western blots using purified goat anti-pepsin antibodies and quantitated using the ELISA method.

The results of the reverse phase HPLC analysis are shown in Table 1. In the first run, pepsin was detected in both the anion exchange membrane regeneration pool at a concentration of 40 μg/ml and in the digested precursor antibody pool after the CPG-pepsin and filtration step at a concentration of 48.3 μg/ml. By adding the total concentration of pepsin in both pools it was determined that 13.4 g of pepsin leached from the CPG-pepsin column during the digestion step in the first run. The data also revealed that the amount of filtration area used to remove leached pepsin was not enough at the flow rates and pH used in the first run. Nevertheless, the membrane was able to remove 21% of the total amount of pepsin leached from the pepsin-CPG column. Since the digested precursor antibody pool contained 10.6 g of leached pepsin that was not removed by the membrane, the purification yields from the pepsin-CPG digestion step and the SPHP step were low; 77 and 53%, respectively. Also, pepsin was detected in the SPHP pool by Western blot analysis.

TABLE 1

	Pepsin concentration
RUN #1	
Pepsin digested Ab pool	48.3 μg/ml
Pepsin digested Ab pool volume	220 L
Total amount of pepsin Ab pool	10.6 g
Membrane regeneration pool	40.4 μg/ml
Membrane regeneration volume	70 L
Total amount of pepsin	2.8 g
Membrane pool	
RUN #2	
Pepsin digested Ab pool	0
Pepsin digested Ab pool volume	630 L
Total amount of pepsin Ab pool	0
Membrane regeneration pool	230 μg/ml
Membrane regeneration volume	10 L
Total amount of pepsin	2.3 g
Membrane pool	

After the final purification step (Phenyl sepharose), pepsin was not detected by ELISA (Table 2) or by Western blot analysis. In the second run, the filtration area of the anion exchange membrane was doubled from 11,000 cm^2 to 22,000 cm^2. Pepsin was detected only in the anion exchange regeneration pool at a concentration of 230 μg/ml. Pepsin was not detected in the digested precursor antibody pool, after the CPG-pepsin digestion and filtration steps. The total amount of pepsin leached by the CPG-pepsin resin was 2.3 g. This value is 17% of the total amount of leached pepsin detected during the first run. Pepsin was not detected by reverse phase, pepsin ELISA or Western blots through the remaining purification steps of the second run. As a result of completely removing pepsin from the digested precursor pool, the purification yields from the pepsin-CPG digestion step and the SPHP were improved to 97 and 90%, respectively.

TABLE 2

Sample	Pepsin Values (mean of 2 reps.) [μg/ml]
Abx pool	<.5, <.5
Q pool run 1	7.4
Q pool run 2	<.5, <.5
SPHP Pool run 1	<.5, <.5
SPHP Pool run 2	<.5, <.5
HIC pool run 1	<.5, <.5
HIC pool run 2	<.5, <.5
Form. product run 1	<.5, <.5
Form. product run 2	<.5, <.5
Placebo formulation	<.5, <.5

The results of these experiments demonstrate that the use of a positively charged membrane in line immediately after the immobilized pepsin digestion step was advantageous. When pepsin was not completely removed by the membrane from the digested precursor antibody pool, decreased yields of functional antibody were obtained. Without being bound to any one theory, this was probably the result of overdigestion by the remaining pepsin in the pool. Furthermore when pepsin is not completely removed by the positively

charged membrane it was detected in the SPHP pool by Western blots. In the second run, leached pepsin was completely removed by the membrane. As a result the recovery yields for the pepsin digestion step and the SPHP cation exchange steps improved. Introduction of the anion exchange membrane improved the anti-CD18 purification process in two fundamental ways. First yields were improved by effectively removing pepsin from the CPG digestion pool, preventing further digestion. Second the overall efficiency and reproducibility of the process was improved by removing pepsin and other negatively charged contaminants early in the process.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 7

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
                20                  25                  30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
                50                  55                  60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                  70                  75

Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
                95                 100                 105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
               110                 115                 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
               125                 130                 135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
               140                 145                 150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
               155                 160                 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
               170                 175                 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
               185                 190                 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
               200                 205                 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
               215                 220                 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
               230                 235                 240

Leu
241
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(2) INFORMATION FOR SEQ ID NO:2:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 214 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Asn
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                  100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
           110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
           125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
           140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
           155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
           170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
           185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
           200                 205                 210

Arg Gly Glu Cys
       214
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Leu Gly Gly Arg Met Lys Gln Leu Glu Asp Lys Val Glu Glu Leu
 1               5                  10                  15

Leu Ser Lys Asn Tyr His Leu Glu Asn Glu Val Ala Arg Leu Lys
            20                  25                  30

Lys Leu Val Gly Glu Arg
            35  36
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Xaa Xaa Xaa Xaa Xaa Xaa
1 5 7

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2143 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC 50

TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC 100

TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG 150

GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC 200

GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT 250

AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT 300

TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG 350

CTCGCCGGGG ATCCTCTAGA GGTTGAGGTG ATTTTATGAA AAAGAATATC 400

GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGCGTA 450

CGCTGATATC CAGATGACCC AGTCCCCGAG CTCCCTGTCC GCCTCTGTGG 500

GCGATAGGGT CACCATCACC TGTCGTGCCA GTCAGGACAT CAACAATTAT 550

CTGAACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA 600

CTATACCTCC ACCCTCCACT CTGGAGTCCC TTCTCGCTTC TCTGGTTCTG 650

GTTCTGGGAC GGATTACACT CTGACCATCA GCAGTCTGCA ACCGGAGGAC 700

TTCGCAACTT ATTACTGTCA GCAAGGTAAT ACTCTGCCGC CGACGTTCGG 750

ACAGGGCACG AAGGTGGAGA TCAAACGAAC TGTGGCTGCA CCATCTGTCT 800

TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCCTCTGTT 850

GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA 900

GGTGGATAAC GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC 950

AGGACAGCAA GGACAGCACC TACAGCCTCA GCAGCACCCT GACGCTGAGC 1000

AAAGCAGACT ACGAGAAACA CAAAGTCTAC GCCTGCGAAG TCACCCATCA 1050

GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA GAGTGTTAAG 1100

CTGATCCTCT ACGCCGGACG CATCGTGGCG CTAGTACGCA AGTTCACGTA 1150

AAAACGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT 1200

TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC GCGTACGCTG 1250

AGGTTCAGCT GGTGGAGTCT GGCGGTGGCC TGGTGCAGCC AGGGGGCTCA 1300

CTCCGTTTGT CCTGTGCAAC TTCTGGCTAC ACCTTTACCG AATACACTAT 1350

GCACTGGATG CGTCAGGCCC CGGGTAAGGG CCTGGAATGG GTTGCAGGGA 1400

TTAATCCTAA AAACGGTGGT ACCAGCCACA ACCAGAGGTT CATGGACCGT 1450

TTCACTATAA GCGTAGATAA ATCCACCAGT ACAGCCTACA TGCAAATGAA 1500

CAGCCTGCGT GCTGAGGACA CTGCCGTCTA TTATTGTGCT AGATGGCGAG 1550

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GCCTGAACTA CGGCTTTGAC GTCCGTTATT TTGACGTCTG GGGTCAAGGA            1600

ACCCTGGTCA CCGTCTCCTC GGCCTCCACC AAGGGCCCAT CGGTCTTCCC            1650

CCTGGCACCC TCCTCCAAGA GCACCTCTGG GGGCACAGCG GCCCTGGGCT            1700

GCCTGGTCAA GGACTACTTC CCCGAACCGG TGACGGTGTC GTGGAACTCA            1750

GGCGCCCTGA CCAGCGGCGT GCACACCTTC CCGGCTGTCC TACAGTCCTC            1800

AGGACTCTAC TCCCTCAGCA GCGTGGTGAC CGTGCCCTCC AGCAGCTTGG            1850

GCACCCAGAC CTACATCTGC AACGTGAATC ACAAGCCCAG CAACACCAAG            1900

GTCGACAAGA AAGTTGAGCC CAAATCTTGT GACAAAACTC ACACATGCCC            1950

GCCGTGCCCA GCACCAGAAC TGCTGGGCGG CCGCATGAAA CAGCTAGAGG            2000

ACAAGGTCGA AGAGCTACTC TCCAAGAACT ACCACCTAGA GAATGAAGTG            2050

GCAAGACTCA AAAAGCTTGT CGGGGAGCGC TAAGCATGCG ACGGCCCTAG            2100

AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA                   2143
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Met Thr Gln Ser
             -5                   1                   5

Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr
          10                  15                  20

Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln
          25                  30                  35

Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser
          40                  45                  50

Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
          55                  60                  65

Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp
          70                  75                  80

Phe Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr
          85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
         100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
         115                 120                 125

Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg
         130                 135                 140

Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
         145                 150                 155

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
         160                 165                 170

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
         175                 180                 185

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
         190                 195                 200
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-continued

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Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
        205                 210             214


(2) INFORMATION FOR SEQ ID NO:7:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 300 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Glu Val Gln Leu Val Glu Ser
             -5                   1               5

Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys
         10                  15                  20

Ala Thr Ser Gly Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met
         25                  30                  35

Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn
         40                  45                  50

Pro Lys Asn Gly Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg
         55                  60                  65

Phe Thr Ile Ser Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln
         70                  75                  80

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala
         85                  90                  95

Arg Trp Arg Gly Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp
        100                 105                 110

Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
        115                 120                 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr
        130                 135                 140

Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe
        145                 150                 155

Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        160                 165                 170

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr
        175                 180                 185

Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr
        190                 195                 200

Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys
        205                 210                 215

Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
        220                 225                 230

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Arg Met Lys
        235                 240                 245

Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr His
        250                 255                 260

Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu Arg
        265                 270                 275     277
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We claim:

1. A method for recovering a polypeptide comprising:

(a) exposing a composition comprising a polypeptide to a reagent which binds to, or modifies, the polypeptide, wherein the reagent is immobilized on a solid phase; and then

(b) passing an effluent comprising the polypeptide eluted from or modified by the immobilized reagent, and any reagent leached from the solid phase, through a filter beating a charge which is opposite to the charge of the reagent in and at the pH of, the composition, so as to remove leached reagent from the effluent.

2. The method of claim 1 wherein the charge characteristics of the polypeptide in the composition in step (b) are such that the polypeptide passes through the filter.

3. The method of claim 1 wherein the filter is positively charged.

4. The method of claim 1 wherein the filter is negatively charged.

5. The method of claim 1 wherein the effluent is passed directly in line through the filter.

6. The method of claim 1 wherein the immobilized reagent is a protease.

7. The method of claim 6 wherein the protease is pepsin.

8. The method of claim 6 wherein the polypeptide exposed to the protease in step (a) is a precursor polypeptide and the protease removes a precursor domain from the polypeptide.

9. The method of claim 8 wherein the precursor domain comprises a leucine zipper.

10. The method of claim 9 wherein the polypeptide is an antibody.

11. The method of claim 10 wherein the antibody is a $F(ab')_2$ fragment.

12. The method of claim 10 wherein the antibody binds CD18.

13. The method of claim 9 wherein the leucine zipper is a yeast GCN4 leucine zipper.

14. The method of claim 9, wherein the precursor polypeptide is an anti-CD18 antibody having the amino acid sequence of SEQ ID NO. 1 for the heavy chain, and SEQ ID NO 2 for the light chain.

15. The method of claim 1, wherein the solid phase comprises controlled pore glass beads.

16. A method for recovering a polypeptide comprising removing a leached reagent from a composition comprising the polypeptide and the leached reagent by passing the composition through a filter bearing a charge opposite to that of the leached reagent at the pH of the composition, wherein the leached reagent was previously immobilized on a solid phase.

* * * * *

EXHIBIT O

US006620918B2

(12) **United States Patent**
Ansaldi et al.

(10) **Patent No.: US 6,620,918 B2**
(45) **Date of Patent: Sep. 16, 2003**

(54) **SEPARATION OF POLYPEPTIDE MONOMERS**

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(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/320,100**

(22) Filed: **May 26, 1999**

(65) **Prior Publication Data**

US 2002/0010319 A1 Jan. 24, 2002

Related U.S. Application Data

(60) Provisional application No. 60/087,602, filed on Jun. 1, 1998.

(51) **Int. Cl.**[7] **C07K 1/18**; C07K 1/20; C07K 1/22; C07K 1/14

(52) **U.S. Cl.** **530/416**; 530/412; 530/413; 530/417; 530/418

(58) **Field of Search** 530/412, 413, 530/416, 417, 418

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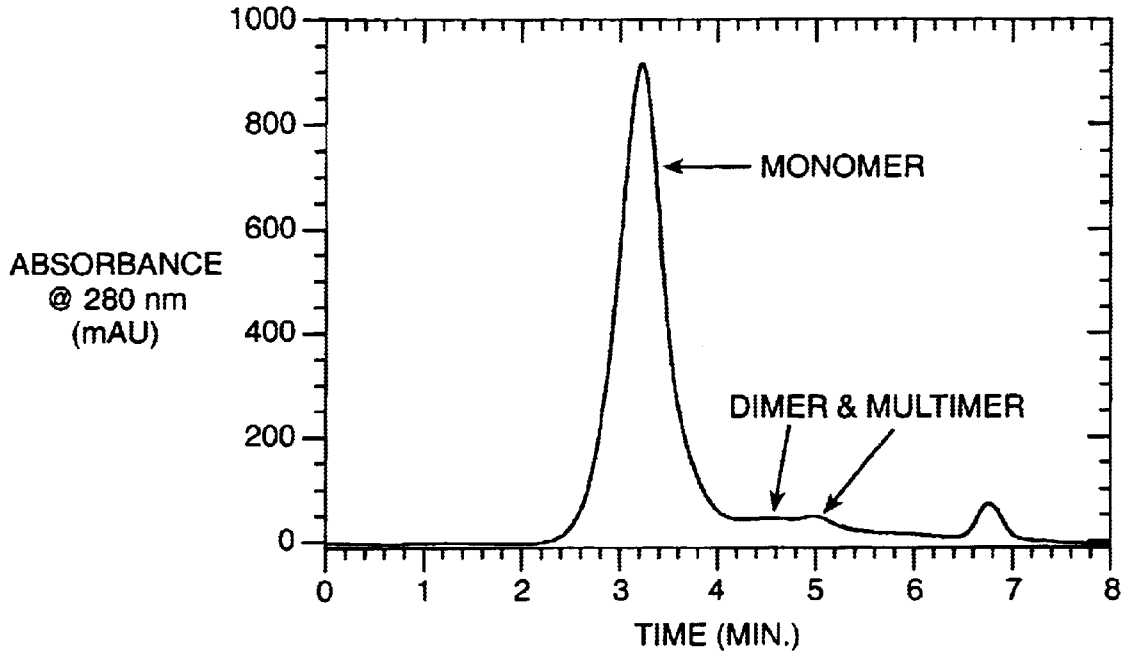
Primary Examiner—Anthony C. Caputa
Assistant Examiner—Anne L. Holleran
(74) *Attorney, Agent, or Firm*—Janet E. Hasak

(57) **ABSTRACT**

A method is disclosed for separating a polypeptide monomer from a mixture comprising dimers and/or multimers. The method comprises applying the mixture to either a cation-exchange chromatography resin or an anion-exchange chromatography resin and eluting the mixture at a gradient of about 0–1 M of an elution salt, wherein the monomer is separated from the dimers and/or multimers present in the mixture.

15 Claims, 8 Drawing Sheets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800
600
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200
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ABSORBANCE
@ 280 nm
(mAU)
MONOMER
DIMER & MULTIMER
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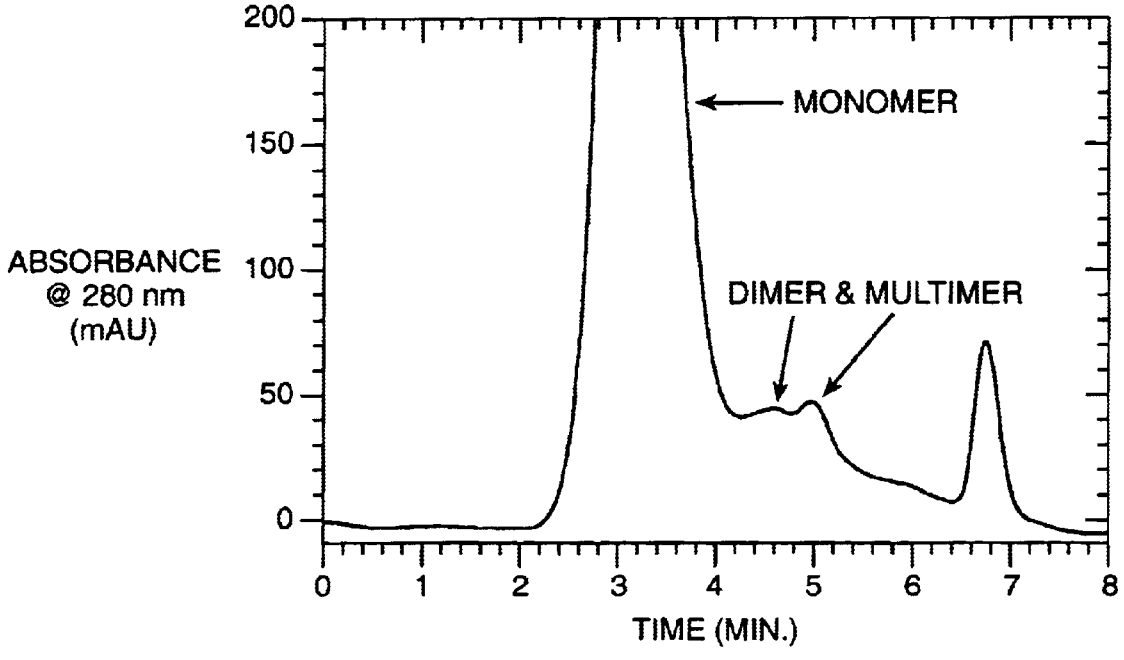
1
2
3
4
5
6
7
8
TIME (MIN.)

FIG._1A


200
150
100
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0
ABSORBANCE
@ 280 nm
(mAU)
MONOMER
DIMER & MULTIMER
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1
2
3
4
5
6
7
8
TIME (MIN.)

FIG._1B

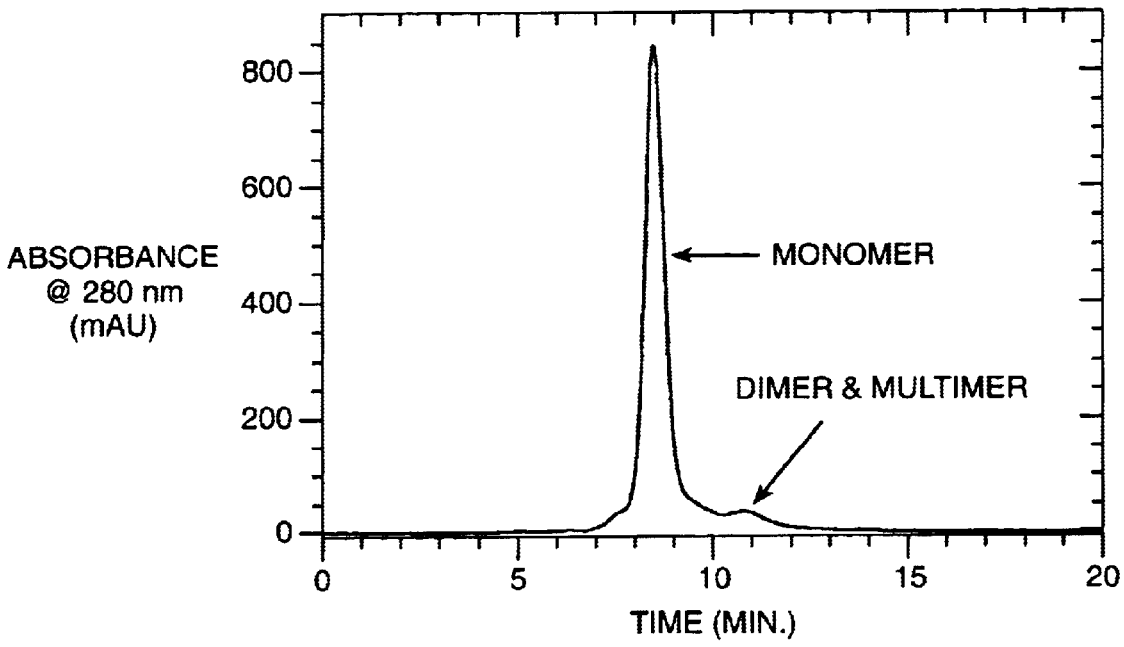


FIG._2A-1

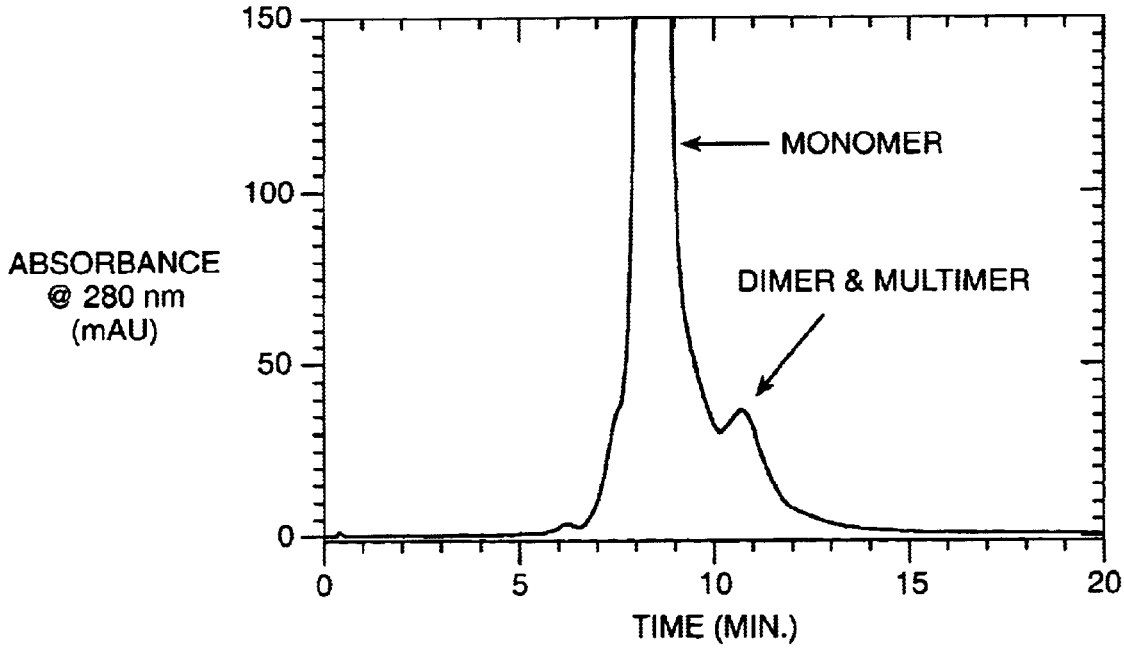


FIG._2A-2

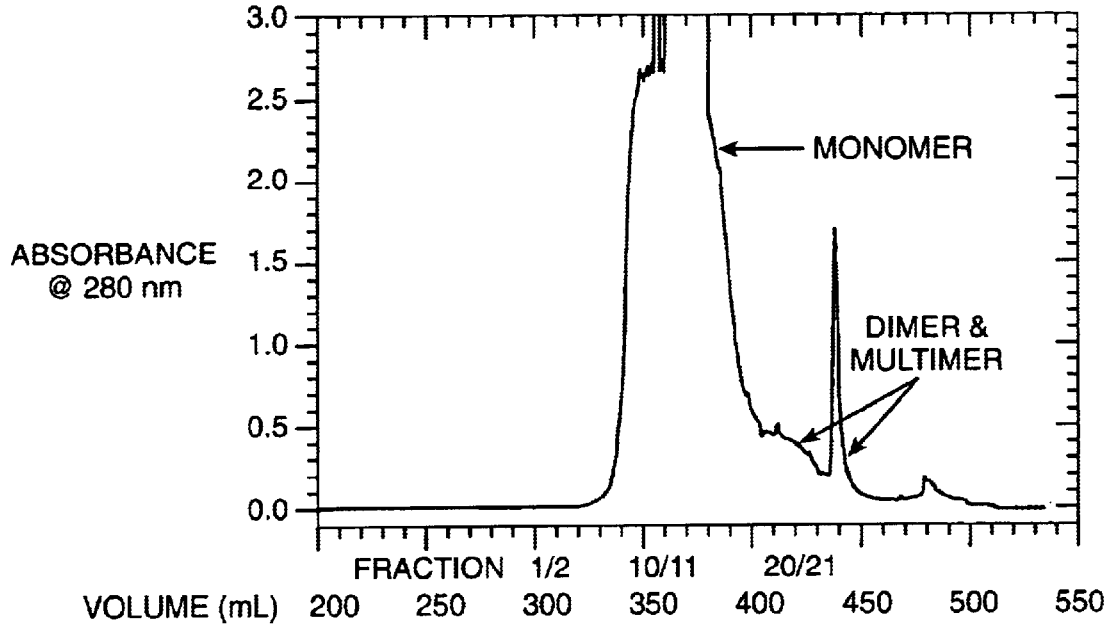

3.0
2.5
2.0
1.5
1.0
0.5
0.0
ABSORBANCE
@ 280 nm
MONOMER
DIMER &
MULTIMER
FRACTION 1/2 10/11 20/21
VOLUME (mL) 200 250 300 350 400 450 500 550

FIG._2B

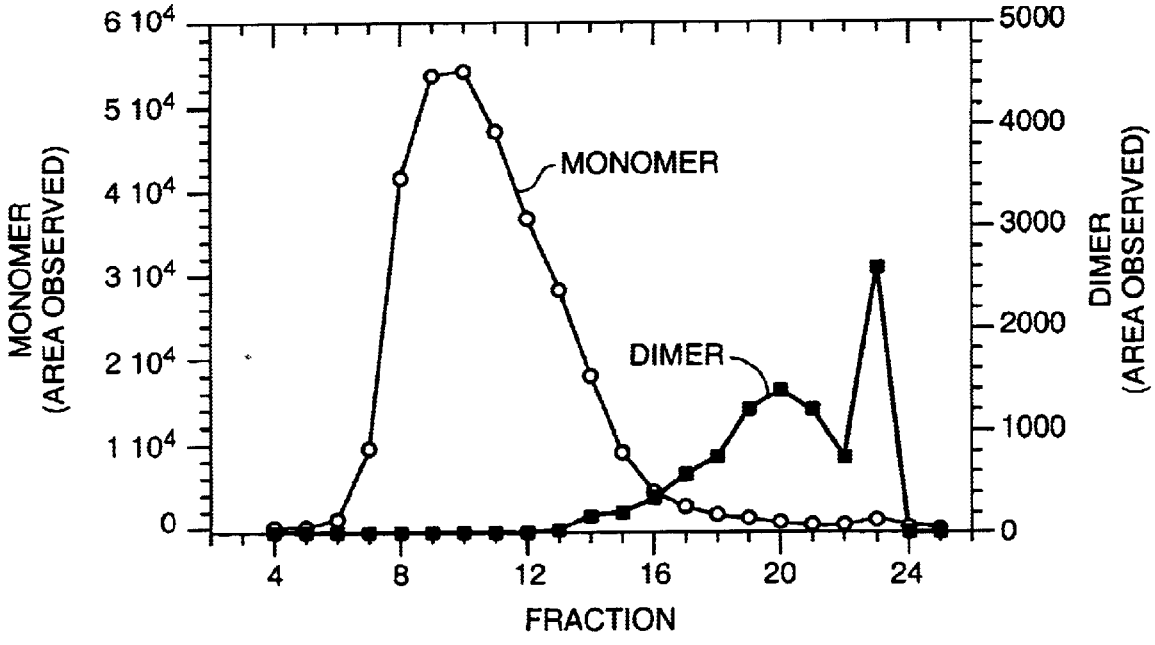
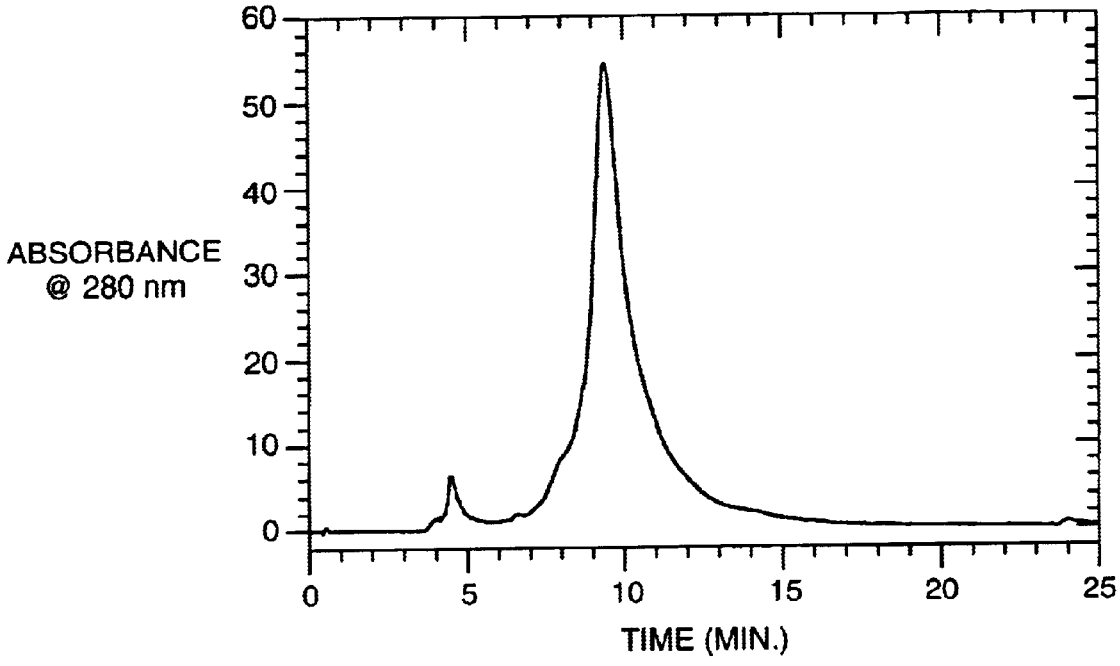

6 10^4
5 10^4
4 10^4
3 10^4
2 10^4
1 10^4
0
MONOMER
(AREA OBSERVED)
5000
4000
3000
2000
1000
0
DIMER
(AREA OBSERVED)
MONOMER
DIMER
4
8
12
16
20
24
FRACTION

FIG._2C


ABSORBANCE
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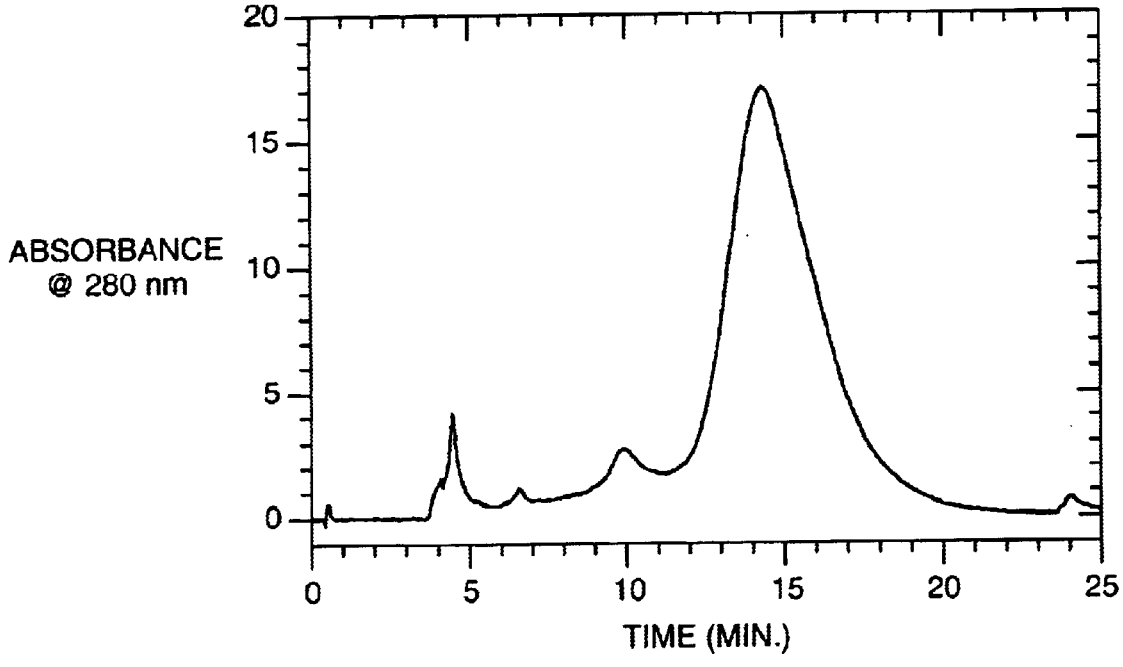
60
50
40
30
20
10
0
0
5
10
15
20
25
TIME (MIN.)

FIG._3A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@ 280 nm
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15
10
5
0
0
5
10
15
20
25
TIME (MIN.)

FIG._3B

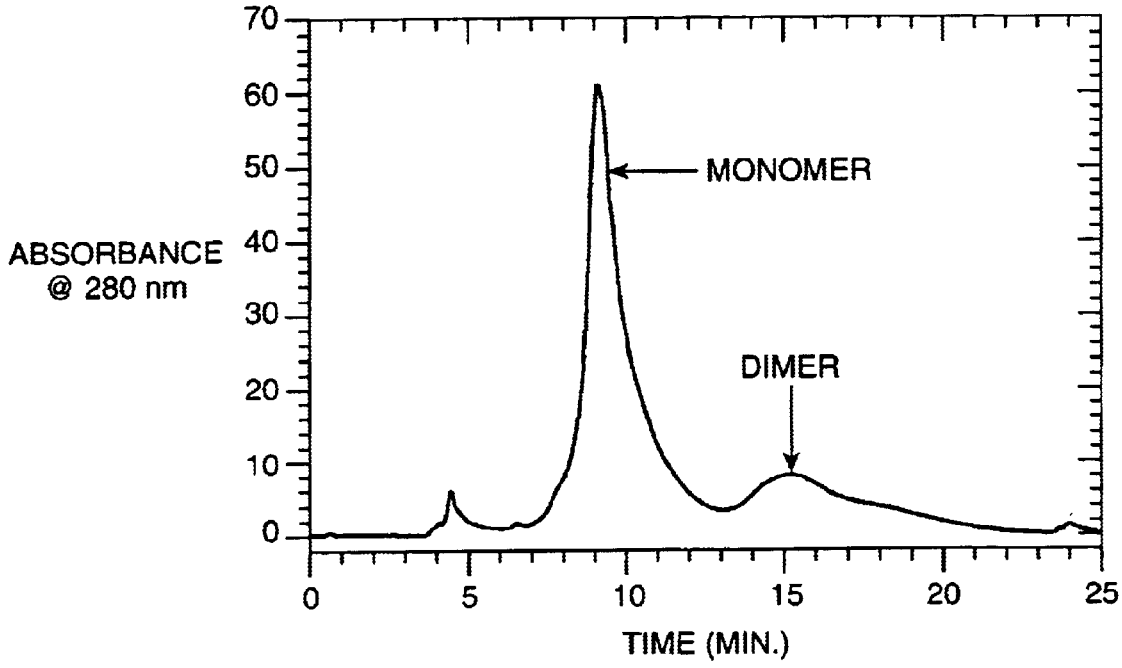
ABSORBANCE
@ 280 nm
70
60
50
40
30
20
10
0
MONOMER
DIMER
0
5
10
15
20
25
TIME (MIN.)

FIG._3C

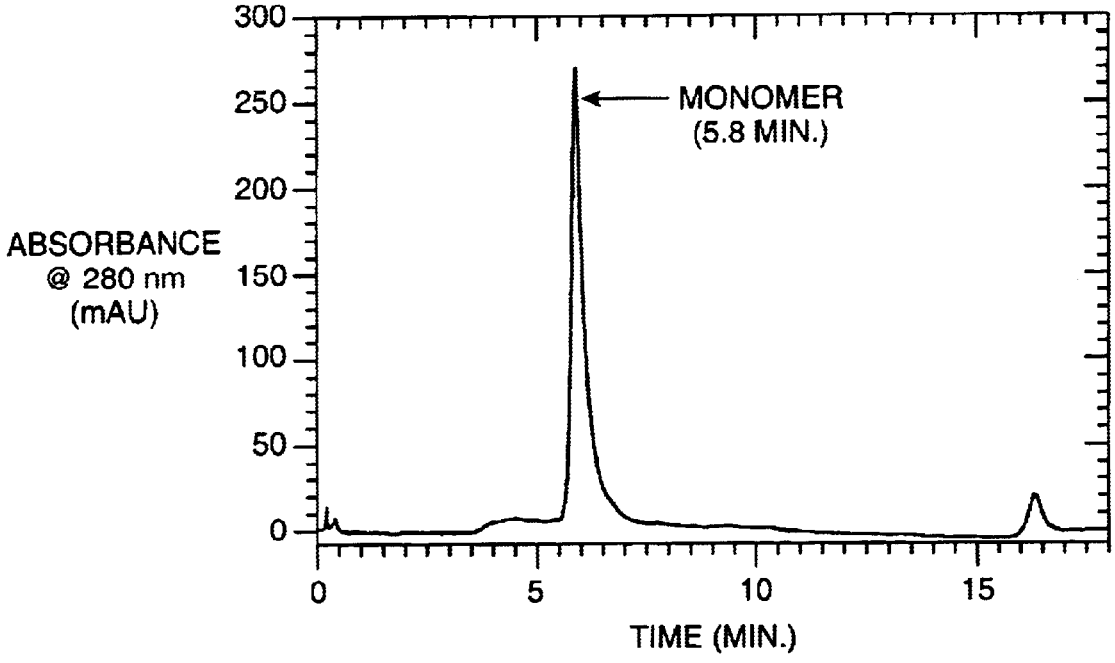
ABSORBANCE
@ 280 nm
(mAU)
300
250
200
150
100
50
0
MONOMER
(5.8 MIN.)
0
5
10
15
TIME (MIN.)

FIG._4A

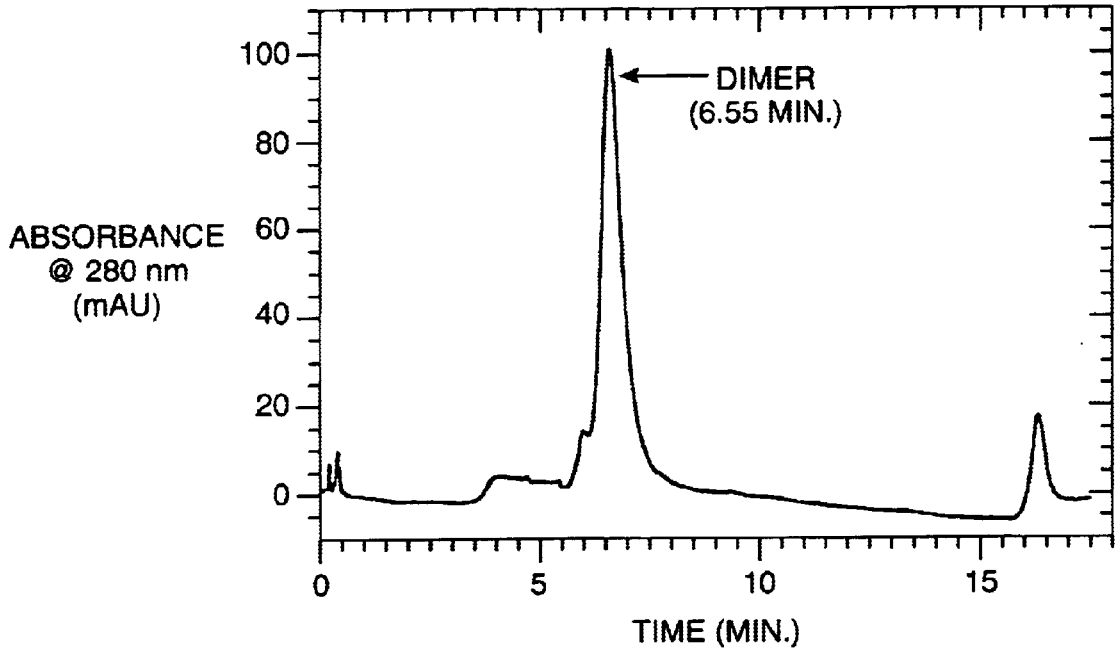
ABSORBANCE
@ 280 nm
(mAU)
100
80
60
40
20
0
DIMER
(6.55 MIN.)
0
5
10
15
TIME (MIN.)

FIG._4B

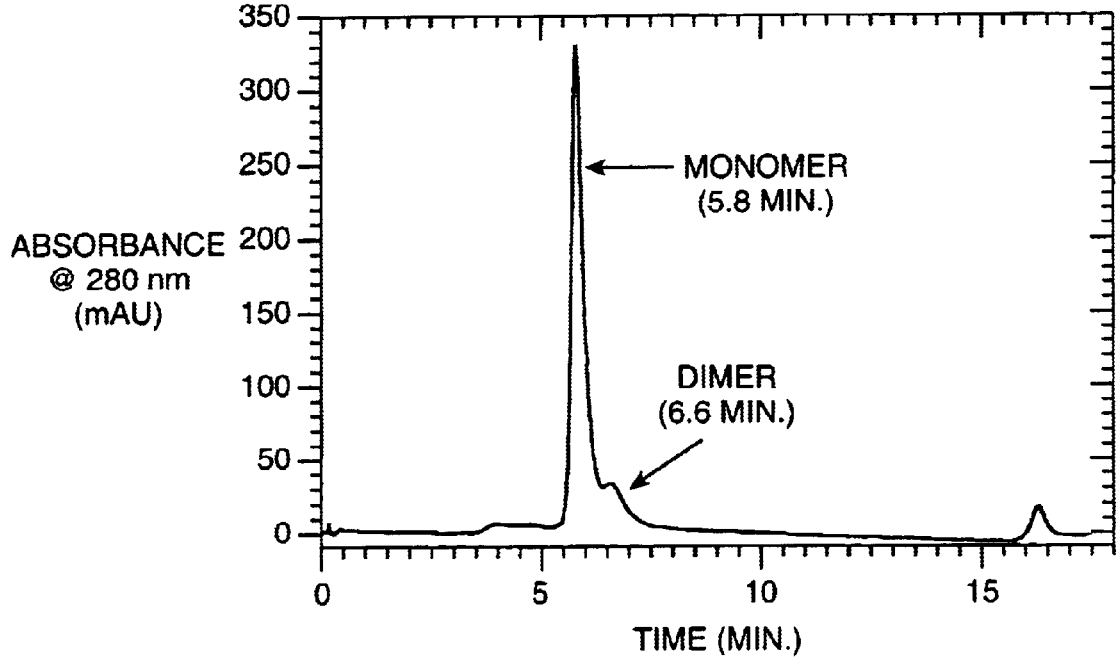
ABSORBANCE
@ 280 nm
(mAU)
350
300
250
200
150
100
50
0
MONOMER
(5.8 MIN.)
DIMER
(6.6 MIN.)
0
5
10
15
TIME (MIN.)

FIG._4C

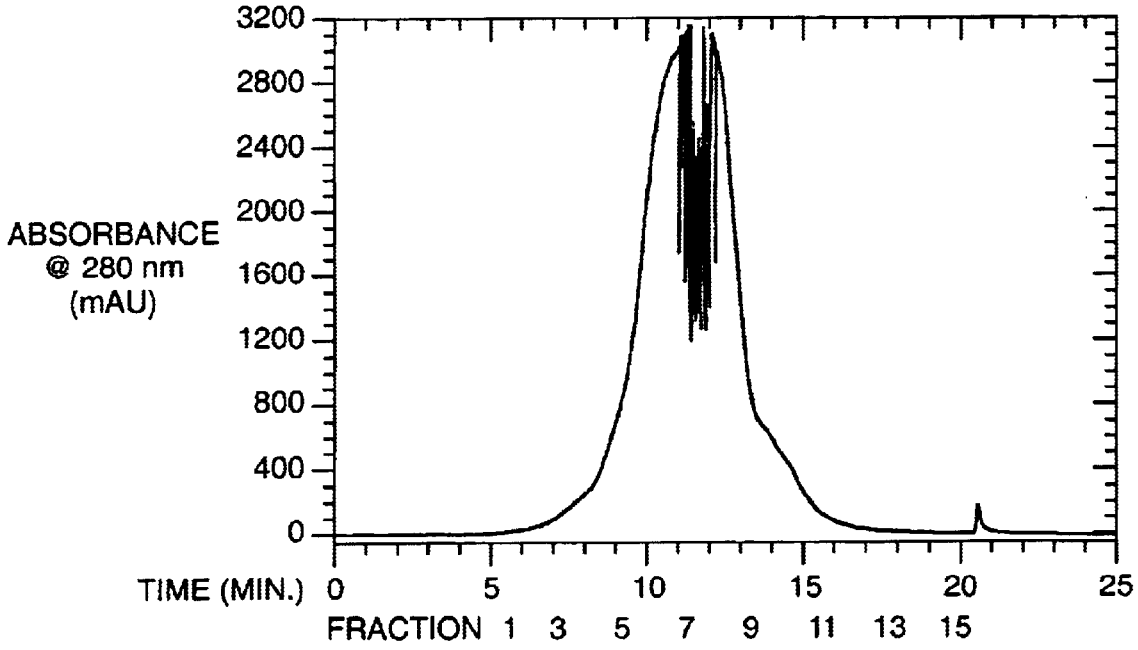
ABSORBANCE
@ 280 nm
(mAU)
3200
2800
2400
2000
1600
1200
800
400
0
TIME (MIN.) 0 5 10 15 20 25
FRACTION 1 3 5 7 9 11 13 15

FIG._5A

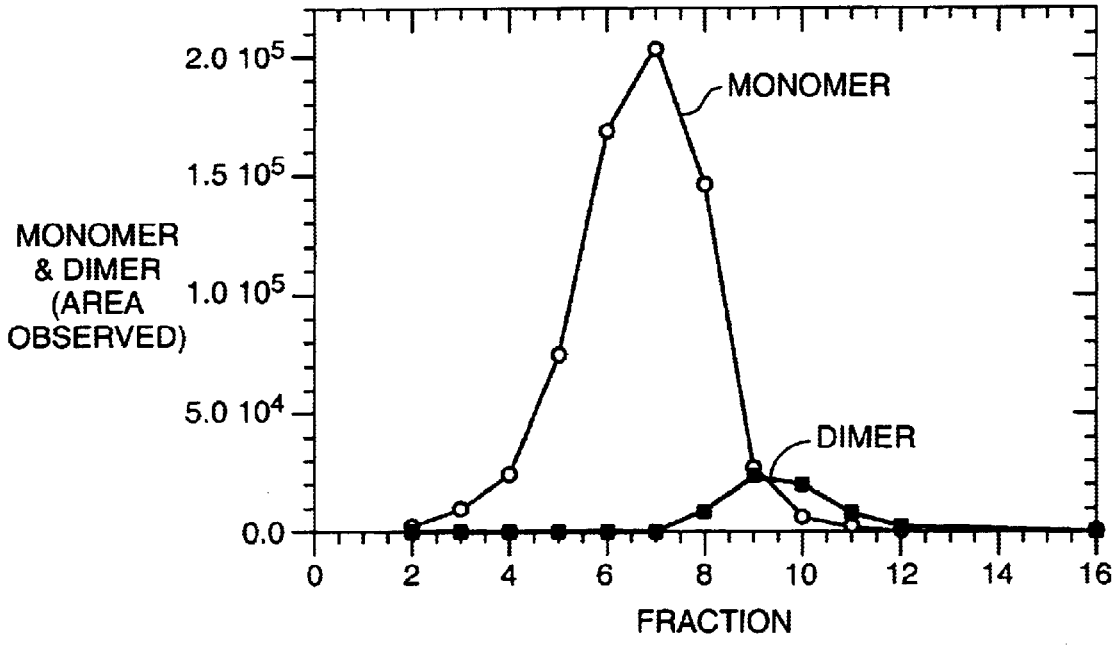
MONOMER
& DIMER
(AREA
OBSERVED)
2.0 10^5
1.5 10^5
1.0 10^5
5.0 10^4
0.0
MONOMER
DIMER
0 2 4 6 8 10 12 14 16
FRACTION

FIG._5B

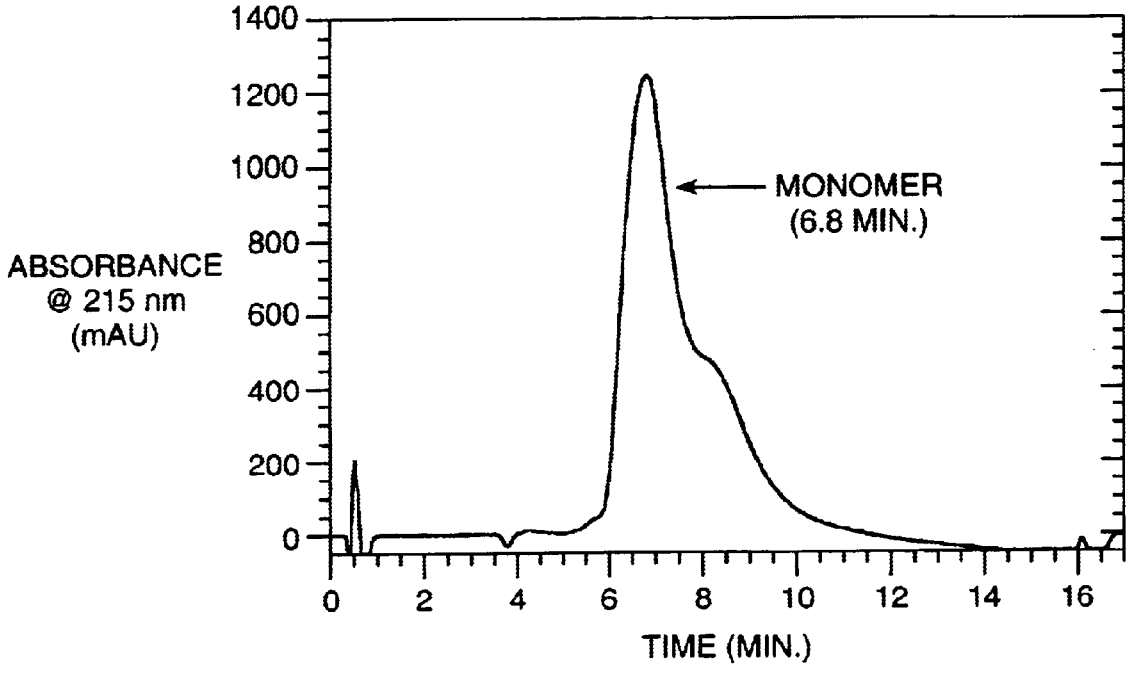
ABSORBANCE
@ 215 nm
(mAU)
1400
1200
1000
800
600
400
200
0
MONOMER
(6.8 MIN.)
0
2
4
6
8
10
12
14
16
TIME (MIN.)

FIG._6A

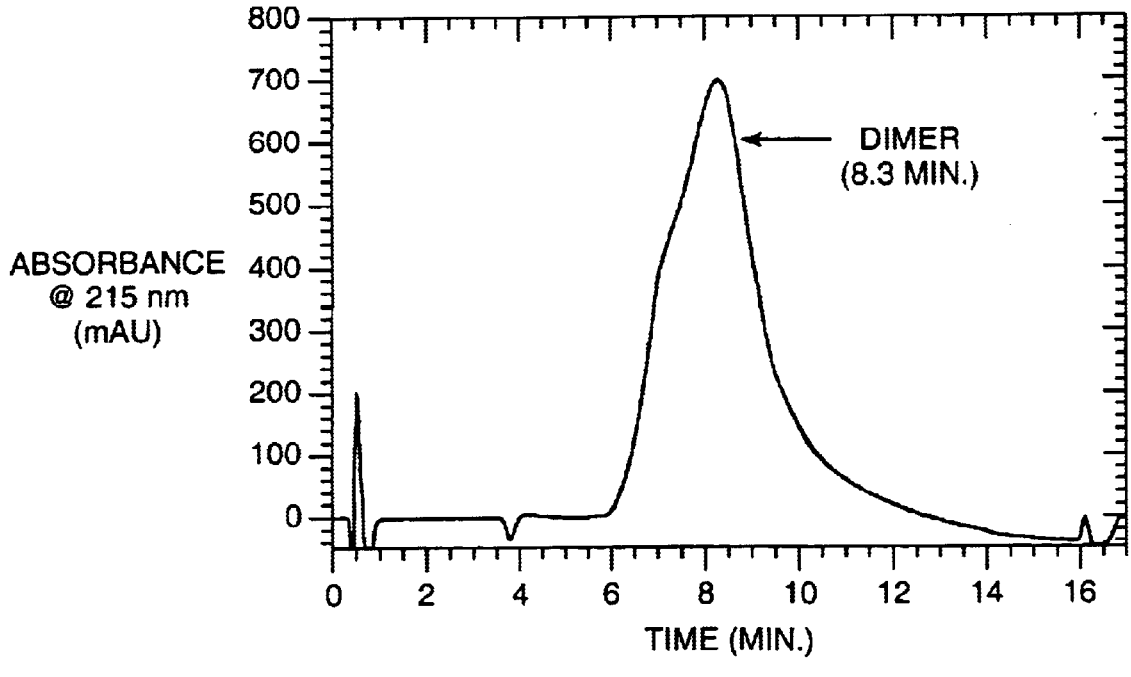
ABSORBANCE
@ 215 nm
(mAU)
800
700
600
500
400
300
200
100
0
DIMER
(8.3 MIN.)
0
2
4
6
8
10
12
14
16
TIME (MIN.)

FIG._6B

SEPARATION OF POLYPEPTIDE MONOMERS

RELATED APPLICATIONS

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application No. 60/087,602 filed Jun. 1, 1998, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a process for separating polypeptide monomers from dimers and/or other multimers using ion-exchange chromatography.

2. Description of Background and Related Art

Attempts to purify authentic, properly folded protein from recombinant hosts have been frustrated due to the tertiary structure of the molecule. In this regard, purification of the recombinantly produced molecule often yields a heterogeneous mixture that consists largely of inactive, misfolded, insoluble, and/or soluble dimers, multimers, and disulfide-linked aggregates. Other aberrant molecules, such as fragments, nicked, oxidized, and glycosylated forms, may also be present. Thus, purification is difficult and yields of the authentic monomer are often low. See, e.g., Elliott et al, *J. Protein Chem.,* 9: 95–104 (1990).

Different techniques have been used to correct these problems. For example, Chang and Swartz, *Protein Folding: in vivo and in vitro* (American Chemical Society, 1993), pp. 178–188 describe a method for solubilizing aggregated IGF-I produced in *E. coli*, using low concentrations of urea and dithiothreitol (DTT) in an alkaline buffer. U.S. Pat. No. 5,231,178 describes a method for the purification of correctly folded, monomeric IGF-I from *P. pastoris* using a combination of cation exchange, hydrophobic interaction, and gel filtration chromatography. WO 96/40776 describes a method for producing authentic properly folded IGF from yeast using a first cation exchange chromatography with the yeast cell medium, denaturing and chromatography, and performing reverse phase high performance liquid chromatography.

Separation of protein and peptide monomers from their dimers, tetramers, and multimers presents a serious challenge to the separations scientist. Size-exclusion chromatography (SEC) and Tangential-Flow Filtration (TFF) (U.S. Pat. Nos. 5,256,294 and 5,490,937) have been used for separating monomers from aggregates but have limitations. SEC can separate monomers from multimers, and in some cases monomers from dimers. The main limitations of SEC are 1) limited load volumes (typically 5% of the bed volume) requiring large columns or multiple cycles, 2) and load protein concentration (low concentration feed stocks require pre-concentration or multiple cycles on the column. Higher protein concentrations can be more viscous, thereby reducing the efficiency of the separation). Historically TFF can separate protein multimers that are ten-fold larger than the monomer. U.S. Pat. No. 5,256,294.

U.S. Pat. Nos. 4,228,154 and 5,250,663 disclose separations of albumin from mixtures. U.S. Pat. No. 4,228,154 describes use of both cation-exchange and anion-exchange chromatography steps for the purification, without separation of monomer from multimers.

There is a need for separating monomers from dimers and multimers that is satisfactory, requires the use of only one ion-exchange step, and does not have the limitations of SEC or TFF.

SUMMARY OF THE INVENTION

Accordingly, this invention provides a method for separating a polypeptide monomer from a mixture comprising dimers and/or multimers, wherein the method comprises applying the mixture to either a cation-exchange or an anion-exchange chromatography resin in a buffer, wherein if the resin is cation-exchange, the pH of the buffer is about 4–7, and wherein if the resin is anion-exchange, the pH of the buffer is about 6–9, and eluting the mixture at a gradient of about 0–1 M of an elution salt, wherein the monomer is separated from the dimers and/or multimers present in the mixture.

In this study it is demonstrated that ion-exchange chromatography—either anion or cation—is an effective means to separate protein or polypeptide monomers from their dimers and/or multimers. Separations are performed using either step or linear gradient elution. Ion exchange has several advantages over the SEC and TFF methods described above. First, separation is independent of polypeptide concentration in the load and therefore no pre-concentration is required. Second, resins can be loaded to greater than 30 mg polypeptide/mL resin and still achieve excellent separations. Third, ion-exchange resins are inexpensive and easy to use. Typical separations achieve enrichment of monomer to greater than 99.5% purity and yields in excess of 90%.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and **1**B show separation of U266 IgE monomer from dimers and multimers on a RESOURCE Q™ anion-exchange column. The column was equilibrated in 25 mM Tris/pH 8, and eluted with a gradient from 0 to 0.5 M sodium chloride over 10 column volumes. FIG. **1**A is full-scale; FIG. **1**B is a close-up view to show the dimers and multimers.

FIGS. **2**A**1**, **2**A**2**, **2**B, and **2**C show separation of anti-IgE monoclonal antibody monomer from dimers and multimers. FIGS. **2**A**1** and **2**A**2** were run on a RESOURCE Q™ anion-exchange column. FIG. **2**A**1** is full-scale; FIG. **2**A**2** is a close-up view to show the dimers and multimers. FIG. **2**B is a run on Q-SEPHAROSE FAST-FLOW™ resin. FIG. **2**C is a plot of monomer and dimer/multimer observed in fractions, where the open dots are monomer and the solid dots are dimer. The monomer and dimer/multimer were determined using a SUPERDEX 200 HR™ 10/30 analytical size-exclusion column (Pharmacia Biotech). In all cases the columns were equilibrated in 25 mM Tris/pH 8. The gradient used in the FIG. **2**A panels was 0 to 0.5 M sodium chloride over 40 column volumes. The gradient used for FIG. **2**B (Q-SEPHAROSE FAST-FLOW™) was 0.05 to 0.2 M NaCl over 10 column volumes.

FIGS. **3**A–C show separation of BSA monomer and dimer on a RESOURCE Q™ anion-exchange column at pH 8. The column was equilibrated in 25 mM Tris/pH 8, and eluted with a gradient from 0.125 to 0.275 M sodium chloride over 40 column volumes. FIG. **3**A is purified monomer, FIG. **3**B is purified dimer, and FIG. **3**C is a commercial preparation of BSA (Bayer) that contains both monomer and dimer.

FIGS. **4**A–C show separation of BSA monomer and dimer on a RESOURCE Q™ anion-exchange column at pH 6. The column was equilibrated in 20 mM sodium phosphate/pH 6, and eluted with a linear gradient from 0 to 0.5 M sodium chloride over 10 column volumes. FIG. **4**A is purified monomer, FIG. **4**B is purified dimer, and FIG. **4**C is a commercial preparation of BSA (Bayer) that contains both monomer and dimer.

FIGS. **5**A and **5**B show separation of anti-IgE monoclonal antibody monomer from dimers and multimers on a RESOURCE S™ cation-exchange column at pH 6. The column was equilibrated in 20 mM sodium phosphate/pH 6, and eluted with a linear gradient from 0 to 0.05 M sodium chloride over 30 column volumes. FIG. **5**A is the chromatogram from the separation, and FIG. **5**B is a plot of monomer and dimer/multimer observed in fractions using the same method described in FIG. **2**, where the open dots are monomer and the solid dots are dimer.

FIGS. **6**A and **6**B show separation of BSA monomer and dimer on a RESOURCE S™ cation-exchange column at pH 4.3. The column was equilibrated in 20 mM sodium acetate/pH 4.3, then eluted with a gradient from 0 to 1 M sodium chloride over 20 column volumes. FIG. **6**A is purified monomer, and FIG. **6**B is purified dimer.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptides are "exogenous," meaning that they are "heterologous," i.e., foreign to the host cell being utilized, such as a human protein produced by *E. coli*. However, they may also be derived from a native source in which they are present naturally.

Examples of mammalian polypeptides include molecules such as, e.g., renin, a growth hormone, including human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; 1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; thrombopoietin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial naturietic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; a serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF; cardiotrophins (cardiac hypertrophy factor) such as cardiotrophin-1(CT-1); platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-1, TGF-2, TGF-3, TGF-4, or TGF-5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD-3, CD-4, CD-8, and CD-19; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; serum albumin, such as human serum albumin (HSA) or bovine serum albumin (BSA); colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; anti-HER-2 antibody; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressing; regulatory proteins; antibodies; and fragments of any of the above-listed polypeptides.

The preferred polypeptides of interest are mammalian polypeptides. Examples of such mammalian polypeptides include enzymes, hormones, cytokines, albumins, chemokines, immunotoxins, viral components, antibodies, neurotrophins, and antigens. Suitable such polypeptides encompass polypeptides such as HSA, BSA, anti-IgE, anti-CD20, anti-IgG, t-PA, gp 120, anti-CD11a, anti-CD18, anti-VEGF, VEGF, TGF-beta, activin, inhibin, anti-HER-2, DNase, IGF-I, IGF-II, brain IGF-I, growth hormone, relaxin chains, growth hormone releasing factor, insulin chains or pro-insulin, NGF, NT-3, BDNF, and urokinase. Particularly preferred mammalian polypeptides include, e.g., t-PA, gp120 (IIIb), anti-HER-2, anti-CD11a, anti-CD18, anti-VEGF, VEGF, BSA, HSA, anti-CD20, anti-IgE, anti-IgG, DNase, IGF-I, IGF-I1, TGF-beta, IGFBP-3, IGFBP-2, IGFBP-1, growth hormone, NGF, NT-3, NT-4, NT-5, and NT-6. The polypeptide is more preferably an antibody or a serum albumin, more preferably, anti-IgE, anti-IgG, anti-Her-2, anti-CD11a, anti-CD18, anti-CD20, anti-VEGF, BSA, or HSA.

For purposes herein, the "mixture" contains monomers and either dimers or multimers or both dimers and multimers. Typically, the mixture is a biological fluid, which denotes any fluid derived from or containing cells, cell components, or cell products. Biological fluids include, but are not limited to, fermentation broth, cell culture supernatants, cell lysates, cleared cell lysates, cell extracts, tissue extracts, blood, plasma, serum, sputum, semen, mucus, milk, and fractions thereof. This definition includes cell-conditioned culture medium, which denotes a nutrient medium in which cells have been cultured and which contains cell products.

For purposes herein, "ion-exchange chromatography resin" refers to chromatography medium for anion- or cation-exchange separation.

As used herein, "elution salt" refers to an alkaline earth, alkali metal, or ammonium salt, i.e., a salt having a cation from the alkaline earth or alkali metal elements or an ammonium cation and having an inorganic or organic (hydrocarbon-based) anion. Examples of such salts include sodium chloride, ammonium chloride, sodium citrate, potassium citrate, potassium chloride, magnesium chloride, calcium chloride, sodium phosphate, calcium phosphate, ammonium phosphate, magnesium phosphate, potassium phosphate, sodium sulfate, ammonium sulfate, potassium sulfate, magnesium sulfate, calcium sulfate, etc. Preferred salts herein are chlorides or sulfates. The most preferred salt herein is sodium chloride.

As used herein, "multimers" refer to n-mers where n is 3–10, i.e., polymers that are not dimers but exclude aggregates. In contrast to multimers, aggregates have a value for n of greater than 10, and/or a molecular weight of greater than 2 million daltons, and/or are species contained in the excluded volume of analytical size-exclusion chromatography columns such as SUPEROSE 6™ (Pharmacia).

Modes for Carrying out the Invention

This invention relates to a method of separating monomers of polypeptides from their dimers or multimers or both. The method involves placing the mixture of monomers and dimers and/or multimers, from whatever source, in an equilibration buffer at a pH in the range of about 4 and 9

depending on whether the resin used for chromatographic separation is a cation- or anion-exchange resin. The resulting mixture is loaded onto either a cation-exchange or anion-exchange chromatography resin to capture all the n-mers (monomers, dimers, trimers, tetramers, etc.) present in the mixture. For ion-exchange column chromatography, ligands of general affinity can be used to achieve the desired selectivities and binding properties. The loading takes place in a buffer at a pH of about 6–9 if the resin is anion-exchange and about 4–7 if the resin is cation-exchange. The exact pH will depend, for example, on the isoelectric point of the polypeptide.

If the resin is a cation-exchange resin, prior to loading the mixture, the matrix can be equilibrated using several column volumes of a dilute, weak acid (e.g., four column volumes of 20 mM acetic acid, pH 3, or of 20 mM phosphoric acid, pH about 2.8). Following equilibration, the mixture is added and the column can be washed one to several times, prior to elution of the mixture, also using a weak acid solution such as a weak acetic acid or phosphoric acid solution. The buffer used for this purpose depends on, e.g., the polypeptide and the anionic or cationic nature of the resin. For anion-exchange, preferably the buffer is TRIS or phosphate buffer; for cation-exchange, the buffer is preferably acetate or phosphate buffer.

Ion-exchange chromatography is typically carried out at a temperature of about 18–25° C., preferably about 20° C. (room temperature). The preferred column loading is about 1 ml resin per 20–30 mg total polypeptide.

Following adsorption of the n-mer molecules to the ion exchanger, the mixture is eluted by contacting the resin with an elution salt having an appropriate ionic strength to displace the monomer from the matrix. An elution salt gradient is used of about 0 to 1 M. The gradient may be linear or stepwise. Preferably the gradient is from about 0 to 500 mM elution salt, more preferably 50 to 200 mM elution salt, and most preferably, 0 to 50 mM elution salt. Preferably the elution salt is a sodium salt, such as sodium chloride, although other elution salts and concentration gradients, known to those of skill in the art, also find use herein. The quantity of elution buffer can vary widely and will generally be in the range of about 2 to 40 column volumes, preferably 10 to 40 column volumes. Following elution, the eluate can be assayed for total monomeric concentration.

Suitable cation-exchange resins herein include a wide variety of materials known in the art, including those capable of binding polypeptides over a wide pH range. For example, carboxymethylated, sulfonated, agarose-based, or polymeric polystyrene/divinyl benzene cation-exchange matrices are particularly preferred. Other useful matrix materials include, but are not limited to, cellulose matrices, such as fibrous, microgranular, and beaded matrices; dextran, polyacrylate, polyvinyl, polystyrene, silica, and polyether matrices; and composites. These matrices include, for example, CM52 CELLULOSE™ (Whatman, Inc.); S-HYPERD™ and CM SPHERODEX™ (Secpracor); SP SEPHAROSE FF™, DEAE SEPHAROSE FF™, CM-SEPHAROSE™, and RESOURCE S™ (Amersham Pharmacia Biotech AB); and JT BAKER CSx™ (J.T. Baker, Inc.), as well as those containing the functional ligand R—SO_3^-, preferably sulfopropyl resins, such as TOYOPEARL SP550C™ (Tosohaas) and FRACTOGEL EMD™ SO_3^--650 (m) (Merck). Other suitable materials for use in cation-exchange chromatography are within the knowledge of those skilled in the art.

Anion-exchange chromatography is carried out using media appropriate therefor, as are known in the art. Suitable media include, e.g., polymeric polystyrene/divinyl benzene resins and agarose-based resins, as well as agarose beads, dextran beads, polystyrene beads, media that comprise an insoluble, particulate support derivatized with tertiary or quaternary amino groups., and supports derivatized with trimethylaminoethyl groups. Examples of suitable such media include DE92™ (diethylaminoethyl cellulose, Whatman); DEAE-CELLULOSE™ (Sigma), BAKERBOND ABX 40 mu™ (J.T. Baker, Inc.); DEAE resins such as FRACTOGEL EMD DEAE-650™ (EM Separations), FRACTOGEL EMD TMAE-650 (S)™ (EM Science, Gibbstown, N.J.), TSK gel DEAE-SPW™ (Tosohaas), DEAE-SEPHAROSE CL-6B™ and chelating SEPHAROSE™ (Amersham Pharmacia Biotech AB), DEAE MERE SEP. 1000™ (Millipore), and DEAE SPHERODEX™ (Sepracor); RESOURCE Q™ and Q SEPHAROSE™ (QSFF) (Amersham Pharmacia Biotech AB); MACRO-PEP Q™ (Bio-Rad Laboratories, Hercules, Calif.); Q-HYPERD™ (BioSepra, Inc., Marlborough, Mass.); and the like. Other suitable anion-exchange chromatography materials, as well as the selection and use of these materials for the present application, are conventional in the art.

Purified fractions of monomer obtained from the ion-exchange chromatography may be further processed by subjecting them to any appropriate technique designed for downstream processing and purification. This will depend largely on the type of polypeptide and its intended use. Only one ion-exchange step is necessary to effect the desired separation of monomer from dimers and/or multimers in a mixture, although the invention does not exclude using more such steps if desired in the upstream or downstream processing of the polypeptide.

The invention will be more filly understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature and patent citations herein are incorporated by reference.

EXAMPLE I

This example shows the separation of anti-IgE monomers and bovine serum albumin monomers from dimers and multimers.

Materials and Methods

Resins

Pharmacia Q-SEPHAROSE FAST FLOW™. 4 mL to 235L bed volumes evaluated

Pharmacia RESOURCE S and RESOURCE Q™: 1 mL prepacked columns

JT Baker CSx™, 0.46×5 cm, 5μ particles

Proteins

A. Humanized anti-IgE monoclonal antibodies (IgG_1) available from Genentech, Inc.: pI~7.5, designated as E25 and E26. WO 93/04173 published Mar. 4, 1993 describes humanized anti-IgE antibodies wherein a murine antibody directed against human IgE (MaE11) was used to provide the CDR regions that were substituted into an IgG1 immunoglobulin framework (rhuMaE25). See also Cacia et al., *Biochemistry,* 35: 1897–1903 (1996) for studies and further descriptions of E-25.

B. Monoclonal anti-IgE antibody prepared from the culture supernatants of an immortalized human myeloma cell line U266B1 (ATCC TIB 196) using affinity chromatography purification on an isolated anti-IgE antibody (Genentech MAE1). Specifically, five BALB/c female mice, age six weeks, were immunized in their foot pads with 10 μg of purified IgE in Ribi's adjuvant. Subsequent injections were

done in the same manner at one and three weeks after the initial immunizations. Three days after the final injection, the inguinal and popliteal lymph nodes were removed and pooled, and a single cell suspension was made by passing the tissue through steel gauze. The cells were fused at a 4:1 ratio with mouse myeloma P3X63-Ag8.653 (ATCC CRL 1580) in high glucose (DMEM) containing 50% w/v polyethylene glycol 4000. Alternatively, the immunizations were done in a similar manner except that 30 μg of IgE per injection were used and IgE fragment 315–347 (Kabat) was assayed as a prefusion boost; or injections were given subcutaneously in two doses of 100 μg and a final booster of 50 μg, and spleen cells were used for the fusions.

The fused cells were then plated at a density of 2×105 per well in 96-well tissue culture plates. After 24 hours HAT selective medium hypoxanthine/aminopterin/thymidine, Sigma, #H0262) was added. Of 1440 wells plated, 365 contained growing cells after HAT selection.

Fifteen days after the fusion, supernatants were tested for the presence of antibodies specific for human IgE using an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed as follows, with all incubations done at room temperature. Test plates (Nunc Immunoplate) were coated for 2 hours with rat anti-mouse IgG (Boehringer Mannheim, #605-500) at 1 μg/ml in 50 mM sodium carbonate buffer, pH 9.6, then blocked with 0.5% bovine serum albumin in phosphate buffered saline (PBS) for 30 minutes, then washed four times with PBS containing 0.05% TWEEN 20™ (PBST). Test supernatants were added and incubated two hours with shaking, then washed four times with PBST. Human IgE (purified from U266 cells as described above) was added at 0.5 μg/ml and incubated for one hour with shaking, then washed four times in PBST. Horseradish-peroxidase-conjugated goat anti-human IgE (Kirkegarrd & Perry Labs, #14-10-04, 0.5 mg/ml) was added at a 1:2500 dilution and incubated for one hour, then washed four times with PBST. The plates were developed by adding 100 μl/well of a solution containing 10 mg of o-phenylenediamine dihydrochloride (Sigma, #P8287) and 10 μl of a 30% hydrogen peroxide solution in 25 ml phosphate citrate buffer, pH 5.0, and incubating for 15 minutes. The reaction was stopped by adding 100 μl/well of 2.5 M sulfuric acid. Data were obtained by reading the plates in an automated ELISA plate reader at an absorbance of 490 nm. For one antibody, 365 supernatants were tested and 100 were specific for human IgE. Similar frequencies of IgE specificity were obtained when screening for the other antibodies.

C. Bovine serum Albumin: pI 4.7 and 4.9 (Radola, *Biochim. Biophys. Acta,* 295: 412–428 (1973))

Bayer Corp. P/N 81-024-2, "Bovine Albumin, Sulfhydryl Modified" (BSA Mix, blocked)

ICN Biomedical Inc. P/N 810013, "Albumin Bovine" (BSA Mix, native)

BSA monomer and dimer prepared in house from Bayer BSA (BSA Monomer and BSA Dimer, respectively)

Chromatography Systems

Hewlett-Packard1090™ HPLC

Pharmacia™ FPLC

Detection at 215 or 280 nm

Buffers: (see Table I for details)

Purified water

Tris•HCl

Sodium acetate

Sodium chloride

Sodium phosphate

Sodium citrate and citric acid

Sample Preparation

Samples were diluted with the buffer used for equilibration (indicated in Table I below) to assure pH and conductivity matched starting column conditions. All samples were 0.2-μm filtered prior to loading.

Chromatography

Samples were introduced to the column using either an automatic or manual injector. All runs were performed at room temperature. Fractions were collected manually or with a PHARMACIA FRAC 100™ collector.

Chromatographic separation performance was evaluated by comparing elution profiles of BSA stock reagent and purified BSA monomer and dimer; the same was done for IgE and the monoclonal antibodies (MAb). Separation of IgE and MAb from their dimers and multimers was further evaluated by analyzing elution fraction using analytical size-exclusion chromatography. Plots of MAb MW forms vs. Fraction number were created. Recovery of IgE and MAb was determined spectrophotometrically by measuring absorbance at 280 nm.

Results

The results are summarized in Table I below.

TABLE I

MONOMER-DIMER/MULTIMER SEPARATIONS

RESIN	PROTEIN	PH	EQUILIBRATION BUFFER	ELUTION	COMMENTS
			Anion-Exchange		
QSFF ™	MAb	8	Tris-HCl	linear gradient: 0 to 500 mM NaCl	Good separation
				linear gradient: 50 to 200 mM NaCl	Best separation
				step gradients to 200, 175, 150, 125 mM NaCl	Separation works
Resource Q ™	MAb	8	Tris-HCl		
Resource Q ™	U266 IgE	8	Tris-HCl		Removed aggregates and multimers
Resource Q ™	BSA Monomer BSA	8	Tris-HCl	linear gradient: 0 to 1 M NaCl, linear gradients: 150	Good separation Excellent

TABLE I-continued

MONOMER-DIMER/MULTIMER SEPARATIONS

RESIN	PROTEIN	PH	EQUILIBRATION BUFFER	ELUTION	COMMENTS
	Dimer			to 550, 250 to 550 mM NaCl	separation
	BSA Mix, native				
	BSA Mix, blocked			step gradients: 0.3/0.6, 0.38/0.6, 0.4 M/0.6 M NaCl	Some separation but fine control required
Resource Q ™	BSA Monomer	6	sodium citrate	linear gradient:	Does not bind in citrate pH 6
	BSA Dimer				Does not bind in citrate pH 6
	BSA Mix, blocked				Does not bind in citrate pH 6
Resource Q ™	BSA Monomer	6	sodium phosphate	linear gradient: 0 to 0.5 M NaCl in 10 CVs	good separations
	BSA Dimer				
	BSA Mix, blocked				
			Cation Exchange		
Resource S ™	BSA Monomer	6	sodium citrate	linear gradient: 0 to 0.5 M NaCl in 10 CVs	does not bind in citrate pH 6
	BSA Dimer				does not bind in citrate pH 6
	MAb				does not bind in citrate pH 6
Resource S ™	MAb	6	sodium phosphate	linear gradient: 0 to 0.05 M NaCl in 20 CVs	equivalent to Q separation
					loaded to 16.5 mg/mL
Resource S ™	BSA Monomer	4.6	NaOAc buffer	linear gradient: 0 to 1 M NaCl/40 CVs	proteins somewhat resolved
	BSA Dimer				
	BSA Mix, blocked				
	BSA Monomer	4.3	NaOAc buffer	linear gradient: 0 to 1 M NaCl/20 CVs	better resolution than pH 4.6
	BSA Dimer				
	BSA Mix, blocked				
JT Baker CSx ™	BSA Monomer	4.6	NaOAc buffer	linear gradient: 0 to 1 M NaCl/12 CVs	proteins somewhat resolved
	BSA Dimer				
JT Baker CSx ™	BSA Monomer	4.3	NaOAc buffer	linear gradient: 0 to 1 M NaCl/12 CVs	proteins somewhat resolved
	BSA Dimer				

Separations were evaluated using polymeric polystyrene/divinyl benzene resins (RESOURCE Q and S™), a silica-based resin (JT BAKER CSX™), and an agarose-based resin (Q-SEPHAROSE FAST FLOW™; QSFF). While separations were accomplished using any of these resins, separations worked especially well on Q-SEPHAROSE FAST FLOW™, RESOURCE Q™, and RESOURCE S™. The separation of BSA monomer and dimer from both suppliers looked very similar, suggesting the "Sulfhydryl Modified" material from Bayer did not alter the protein such that the species were easier to separate. It can be seen that phosphate buffer at pH 6 worked well, but no protein bound to the cation- or anion-exchange columns when 20 mM citrate buffer at pH 6 was used as equilibration buffer. Citrate buffer would be expected to work for both anion- and cation-exchange at a lower concentration, e.g., about 5 mM.

Recovery of monomeric IgE and MAbs to IgE on anion-exchange resins was typically greater than 90% at greater than 99.5% purity. FIGS. **1**A and **1**B show anion-exchange (RESOURCE™ Q) chromatograms in the separation of IgE monomers from dimers and multimers. FIGS. **2**A**1** and **2**A**2** show anion-exchange (RESOURCE™ Q) chromatograms in the separation of anti-IgE MAb monomers from dimers and multimers. FIG. **2**B shows an anion-exchange (Q-SEPHAROSE FAST-FLOW™) chromatogram in the separation of anti-IgE MAb monomers from dimers and multimers. SEC (SUPERDEX 200 HR 10/30™) was used as an analytical method to determine the amount of monomer and multimer in samples from the ion-exchange separation, and FIG. **2**C shows the SEC analysis of fractions from FIG. **2**B. Separation of BSA monomer from dimer was readily achieved on anion-exchange resins at pH 8 and pH 6. See FIGS. **3**A–C and **4**A–C for chromatograms in the separation of BSA monomers from dimers and multimers by anion-exchange (RESOURCE™ Q) at pH 8 (Tris buffer) and at pH 6 (phosphate buffer), respectively.

Recovery and purity of MAb monomer from the cation-exchange resin was comparable to that of the anion-exchange resin. FIGS. **5**A–B show cation-exchange (RESOURCE™ S) chromatograms in the separation of anti-IgE MAb monomers from dimers and multimers at pH 6 (phosphate buffer). Separations of BSA on cation-exchange resins could be performed at pH 4.6 and 4.3, 4.3 being somewhat better. FIGS. **6**A–B show cation-exchange (RESOURCE™ S) chromatograms in the separation of BSA monomers from dimers and multimers at pH 4.3 (acetate buffer).

In summary, mixtures of polypeptide mers were subjected to cation- or anion-exchange chromatography using a variety of resins and under a variety of pH and elution salt conditions, and successful separation was achieved. Based on results from four proteins with basic and acidic isoelectric points (two IgG, MAbs, IgE and serum albumin), the method demonstrates general applicability to separation of polypeptide monomers from their dimers and multimers.

What is claimed is:

1. A method for purifying polypeptide monomers from a mixture consisting essentially of said polypeptide monomers, and dimers or multimers of said polypeptide monomers or both dimers and multimers of said polypeptide monomers, wherein the method consists essentially of applying the mixture to a cation-exchange or anion-exchange chromatography resin in a buffer, wherein if the resin is cation-exchange, the pH of the buffer is about 4–7, and wherein if the resin is anion-exchange, the pH of the buffer is about 6–9, and eluting the mixture at a gradient of about 0–1 M of an elution salt, wherein the monomer is purified from the dimers or multimers or both present in the mixture, and wherein the purified monomer has a purity of greater than 99.5% and the monomer yield is greater than 90%.

2. The method of claim **1** wherein the polypeptide is a serum albumin.

3. The method of claim **1** wherein the polypeptide is anti-IgE, anti-IgG, anti-Her-2, anti-CD11a, anti-CD18, anti-CD20, anti-VEGF, or IgE.

4. The method of claim **2** wherein the serum albumin is bovine serum albumin.

5. The method of claim **1** wherein the ion-exchange resin is a cation-exchange resin.

6. The method of claim **1** wherein the ion-exchange resin is an anion-exchange resin.

7. The method of claim **1** wherein the gradient is linear.

8. The method of claim **1** wherein the gradient is stepwise.

9. The method of claim **1** wherein the elution salt is a sodium salt.

10. The method of claim **9** wherein the elution salt is sodium chloride.

11. The method of claim **1** wherein the gradient is from 0 to 500 mM elution salt.

12. The method of claim **1** wherein the gradient is from 50 to 200 mM elution salt.

13. The method of claim **1** wherein the gradient is from 0 to 50 mM elution salt.

14. The method or claim **1** wherein the polypeptide is an antibody.

15. The method of claim **1** wherein the polypeptide is a monoclonal antibody.

* * * * *

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(54) **SEPARATION OF POLYPEPTIDE MONOMERS**

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(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

A method is disclosed for separating a polypeptide monomer from a mixture comprising dimers and/or multimers. The method comprises applying the mixture to either a cation-exchange chromatography resin or an anion-exchange chromatography resin and eluting the mixture at a gradient of about 0–1 M of an elution salt, wherein the monomer is separated from the dimers and/or multimers present in the mixture.

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EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

Claims **2**–**4** and **14**–**15** are cancelled.

Claim **1** is determined to be patentable as amended.

Claims **5**–**13**, dependent on an amended claim, are determined to be patentable.

1. A method for purifying polypeptide monomers from a mixture consisting essentially of said polypeptide monomers, and dimers or multimers of said polypeptide monomers or both dimers and multimers of said polypeptide monomers, wherein the method consists essentially of applying the mixture to a cation-exchange or anion-exchange chromatography resin in a buffer, wherein if the resin is cation-exchange, the pH of the buffer is about 4–7, and wherein if the resin is anion-exchange, the pH of the buffer is about 6–9, and eluting the mixture at a gradient of about 0–1 M of an elution salt, wherein the monomer is purified from the dimers or multimers or both present in the mixture, and wherein the purified monomer has a purity of greater than 99.5% and the monomer yield is greater than 90%, *wherein the polypeptide is anti-IgE, anti-IgG, anti-Her-2, anti-CD11a, anti-CD18, anti-CD20, anti-VEGF, or IgE*.

* * * * *

EXHIBIT P

US008512983B2

(12) **United States Patent**
Gawlitzek et al.

(10) **Patent No.: US 8,512,983 B2**
(45) **Date of Patent: Aug. 20, 2013**

(54) **PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA**

(76) Inventors: **Martin Gawlitzek**, Redwood City, CA (US); **Shun Luo**, Irvine, CA (US); **Christina Teresa Petraglia**, San Ramon, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 151 days.

(21) Appl. No.: **12/852,377**

(22) Filed: **Aug. 6, 2010**

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(52) **U.S. Cl.**
USPC **435/69.1**

(58) **Field of Classification Search**
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See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates generally to glutamine-free cell culture media supplemented with asparagine. The invention further concerns the production of recombinant proteins, such as antibodies, in asparagine-supplemented glutamine-free mammalian cell culture.

25 Claims, 25 Drawing Sheets

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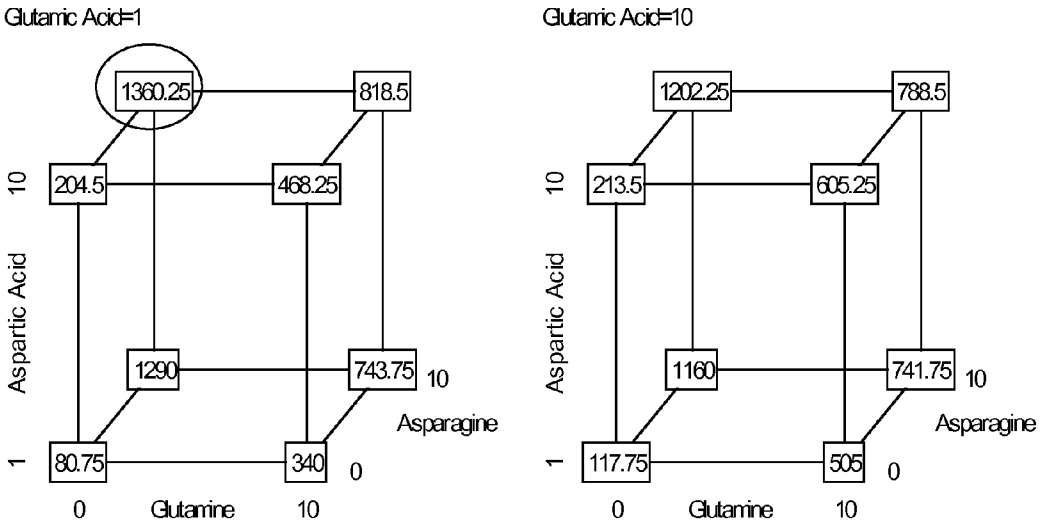

Glutamic Acid=1
1360.25
818.5
204.5
468.25
1290
743.75
80.75
340
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0
Glutamic Acid=10
1202.25
788.5
213.5
605.25
1160
741.75
117.75
505
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0

FIG. 1

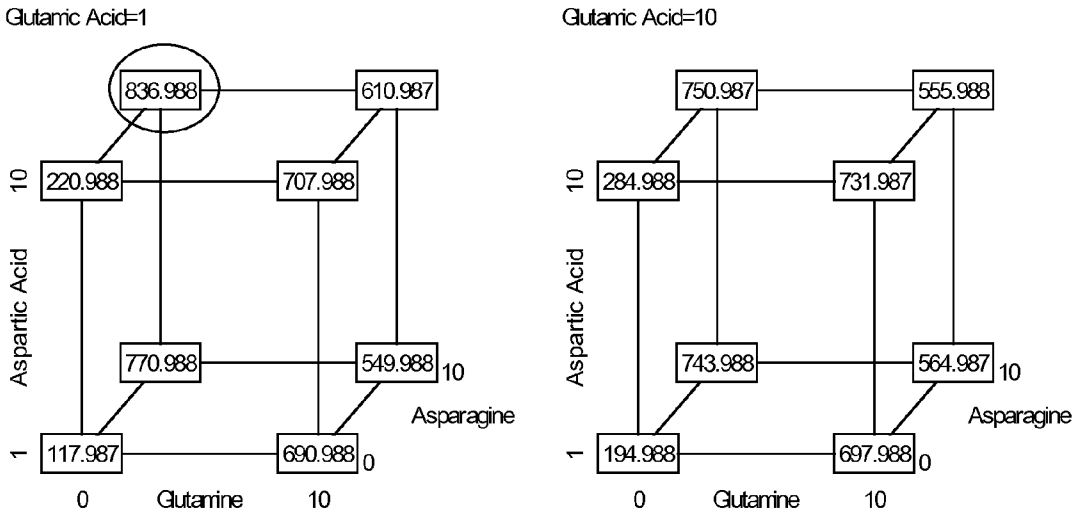

Glutamic Acid=1
836.988
610.987
220.988
707.988
770.988
549.988
117.987
690.988
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0
Glutamic Acid=10
750.987
555.988
284.988
731.987
743.988
564.987
194.988
697.988
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0

FIG. 2

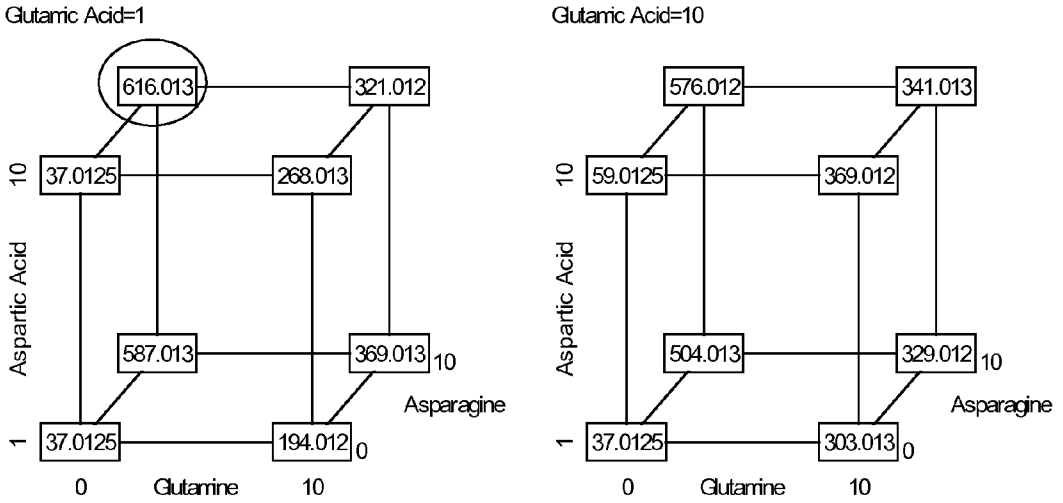

Glutamic Acid=1
616.013
321.012
10
37.0125
268.013
Aspartic Acid
587.013
369.013
10
Asparagine
1
37.0125
194.012
0
0
Glutamine
10
Glutamic Acid=10
576.012
341.013
10
59.0125
369.012
Aspartic Acid
504.013
329.012
10
Asparagine
1
37.0125
303.013
0
0
Glutamine
10

FIG. 3

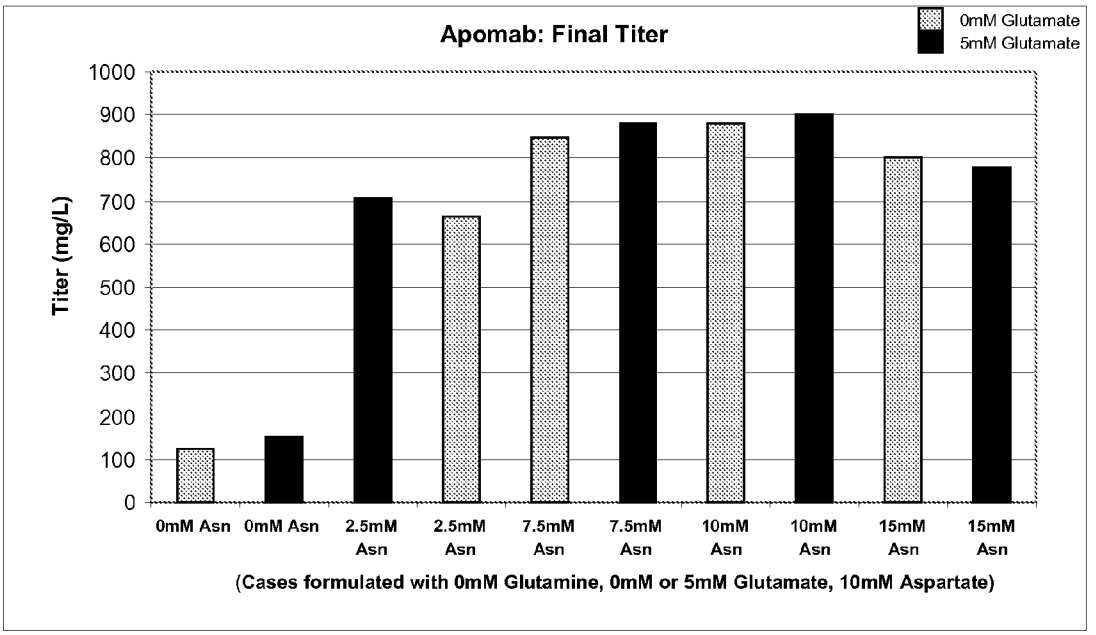
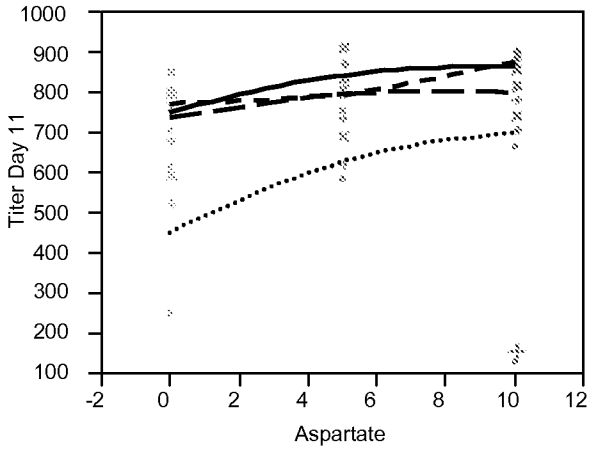

Apomab: Final Titer
0mM Glutamate
5mM Glutamate
Titer (mg/L)
1000
900
800
700
600
500
400
300
200
100
0
0mM Asn
0mM Asn
2.5mM Asn
2.5mM Asn
7.5mM Asn
7.5mM Asn
10mM Asn
10mM Asn
15mM Asn
15mM Asn
(Cases formulated with 0mM Glutamine, 0mM or 5mM Glutamate, 10mM Aspartate)

FIG. 4

Bivariate Fit of Titer Day 11 By Aspartate Glutamine=0



Smoothing Spline Fit, lambda=1 Asparagine==2.5
Smoothing Spline Fit, lambda=1 Asparagine==7.5
Smoothing Spline Fit, lambda=1 Asparagine==10
Smoothing Spline Fit, lambda=1 Asparagine==15

Smoothing Spline Fit, lambda=1 Asparagine==2.5

R-Square	0.573894
Sum of Squares Error	73461.63

Smoothing Spline Fit, lambda=1 Asparagine==7.5

R-Square	0.65596
Sum of Squares Error	11556.31

Smoothing Spline Fit, lambda=1 Asparagine==10

R-Square	0.408718
Sum of Squares Error	25684.65

Smoothing Spline Fit, lambda=1 Asparagine==15

R-Square	0.220438
Sum of Squares Error	26400.64

FIG. 5

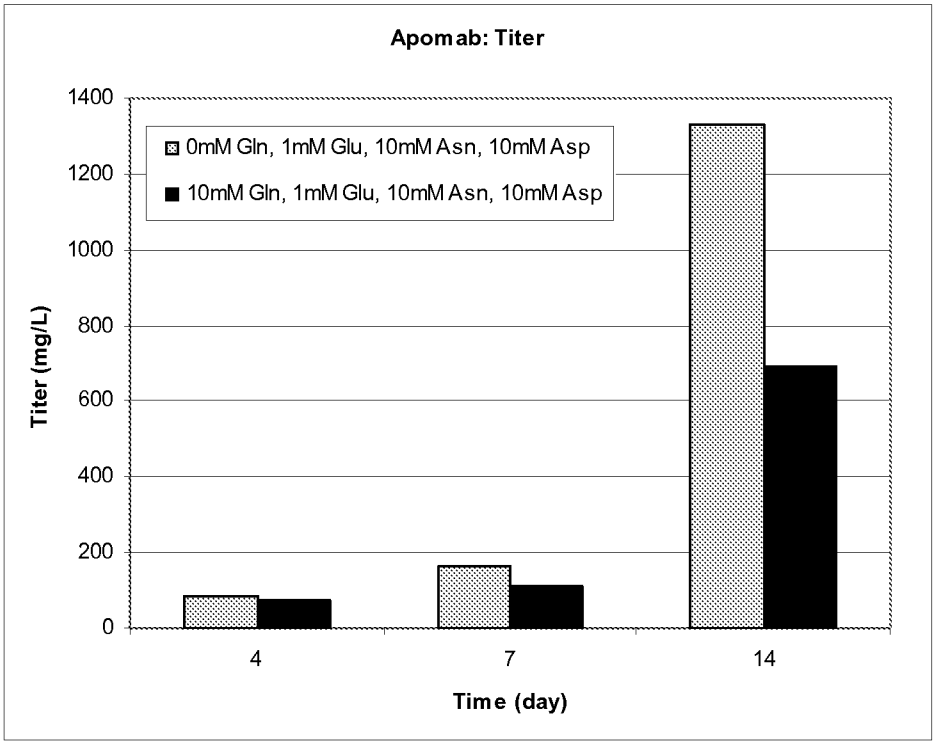

Apomab: Titer
1400
1200
1000
800
600
400
200
0
Titer (mg/L)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

FIG. 6A

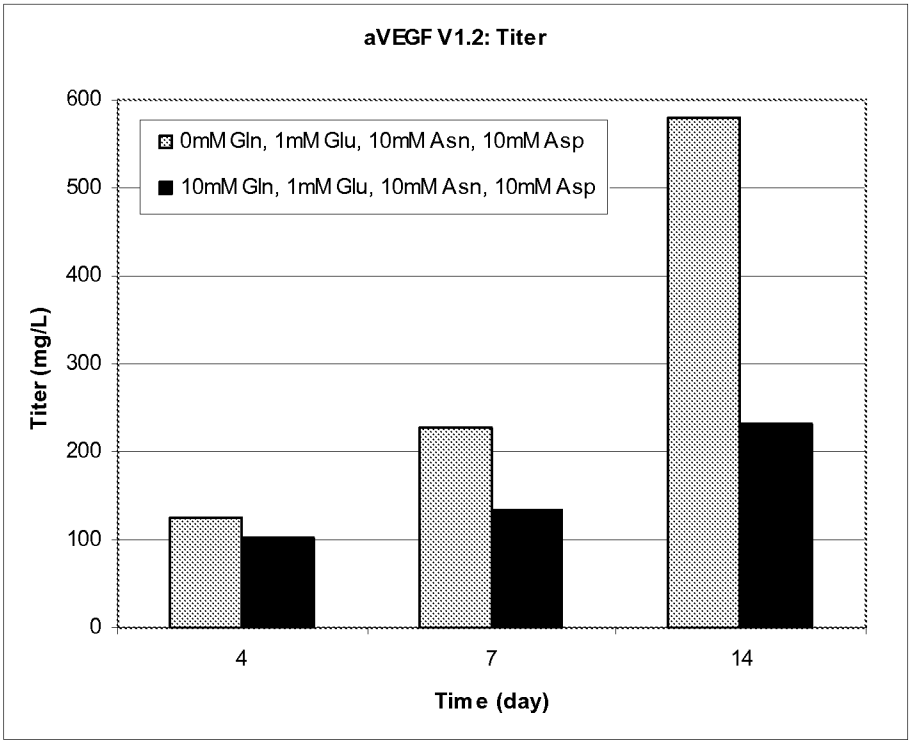

aVEGF V1.2: Titer
600
500
400
300
200
100
0
Titer (mg/L)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

FIG. 6B

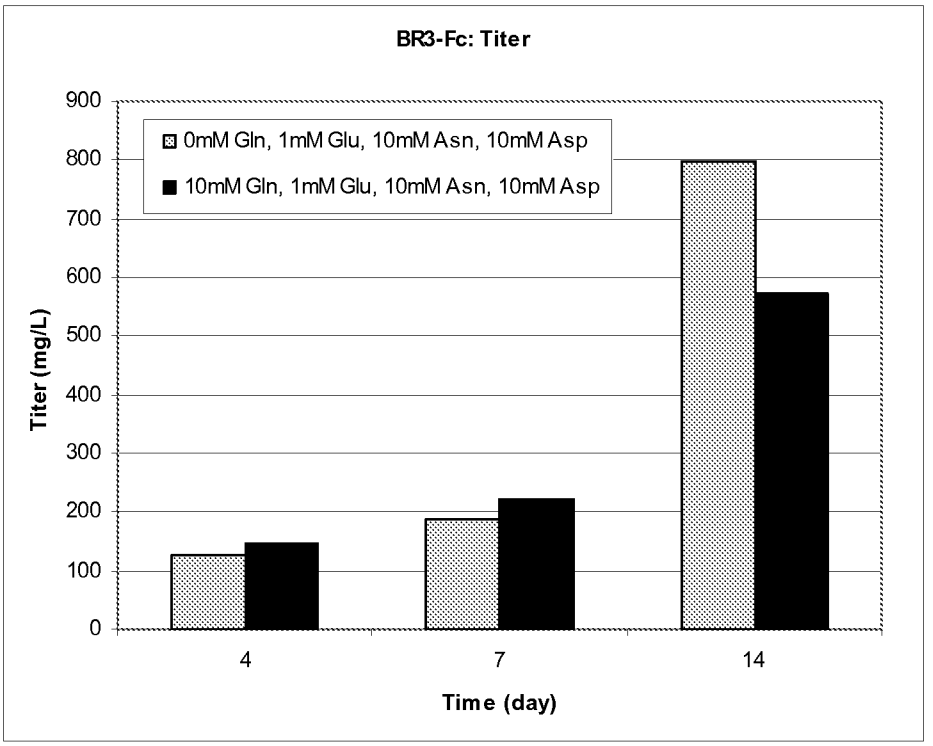

BR3-Fc: Titer
900
800
700
600
500
400
300
200
100
0
Titer (mg/L)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

FIG. 6C

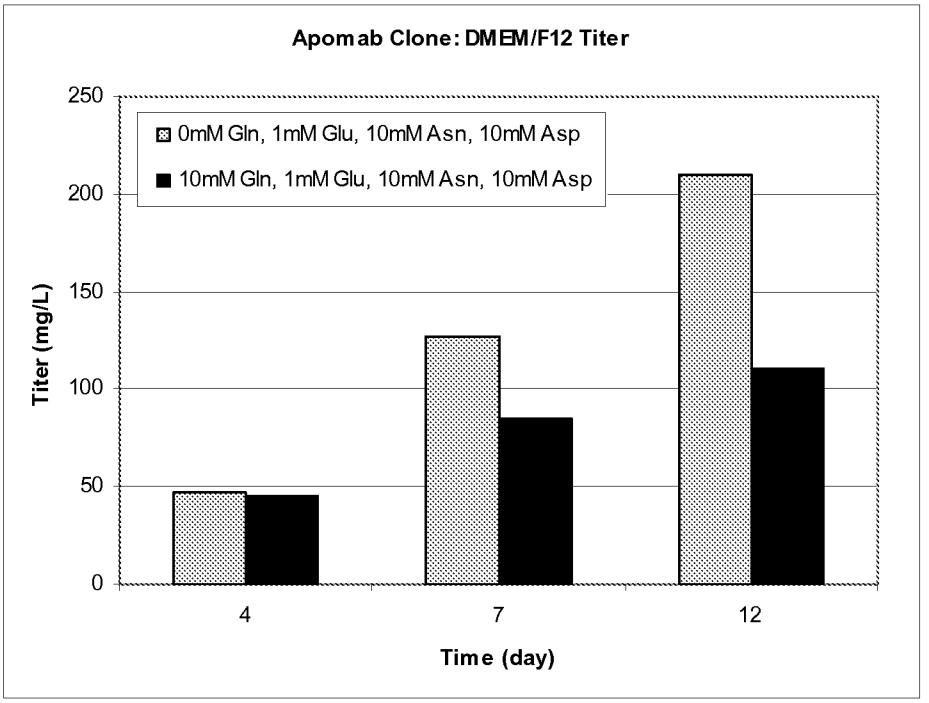

Apomab Clone: DMEM/F12 Titer
250
200
150
100
50
0
Titer (mg/L)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
12
Time (day)

FIG. 7A

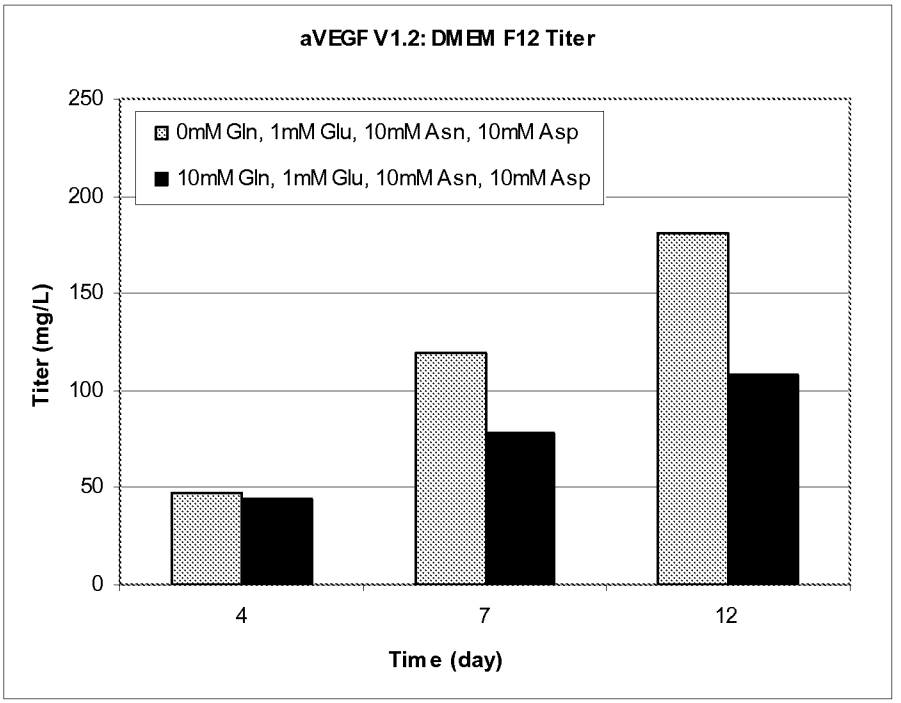

aVEGF V1.2: DMEM F12 Titer
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Titer (mg/L)
250
200
150
100
50
0
4
7
12
Time (day)

FIG. 7B

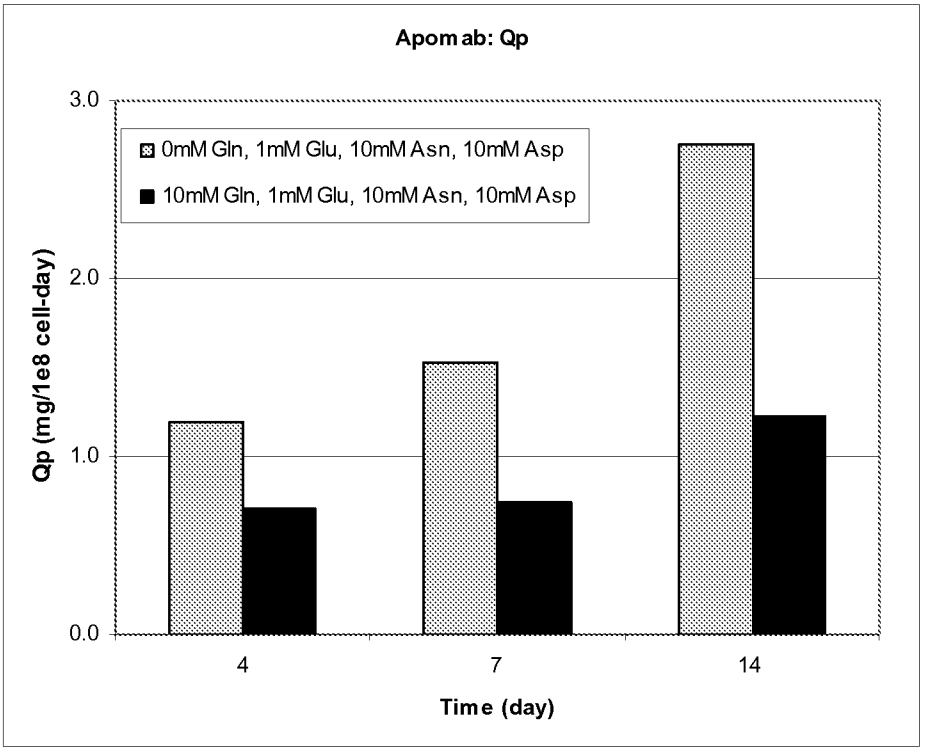

Apomab: Qp
3.0
2.0
1.0
0.0
Qp (mg/1e8 cell-day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

FIG. 8A

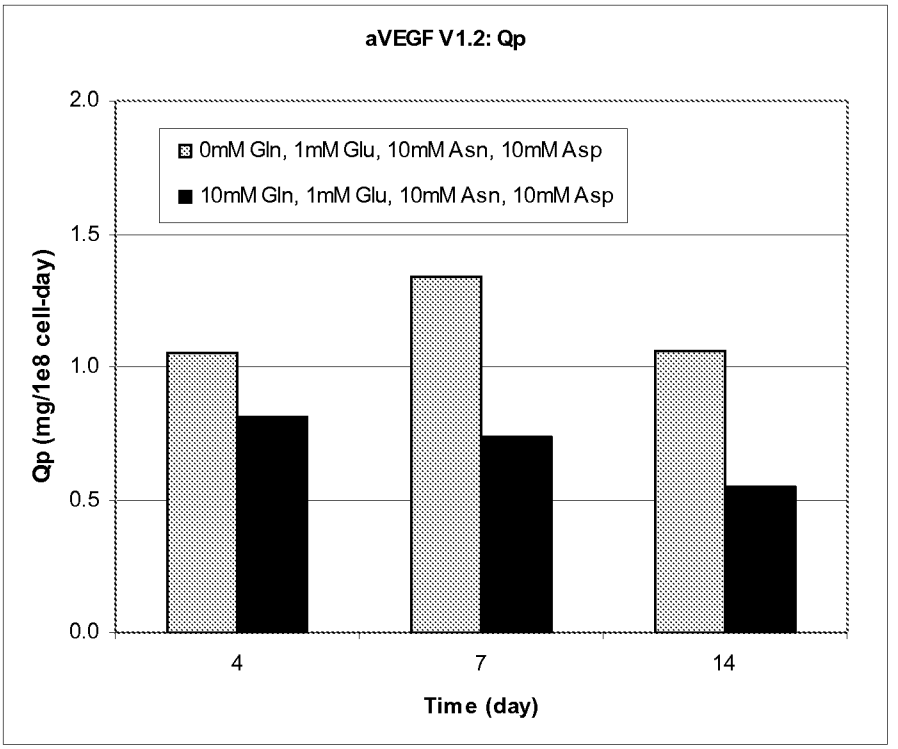

aVEGF V1.2: Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
2.0
1.5
1.0
0.5
0.0
4
7
14
Time (day)

FIG. 8B

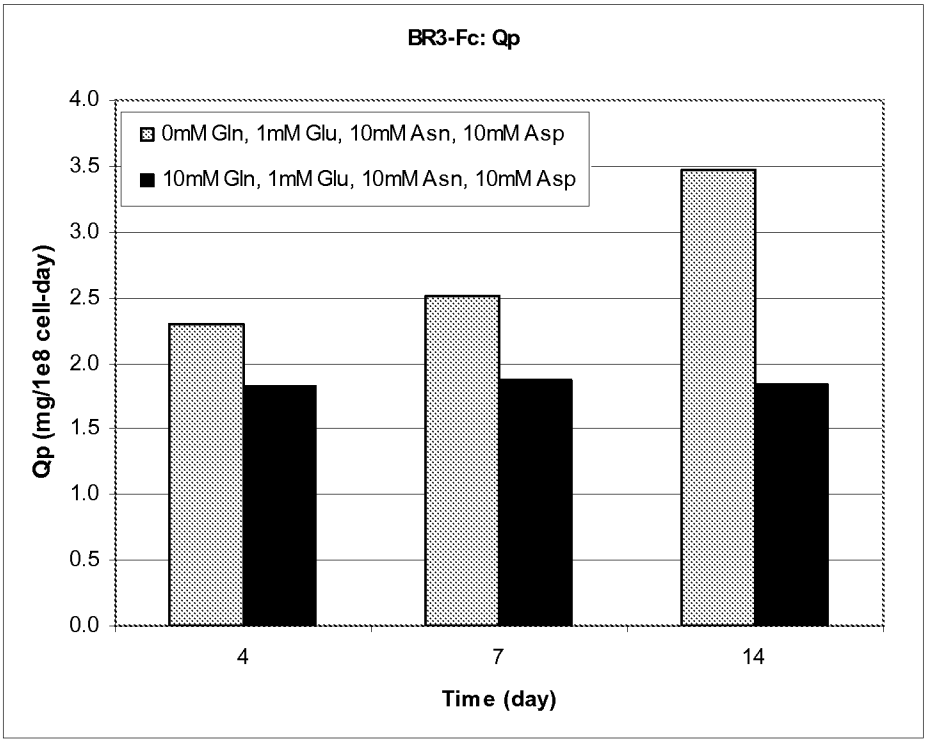

BR3-Fc: Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
7
14
Time (day)

FIG. 8C

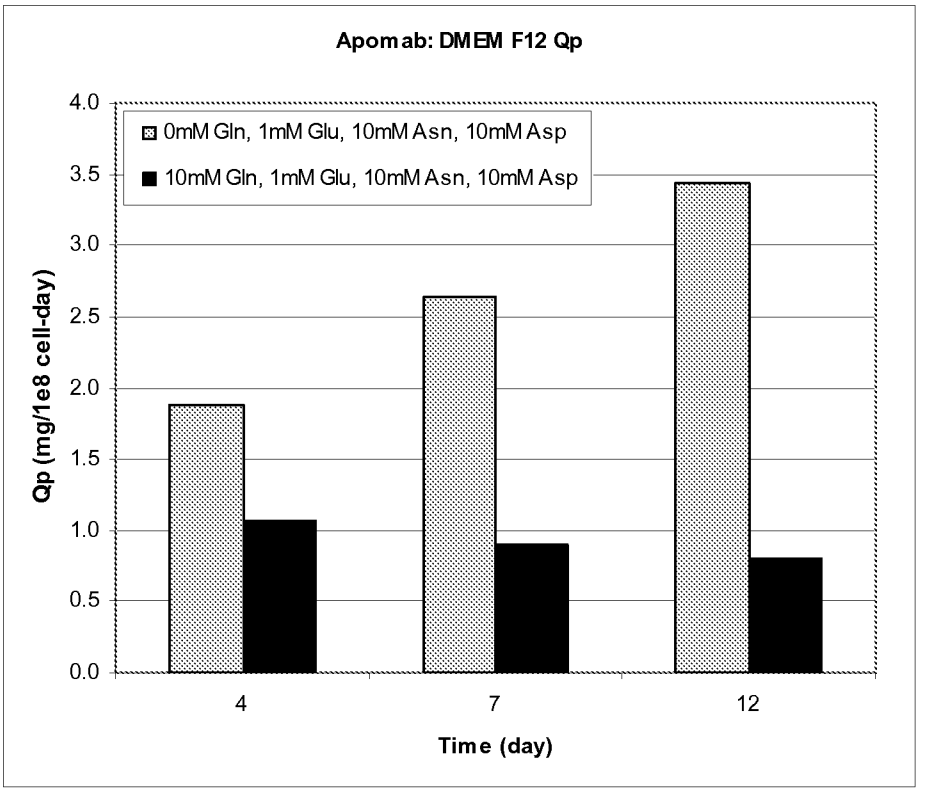

Apomab: DMEM F12 Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
7
12
Time (day)

FIG. 9A

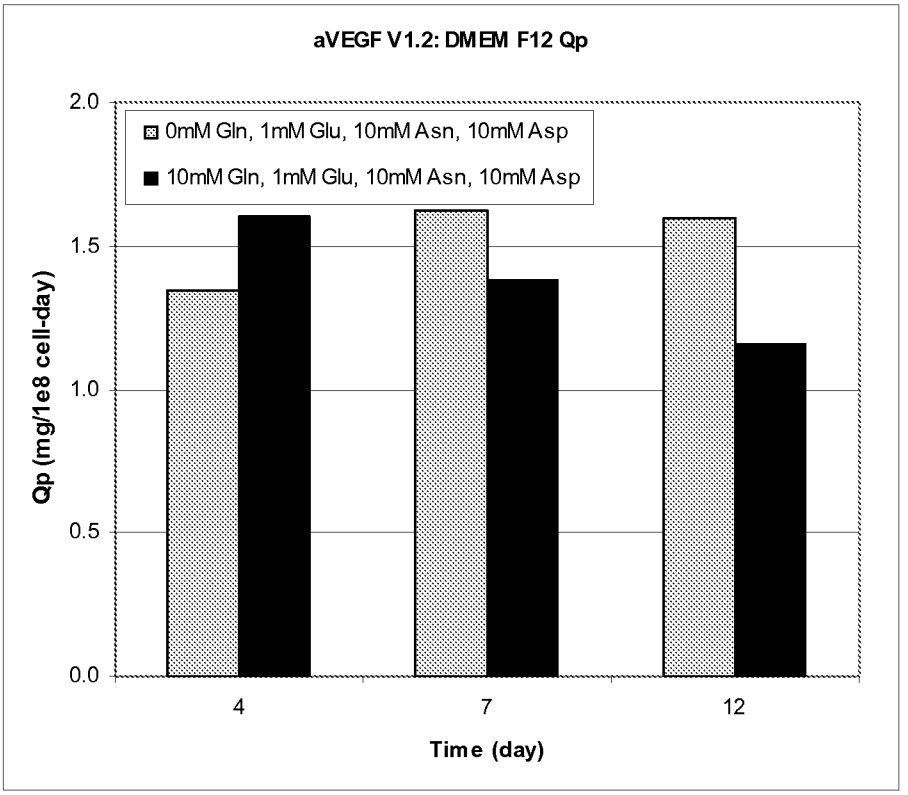

aVEGF V1.2: DMEM F12 Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
2.0
1.5
1.0
0.5
0.0
4
7
12
Time (day)

FIG. 9B

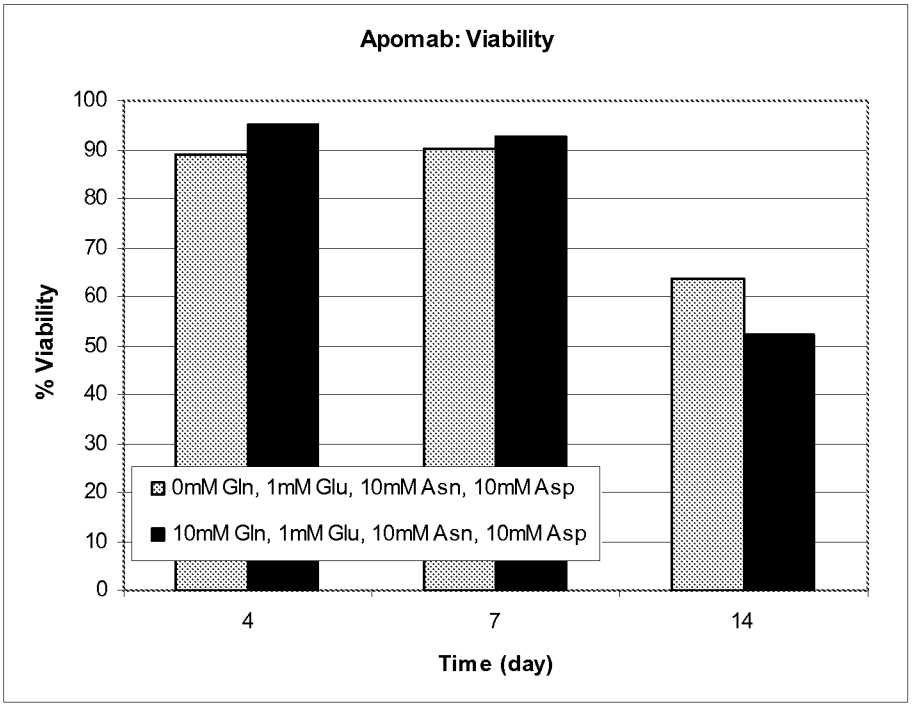

Apomab: Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
14
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

FIG. 10A

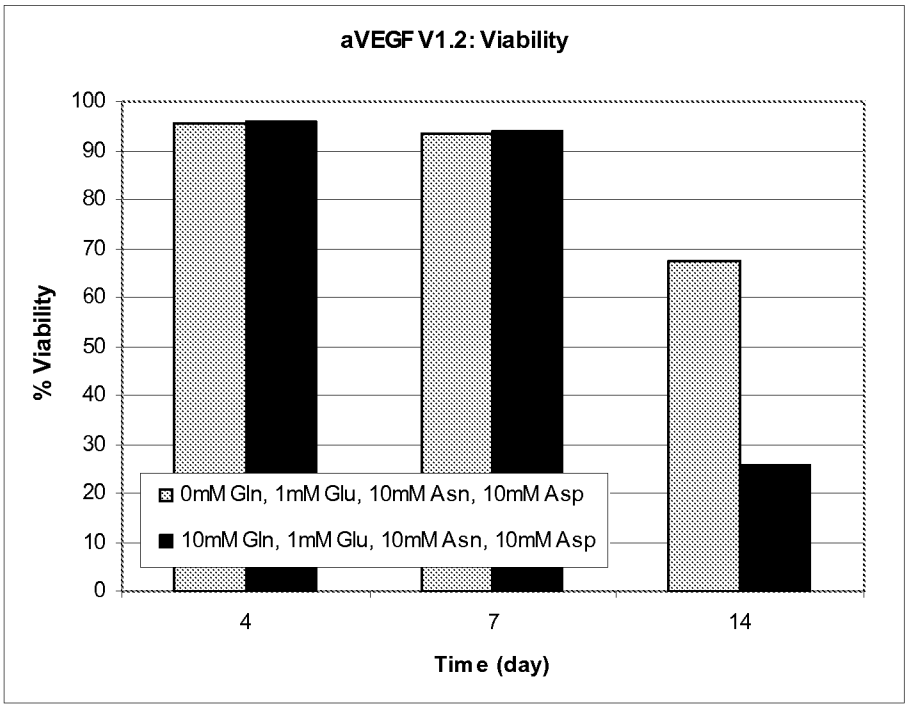

aVEGF V1.2: Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
14
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

FIG. 10B

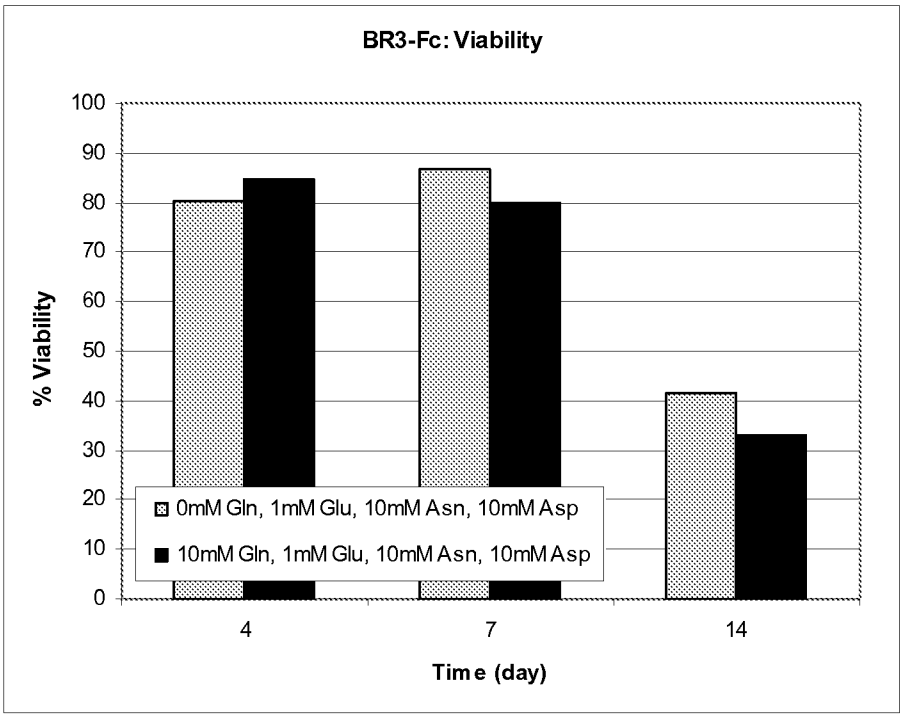

BR3-Fc: Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
14
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

FIG. 10C

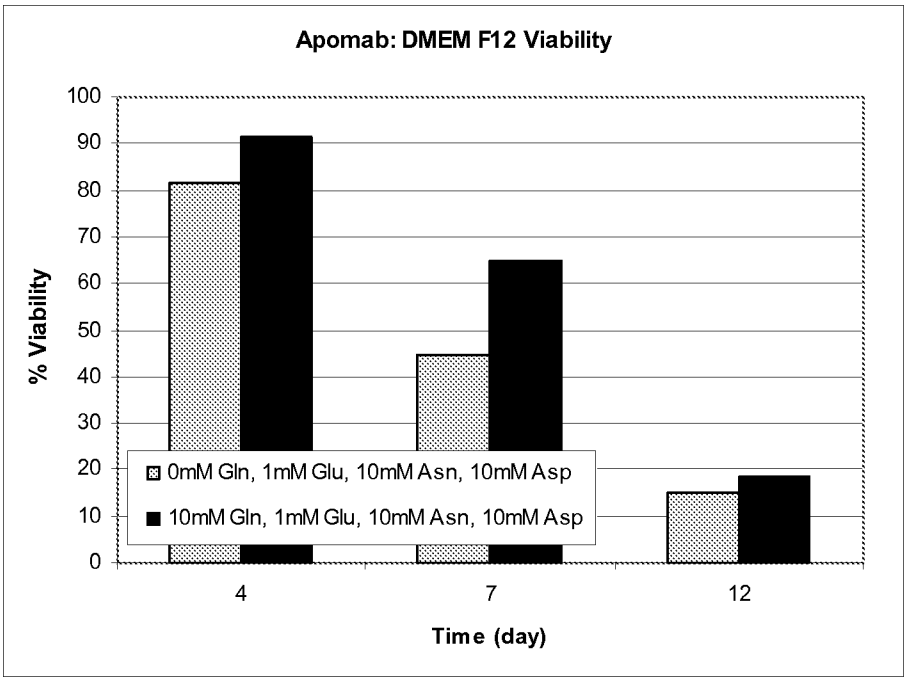

Apomab: DMEM F12 Viability
100
90
80
70
60
50
40
30
20
10
0
% Viability
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
12
Time (day)

FIG. 11A

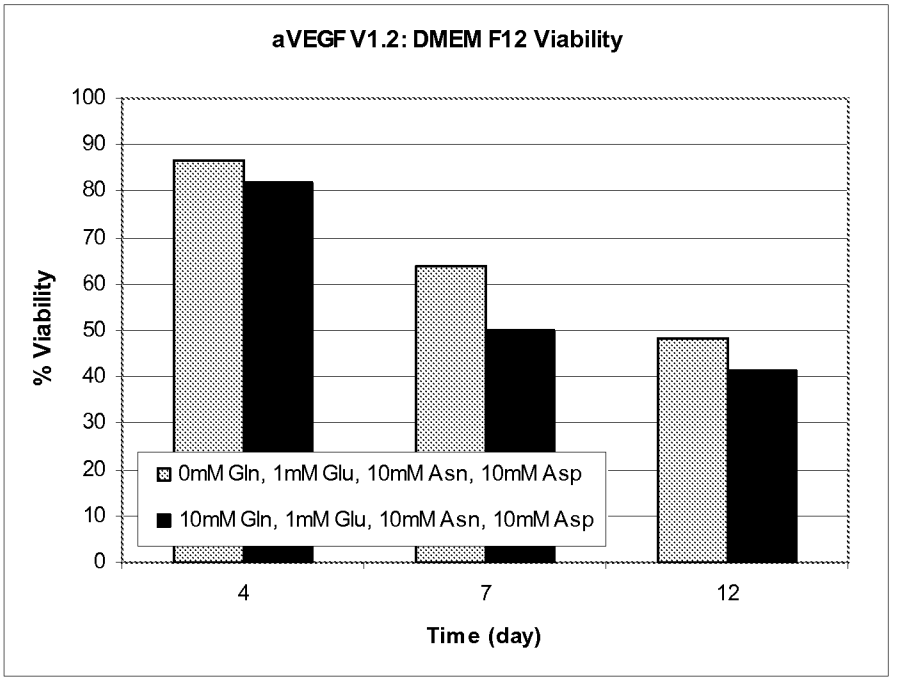

aVEGF V1.2: DMEM F12 Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
12
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

FIG. 11B

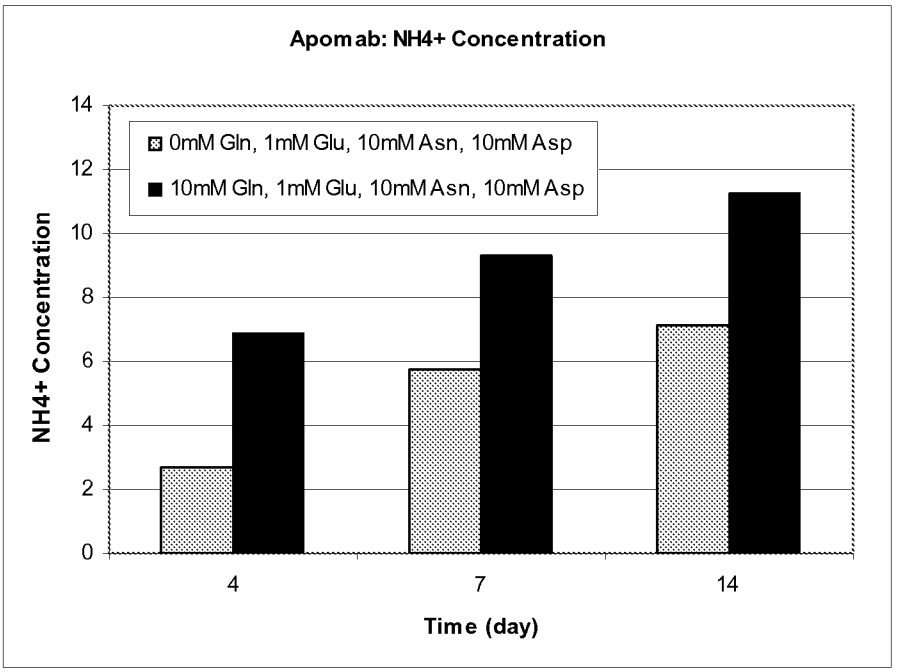

Apomab: NH4+ Concentration
NH4+ Concentration
0
2
4
6
8
10
12
14
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

FIG. 12A

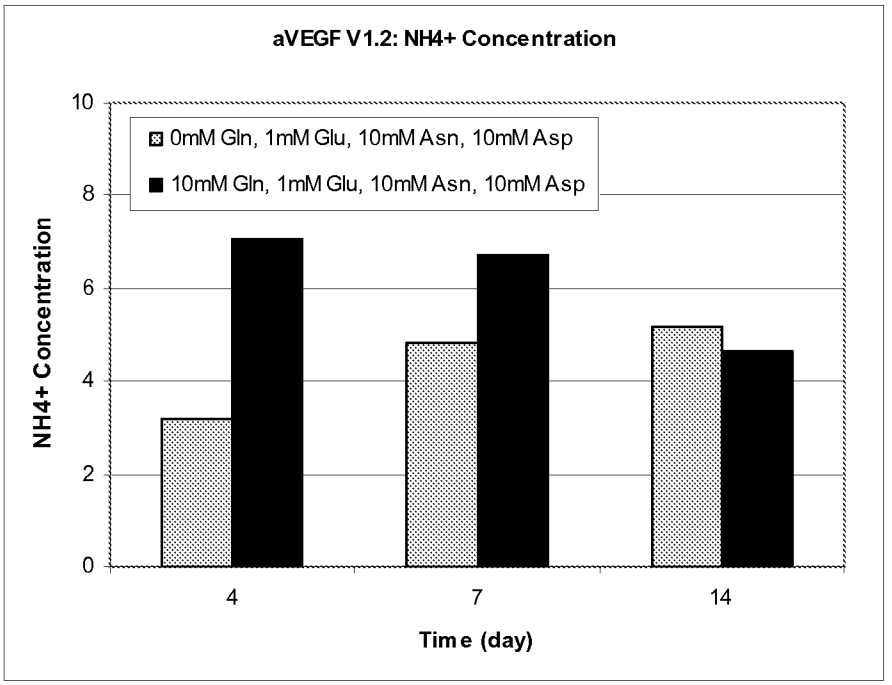

aVEGF V1.2: NH4+ Concentration
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
NH4+ Concentration
10
8
6
4
2
0
4
7
14
Time (day)

FIG. 12B

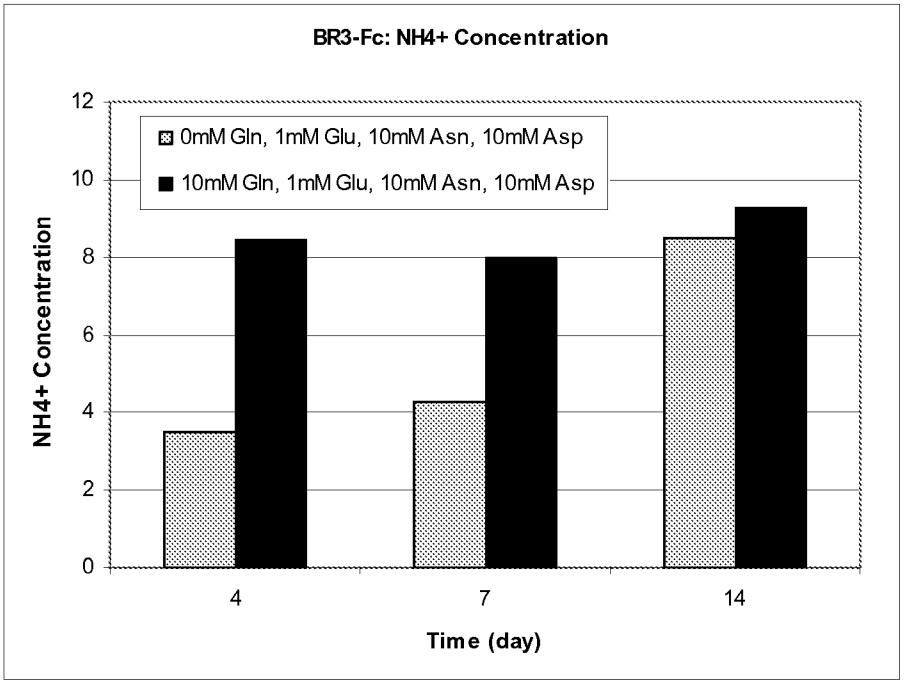

BR3-Fc: NH4+ Concentration
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
NH4+ Concentration
0
2
4
6
8
10
12
4
7
14
Time (day)

FIG. 12C

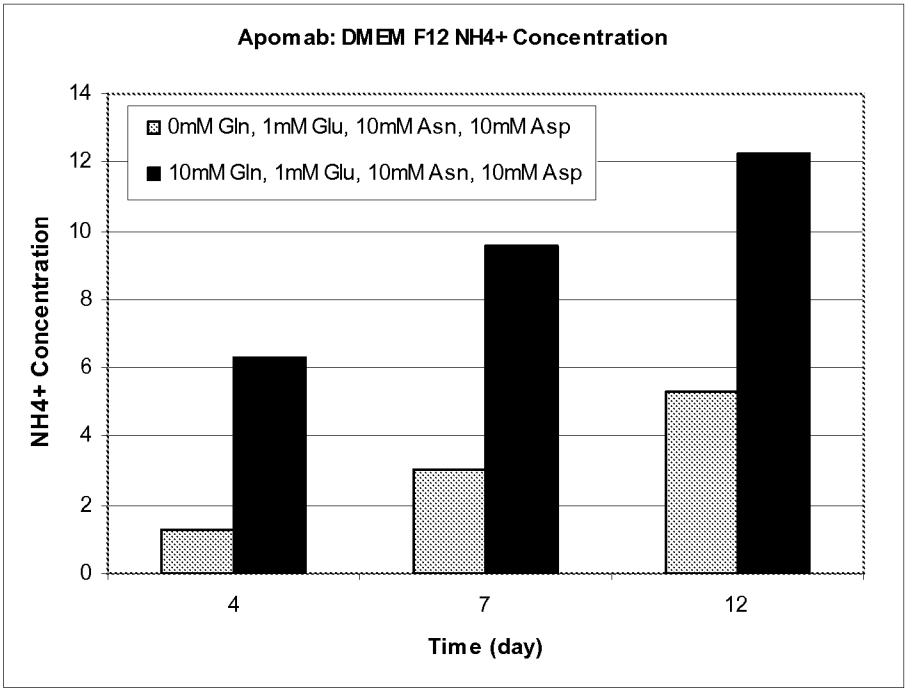

Apomab: DMEM F12 NH4+ Concentration
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
NH4+ Concentration
0
2
4
6
8
10
12
14
4
7
12
Time (day)

FIG. 13A

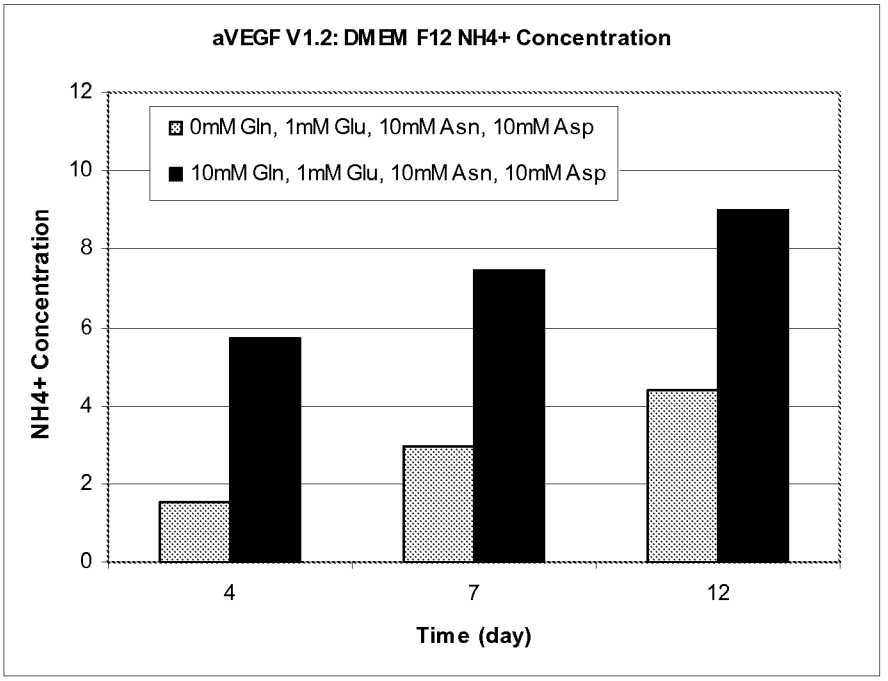

aVEGF V1.2: DMEM F12 NH4+ Concentration
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
NH4+ Concentration
0
2
4
6
8
10
12
4
7
12
Time (day)

FIG. 13B

PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA

RELATED APPLICATIONS

This application claims the benefit under 35 USC §119 to U.S. Provisional Application 61/232,889 filed Aug. 11, 2009.

FIELD OF THE INVENTION

The present invention relates generally to glutamine-free cell culture media. The invention further concerns the production of recombinant proteins, such as antibodies, in glutamine-free mammalian cell culture.

BACKGROUND OF THE INVENTION

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. It is conventional to have glutamine in cell culture media during recombinant production of heterologous proteins, including antibodies. L-glutamine is an essential amino acid, which is considered the primary energy and nitrogen sources for cells in culture. Most commercially available media are formulated with free L-glutamine which is either included in the basal formula or added to liquid media formulations at the time of use. Thus, all mammalian cell culture media contain glutamine except those for glutamine synthetase transfected cell lines, such as GS NS0 and GS CHO cell lines, where the cells themselves produce the glutamine needed for growth. Glutamine is widely used at various concentrations typically from 1 to 20 mM in base media and much higher concentration in feeds for fed-batch process. For example, the concentration of L-glutamine is 0.5 mM in Ames' Medium and 10 mM in MCDP Media 131. DMEM/Ham's Nutrient Mixture F-12 (50:50) is often used as a starting formulation for proprietary media used with Chinese Hamster Ovary (CHO) cells. L-glutamine in DMEM/Ham's Nutrient Mixture F-12 is 2.5 mM. L-glutamine concentration in Serum-Free/Protein Free Hybridoma Medium is 2.7 mM. L-glutamine in DMEM, GMEM, IMDM and H-Y medium is 4 mM, of which IMDM is often used as a starting formulation for proprietary hybridoma cell culture media. It is generally held that hybridoma cells grow better in concentrations of L-glutamine that are above the average levels found in media. (Dennis R. Conrad, Glutamine in Cell Culture, Sigma-Aldrich Media Expert)

It was shown that glutamine is the main source of ammonia accumulated in cell culture (see review by Markus Schneider, et. al. 1996, *Journal of Biotechnology* 46:161-185). Thus, lowering glutamine in cell culture media significantly reduced the accumulation of NH_4^+ level, resulting in lower cytotoxicity (see Markus Schneider, et. al. 1996, supra). Reduced NH_4^+ cytotoxicity resulted in higher cell viability, thus extended culture longevity. Based on an estimated glutamine consumption study using CHO cells, it was suggested that cells may consume glutamine at a rate of 0.3-0.4 mM per day (Miller, et. al. 1988, Biotechnol. Bioeng. 32: 947-965). Altamirano et al. (2001, *J. Biotechnol.* 110:171-9) studied the effect of glutamine replacement by glutamate and the balance between glutamate and glucose metabolism on the redistribution of CHO cells producing recombinant human tissue plasminogen activator (rhut-PA). When glutamine was replaced with glutamate and balanced with glucose catabolism (carbon and nitrogen ratio, C/N ratio), cell metabolism was found redistributed and forced to utilize carbon and energy source more favorably to production of rhut-PA. It was also reported that CHO cells in adherent cultures can grow in the absence of added glutamine due to endogenous glutamine synthetase activity that allowed cells to synthesize glutamine from glutamic acid in the medium (Sanfeliu and Stephanopoulos, 1999, *Biotechnol. Bioeng.* 64:46-53). However, compared to control cultures in glutamine-containing media, the cell growth rate in glutamine-free media was slower with an increased fraction of cells distributed in the G0/G1 phase. The depletion of both glutamine and glutamic acid did cause cell death.

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the unexpected finding that not only can recombinant proteins be produced in a mammalian host cell using a glutamine-free production medium without any significant adverse effect, in fact the use of a glutamine-free medium in the production phase significantly increases cell viability, culture longevity, specific productivity and/or the final recombinant protein titer.

The present invention is also based on the unexpected finding that the addition of asparagine to a glutamine-free production medium can further enhance the cell viability, culture longevity, specific productivity and/or the final recombinant protein titer in a mammalian host cell using a glutamine-free production medium without any significant adverse effect.

In one aspect, the invention concerns a process for producing a polypeptide in a mammalian host cell expressing said polypeptide, comprising culturing the mammalian host cell in a production phase of the culture in a glutamine-free production culture medium supplemented with asparagine.

In one embodiment, the mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

In another embodiment, the mammalian host cell is a $dhfr^-$ CHO cell.

In yet another embodiment, the production medium is serum-free.

In a further embodiment, the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

In a still further embodiment, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

In all embodiments, the production phase may, for example, be a batch or fed batch culture phase.

In all embodiments, the process may further comprise the step of isolating said polypeptide.

In a further embodiment, isolation may be followed by determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

In a still further embodiment, at least one of the cell viability, culture longevity, specific productivity and final recom-

binant protein titer is increased relative to the same polypeptide produced in a glutamine-containing production medium of the same composition.

In a further aspect, the invention concerns a ready-to-use glutamine-free cell culture medium for the production of a polypeptide in a production phase.

In yet another embodiment, the polypeptide is a mammalian glycoprotein.

In other embodiments, the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', $F(ab')_2$, scFv, $(scFv)_2$, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

In a still further embodiment, the antibody or antibody fragment is chimeric, humanized or human.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $a43_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-αvβ3 antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In other embodiments, the therapeutic antibody is an anti-BR3 antibody or BR3-Fc immunoadhesin.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD 18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell. such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

These and other aspects will be apparent from the description below, including the Examples and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Apomab antibody cube plot analysis of titer results from a Full Factorial Design of Experiment (DOE) evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **2**. BR3-Fc immunoadhesin cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **3**. anti-VEGF antibody cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **4**. Effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

FIG. **5**. Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free

and low Glutamate conditions. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

FIGS. **6**. A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **7**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM F12 medium.

FIGS. **8**A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **9**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

FIGS. **10**A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. Cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **11**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. In DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium. Viability was higher for Apomab antibody, but lower for anti-VEGF antibody compared to Glutamine containing medium.

FIGS. **12** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation Ammonia was usually lower in Glutamine-free cultures compared to Glutamine-containing cultures.

FIGS. **13**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation. Ammonia was significantly reduces in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The terms "cell culture medium", "culture medium", and "nutrient mixture" refer to a nutrient solution used for growing mammalian cells that typically provides at least one component from one or more of the following categories:

1) an energy source, usually in the form of a carbohydrate such as glucose;

2) some or all of the essential amino acids, and usually the basic set of twenty amino acids plus cystine;

3) vitamins and/or other organic compounds typically required at low concentrations;

4) free fatty acids; and

5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range.

The nutrient mixture may optionally be supplemented with one or more component from any of the following categories:

1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor;

2) salts and buffers as, for example, calcium, magnesium, and phosphate; and

3) nucleosides such as, for example, adenosine and thymidine.

The cell culture medium is generally "serum free" when the medium is essentially free of serum from any mammalian source (e.g. fetal bovine serum (FBS)). By "essentially free" is meant that the cell culture medium comprises between about 0-5% serum, preferably between about 0-1% serum, and most preferably between about 0-0.1% serum. Advantageously, serum-free "defined" medium can be used, wherein the identity and concentration of each of the components in the medium is known (i.e., an undefined component such as bovine pituitary extract (BPE) is not present in the culture medium).

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed (host) cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "animal host cell," "animal cell," "animal recombinant host cell," and the like, encompasses invertebrate, non-mammalian vertebrate (e.g., avian, reptile and amphibian) and mammalian cells. Examples of invertebrate cells include the following insect cells: *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori*. See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in *Genetic Engineering*, Setlow, J. K. et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature,* 315:592-594 (1985).

The term "mammalian host cell," "mammalian cell," "mammalian recombinant host cell," and the like, refer to cell lines derived from mammals that are capable of growth and survival when placed in either monolayer culture or in suspension culture in a medium containing the appropriate nutrients and growth factors. The necessary nutrients and growth factors for a particular cell line are readily determined empirically without undue experimentation, as described for example in *Mammalian Cell Culture* (Mather, J. P. ed., Plenum Press, N. Y. (1984)), and by Barnes and Sato (*Cell,* 22:649 (1980)). Typically, the cells are capable of expressing and secreting large quantities of a particular protein of interest (typically a recombinant protein) into the culture medium, and are cultured for this purpose. However, the cells may be cultured for a variety of other purposes as well, and the scope of this invention is not limited to culturing the cells only for production of recombinant proteins. Examples of suitable mammalian cell lines, capable of growth in the media of this

invention, include monkey kidney CVI line transformed by SV40 (COS-7, ATCC® CRL 1651); human embryonic kidney line 293S (Graham et al., *J. Gen. Virolo.,* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC® CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23:243 (1980)); monkey kidney cells (CVI-76, ATCC® CCL 70); African green monkey kidney cells (VERO-76, ATCC® CRL-1587); human cervical carcinoma cells (HELA, ATCC® CCL 2); canine kidney cells (MDCK, ATCC® CCL 34); buffalo rat liver cells (BRL 3A, ATCC® CRL 1442); human lung cells (W138, ATCC® CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC® CCL 5I); rat hepatoma cells (HTC, MI.54, Baumann et al., J. Cell Biol., 85:1 (1980)); and TR-1 cells (Mather et al., *Annals N.Y. Acad. Sci.,* 383:44 (1982)) and hybridoma cell lines. Chinese hamster ovary cells (Urlab and Chasin, *Proc. Natl. Acad. Sci. USA,* 77:4216 (1980)) are a preferred cell line for practicing this invention. CHO cells suitable for use in the methods of the present invention have also been described in the following documents: EP 117,159, published Aug. 29, 1989; U.S. Pat. Nos. 4,766,075; 4,853,330; 5,185,259; Lubiniecki et al., in *Advances in Animal Cell Biology and Technology for Bioprocesses*, Spier et al., eds. (1989), pp. 442-451. Known CHO derivatives suitable for use herein include, for example, CHO/-DHFR (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 (1980)), CHO-K1 DUX B11 (Simonsen and Levinson, *Proc. Natl. Acad. Sci. USA* 80: 2495-2499 (1983); Urlaub and Chasin, supra), and dp 12.CHO cells (EP 307,247 published Mar. 15, 1989). Preferred host cells include CHO-K1 DUX B11 and dp 12.CHO cells.

"dhfr$^-$ CHO cell" refers to a dihydrofolate reductase (DHFR) deficient CHO cell. Production of recombinant proteins in mammalian cells has allowed the manufacture of a number of large, complex glycosylated polypeptides for clinical applications. Chinese hamster ovary (CHO) DHFR– cells and the amplifiable selectable marker DHFR are routinely used to establish cell lines that produce clinically useful amounts of product. (Urlab, G. and Chasin, L. A. (1980) Proc. Natl Acad. Sci. USA, 77, 4216-4220; Kaufman, R. J. and Sharp, P. (1982) J. Mol. Biol., 159, 601-621; Gasser, C. S., Simonsen, C. S., Schilling, J. W. and Schmike, R. T. (1982) Proc. Natl Sci. USA, 79, 6522-6526)

By "phase" is meant a certain phase of culturing of the cells as is well recognized by the practitioner.

"Growth phase" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are generally rapidly dividing. During this phase, cells are cultured for a period of time, usually between 1-4 days, and under such conditions that cell growth is maximized. The growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. During the growth phase, cells are cultured in nutrient medium containing the necessary additives generally at about 30-40° C., preferably about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line. Cells are maintained in the growth phase for a period of between about one and four days, usually between about two and three days.

"Transition phase" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as temperature are shifted from growth conditions to production conditions.

"Production phase" of the cell culture refers to the period of time during which cell growth has plateaued. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product.

The phrase "fed batch cell culture" when used herein refers to a batch culture wherein the animal (e.g. mammalian) cells and culture medium are supplied to the culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. Fed batch culture includes "semi-continuous fed batch culture" wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple "batch culture" in which all components for cell culturing (including the animal cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc and the culture medium is continuously or intermittently introduced and removed from the culturing vessel). However, removal of samples for testing purposes during fed batch cell culture is contemplated.

When used herein, the term "glutamine" refers to the amino acid L-glutamine (also known as "Gln" and "Q" by three-letter and single-letter designation, respectively) which is recognized as both an amino acid building block for protein synthesis and as an energy source in cell culture. Thus, the terms "glutamine" and "L-glutamine" are used interchangeably herein.

The word "glucose" refers to either of α-D-glucose or β-D-glucose, separately or in combination. It is noted that α and β glucose forms are interconvertible in solution.

The expression "osmolality" is a measure of the osmotic pressure of dissolved solute particles in an aqueous solution. The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water (1 mOsm/kg H_2O at 38° C. is equivalent to an osmotic pressure of 19 mm Hg). "Osmolarity" refers to the number of solute particles dissolved in 1 liter of solution. Solutes which can be added to the culture medium so as to increase the osmolality thereof include proteins, peptides, amino acids, non-metabolized polymers, vitamins, ions, salts, sugars, metabolites, organic acids, lipids, etc. In the preferred embodiment, the concentration of amino acids and NaCl in the culture medium is increased in order to achieve the desired osmolality ranges set forth herein. When used herein, the abbreviation "mOsm" means "milliosmoles/kg H_2O".

The term "cell density" as used herein refers to that number of cells present in a given volume of medium.

The term "cell viability" as used herein refers to the ability of cells in culture to survive under a given set of culture conditions or experimental variations. The term as used herein also refers to that portion of cells which are alive at a particular time in relation to the total number of cells, living and dead, in the culture at that time.

The terms "amino acids" and "amino acid" refer to all naturally occurring alpha amino acids in both their D and L stereoisomeric forms, and their analogs and derivatives. An analog is defined as a substitution of an atom in the amino acid with a different atom that usually has similar properties. A derivative is defined as an amino acid that has another molecule or atom attached to it. Derivatives would include, for

example, acetylation of an amino group, amination of a carboxyl group, or oxidation of the sulfur residues of two cysteine molecules to form cystine.

The term "protein" is meant to refer to a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term "therapeutic protein" or "therapeutic polypeptide" refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. "Manufacturing scale" production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

As used herein, "polypeptide of interest" refers generally to peptides and proteins having more than about ten amino acids. The polypeptides may be homologous to the host cell, or preferably, may be exogenous, meaning that they are heterologous, i.e., foreign, to the host cell being utilized, such as a human protein produced by a non-human mammalian, e.g., Chinese Hamster Ovary (CHO) cell. Preferably, mammalian polypeptides (polypeptides that were originally derived from a mammalian organism) are used, more preferably those which are directly secreted into the medium. The term "polypeptide" or "polypeptide of interest" specifically includes antibodies, in particular, antibodies binding to mammalian polypeptides, such as any of the mammalian polypeptides listed below or fragments thereof, as well as immunoadhesins (polypeptide-Ig fusion), such as those comprising any of the mammalian polypeptides listed below, or fragments thereof.

Examples of mammalian polypeptides include, without limitation, transmembrane molecules (e.g. receptors) and ligands such, as growth factors. Exemplary polypeptides include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; interferon such as interferon-α, -β, and -γ; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA), including t-PA variants; bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as β-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER1 (EGFR), HER2, HER3 or HER4 receptor; Apo2L/TRAIL, hedgehog, mitogen activated protein kinase (MAPK), and fragments of any of the above-listed polypeptides. Apo2L (TRAIL) and is variants are disclosed, for example, in U.S. Application Publication No. 20040186051. Anti-VEGF antibodies are disclosed, for example, in U.S. Pat. Nos. 8,994,879; 7,060,269; 7,169,901; and 7,297,334. Anti-CD20 antibodies are disclosed, for example, in U.S. Application Publication No. 20060246004. The BR3 polypeptide, anti-BR3 antibodies and BR3-Fc immunoadhesins are described, for example, in U.S. Application Publication No. 20050070689.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin") with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

As noted above, in certain embodiments, the protein is an antibody. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region or intact monoclonal antibodies), antibody compositions with polyepitopic specificity, polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibod-

ies) formed from at least two intact antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, F(ab')2, and Fv).

Unless indicated otherwise, the expression "multivalent antibody" is used throughout this specification to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is typically engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fe" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward et al., *Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) F(ab')2 fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (e.g. single chain Fv; scFv) (Bird et al., *Science* 242: 423-426 (1988); and Huston et al., *PNAS* (*USA*) 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, e.g., EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al. *Protein Eng.* 8(10):1057 1062 (1995); and U.S. Pat. No. 5,641,870).

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; W093/1161; Hudson et al., (2003) Nat. Med. 9:129-134; and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) Nat. Med. 9:129-134.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. Monoclonal antibodies are highly specific, being directed against a single antigen. In certain embodiments, a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody,

etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler and Milstein, *Nature,* 256:495-97 (1975); Hongo et al., *Hybridoma,* 14 (3): 253-260 (1995), Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage-display technologies (see, e.g., Clackson et al., *Nature,* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1991); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, e.g., Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409. See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.,* 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, e.g., immunized xenomice (see, e.g., U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li et al., *Proc. Natl. Acad. Sci. USA,* 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology. The humanized antibody may also include a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. *Nature Biotechnology* 14:309-314 (1996): Sheets et al. *PNAS* (*USA*) 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro). See, e.g., Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.,* 147 (1):86-95 (1991); and U.S. Pat. No. 5,750,373.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., Proc Nat. Acad. Sci. USA 91:3809-3813 (1994); Schier et al., Gene 169:147-155 (1995); Yelton et al., J. Immunol. 155:1994-2004 (1995); Jackson et al., J. Immunol. 154(7):3310-9 (1995); and Hawkins et al., J. Mol. Biol. 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a beta-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences *of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region," "HVR," or "HV," when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. For example, the term hypervariable region refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, e.g., Xu et al., *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, N.J., 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, e.g., Hamers-Casterman et al., *Nature* 363:446-448 (1993); Sheriff et al., *Nature Struct. Biol.* 3:733-736 (1996).

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and Mol. Immunology, 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The term "Fc region" is used to define the C-terminal region of an immunoglobulin heavy chain which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain.

By "Fc region chain" herein is meant one of the two polypeptide chains of an Fc region.

The "CH2 domain" of a human IgG Fc region (also referred to as "Cg2" domain) is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, *Molec. Immunol.* 22:161-206 (1985). The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain.

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region. The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced "protroberance" in one chain thereof and a corresponding introduced "cavity" in the other chain thereof; see U.S. Pat. No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to make multispecific (e.g. bispecific) antibodies as herein described.

"Hinge region" herein may be a native sequence hinge region or a variant hinge region. The two polypeptide chains of a variant hinge region generally retain at least one cysteine residue per polypeptide chain, so that the two polypeptide chains of the variant hinge region can form a disulfide bond between the two chains. The preferred hinge region herein is a native sequence human hinge region, e.g. a native sequence human IgG1 hinge region.

A "functional Fc region" possesses at least one "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native

sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

An "intact" antibody is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2 and C_H3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

A "parent antibody" or "wild-type" antibody is an antibody comprising an amino acid sequence which lacks one or more amino acid sequence alterations compared to an antibody variant as herein disclosed. Thus, the parent antibody generally has at least one hypervariable region which differs in amino acid sequence from the amino acid sequence of the corresponding hypervariable region of an antibody variant as herein disclosed. The parent polypeptide may comprise a native sequence (i.e. a naturally occurring) antibody (including a naturally occurring allelic variant), or an antibody with pre-existing amino acid sequence modifications (such as insertions, deletions and/or other alterations) of a naturally occurring sequence. Throughout the disclosure, "wild type," "WT," "wt," and "parent" or "parental" antibody are used interchangeably.

As used herein, "antibody variant" or "variant antibody" refers to an antibody which has an amino acid sequence which differs from the amino acid sequence of a parent antibody. Preferably, the antibody variant comprises a heavy chain variable domain or a light chain variable domain having an amino acid sequence which is not found in nature. Such variants necessarily have less than 100% sequence identity or similarity with the parent antibody. In a preferred embodiment, the antibody variant will have an amino acid sequence from about 75% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the parent antibody, more preferably from about 80% to less than 100%, more preferably from about 85% to less than 100%, more preferably from about 90% to less than 100%, and most preferably from about 95% to less than 100%. The antibody variant is generally one which comprises one or more amino acid alterations in or adjacent to one or more hypervariable regions thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification. In certain embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will typically possess, e.g., at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, or at least about 90% sequence identity therewith, or at least about 95% sequence or more identity therewith.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or U.S. Pat. No. 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS* (*USA*) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. In certain embodiments, the cells express at least FcγRIII and perform ADCC effector function(s). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being generally preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, e.g., Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein.

The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward., *Immunol. Today* 18(12):592-598 (1997); Ghetie et al., *Nature Biotechnology,* 15(7):637-640 (1997); Hinton et al., *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).

Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the

polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, e.g., Shields et al. *J. Biol. Chem.* 9(2):6591-6604 (2001).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (Clq) to antibodies (of the appropriate subclass), which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed. Polypeptide variants with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased C1q binding capability are described, e.g., in U.S. Pat. No. 6,194,551 B1 and WO 1999/51642. See also, e.g., Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al, *J. Mol. Biol.* 226:889-896 (1992).

The term "therapeutic antibody" refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell's death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A "biologically functional fragment" of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

Therapeutic antibodies of particular interest include those in clinical oncological practice or development such as commercially available AVASTIN® (bevacizumab), HERCEPTIN® (trastuzumab), LUCENTIS® (ranibizumab), RAPTIVA® (efalizumab), RITUXAN® (rituximab), and XOLAIR® (omalizumab), as well as, anti-amyloid beta (Abeta), anti-CD4 (MTRX1011A), anti-EGFL7 (EGF-like-domain 7), anti-IL13, Apomab (anti-DR5-targeted pro-apoptotic receptor agonist (PARA), anti-BR3 (CD268, BLyS receptor 3, BAFF-R, BAFF Receptor), anti-beta 7 integrin subunit, dacetuzumab (Anti-CD40), GA101 (anti-CD20 monoclonal antibody), MetMAb (anti-MET receptor tyrosine kinase), anti-neuropilin-1 (NRP1), ocrelizumab (anti-CD20 antibody), anti-OX40 ligand, anti-oxidized LDL (oxLDL), pertuzumab (HER dimerization inhibitors (HDIs), and. rhuMAb IFN alpha.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a

biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The term "diagnostic protein" refers to a protein that is used in the diagnosis of a disease.

The term "diagnostic antibody" refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A "biologically functional fragment" of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

"Purified" means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained. The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An "essentially pure" protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An "essentially homogeneous" protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

As used herein, "soluble" refers to polypeptides that, when in aqueous solutions, are completely dissolved, resulting in a clear to slightly opalescent solution with no visible particulates, as assessed by visual inspection. A further assay of the turbidity of the solution (or solubility of the protein) may be made by measuring UV absorbances at 340 nm to 360 nm with a 1 cm path-length cell where turbidity at 20 mg/ml is less than 0.05 absorbance units.

An "isolated" antibody or polypeptide is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "Protein A" and "ProA" are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, Pharmacia and Fermatech. Protein A is generally immobilized on a solid phase support material. The term "ProA" also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term "chromatography" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term "affinity chromatography" and "protein affinity chromatography" are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms "non-affinity chromatography" and "non-affinity purification" refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia). A "mixed mode ion exchange resin" refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J.T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

The "intermediate buffer" is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

B. Exemplary Methods and Materials for Carrying Out the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); PCR: *A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells*, 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunochemistry in Practice*, 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering*, 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology*.

1. Recombinant Production of Proteins in Mammalian Host Cells Using a Glutamine-Free Cell Culture Medium

The present invention concerns the large-scale recombinant production of proteins in mammalian host cells, using a glutamine-free cell culture medium supplemented with asparagine. Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells have been approved by regulatory agencies for the production of biopharmaceutical products, including therapeutic antibodies. Of these, Chinese Hamster Ovary Cells (CHO) are among the most commonly used industrial hosts, which are widely employed for the production of heterologous proteins. Thus, methods for the large-scale production of antibodies in CHO, including dihydrofolate reductase negative (DHFR−) CHO cells, are well known in the art (see, e.g. Trill et al., *Curr. Opin. Biotechnol.* 6(5):553-60 (1995) and U.S. Pat. No. 6,610,516).

As a first step, the nucleic acid (e.g., cDNA or genomic DNA) encoding the desired recombinant protein may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below. Optional signal sequences, origins of replication, marker genes, enhancer elements and transcription terminator sequences that may be employed are known in the art and described in further detail in PCT Publication WO 97/25428.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the protein-encoding nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the

presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the desired protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in PCT Publication No. WO97/25428.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* cells, such as *E. coli* K12 strain 294 (ATCC® 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced using standard techniques known in the art. (See, e.g., Messing et al., *Nucleic Acids Res.* 1981, 9:309; Maxam et al., Methods in Enzymology 1980, 65:499).

Expression vectors that provide for the transient expression in mammalian cells may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector (Sambrook et al., supra). Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of a desired heterologous protein in recombinant vertebrate cell culture are described in Gething et al., *Nature* 1981, 293:620-625; Mantei et al., *Nature* 1979, 281:40-46; EP 117,060; and EP 117,058.

For large-scale production, according to the present invention mammalian host cells are transfected and preferably transformed with the above-described expression vectors and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described (Shaw et al., Gene 1983, 23:315 and PCT Publication No. WO 89/05859). In addition, plants may be transfected using ultrasound treatment, PCT Publication No. WO 91/00358 published 10 Jan. 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method (Graham and van der Eb, *Virology* 1978, 52:456-457) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. For various techniques for transforming mammalian cells, see also Keown et al. *Methods in Enzymology* 1990, 185:527-537 and Mansour et al. *Nature* 1988, 336:348-352.

During large-scale production, to begin the production cycle usually a small number of transformed recombinant host cells is allowed to grow in culture for several days. Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks to begin the production phase, and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Harvesting usually includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, there is room fur further improvements in the large-scale commercial production of recombinant proteins, such as antibodies. Thus, increases in cell viability, longevity and specific productivity of mammalian host cell cultures, and improvements in the titer of the recombinant proteins produced have a genuine impact on the price of the recombinant protein produced, and, in the case of therapeutic proteins, the price and availability of drug products.

The present invention concerns an improved method for the production of heterologous proteins in mammalian cell culture, using a glutamine-free culture medium with added asparagine in the production phase of the cell culture process. The culture media used in the process of the present invention can be based on any commercially available medium for recombinant production of proteins in mammalian host cells, in particular CHO cells.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be

included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. In addition, the culture media of the present invention can be based any of the media described in Ham and McKeehan, Meth. Enz., 58: 44 (1979); Barnes and Sato, Anal. Biochem., 102: 255 (1980); U.S. Pat. No. 4,767,704; U.S. Pat. No. 4,657,866; U.S. Pat. No. 4,927,762; U.S. Pat. No. 5,122,469 or U.S. Pat. No. 4,560,655; WO 90/03430; and WO 87/00195, provided that glutamine is omitted as an ingredient.

Under Glutamine-free conditions Asparagine is required since mammalian cells can synthesize Asparagine only in presence of Glutamine. Asparagine is synthesized by amide transfer from Glutamine in the presence of Asparagine synthetase. The Asparagine is preferably added to the culture medium at a concentration in the range of 2.5 mM to 15 mM. In various embodiments of the present invention, the preferred concentration of Asparagine should be at least 2.5 mM. In preferred embodiments, the asparagine is added at a concentration of 10 mM.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in, and can be adapted for the production of recombinant proteins using the cell culture media herein.

The necessary nutrients and growth factors for the medium, including their concentrations, for a particular cell line, are determined empirically without undue experimentation as described, for example, in *Mammalian Cell Culture*, Mather, ed. (Plenum Press: NY, 1984); Barnes and Sato, *Cell,* 22: 649 (1980) or *Mammalian Cell Biotechnology: A Practical Approach*, M. Butler, ed. (IRL Press, 1991). A suitable medium contains a basal medium component such as a DMEM/HAM F-12-based formulation (for composition of DMEM and HAM F12 media and especially serum-free media, see culture media formulations in American Type Culture Collection Catalogue of Cell Lines and Hybridomas, Sixth Edition, 1988, pages 346-349), with modified concentrations of some components such as amino acids, salts, sugar, and vitamins, and optionally containing glycine, hypoxanthine, and thymidine; recombinant human insulin, hydrolyzed peptone, such as PRIMATONE HS™ or PRIMATONE RL™ (Sheffield, England), or the equivalent; a cell protective agent, such as PLURONIC F68™ or the equivalent pluronic polyol; GENTAMYCIN™; and trace elements. The formulations of medium as described in U.S. Pat. No. 5,122,469, characterized by the presence of high levels of certain amino acids, as well as PS-20 as described below, are particularly appropriate.

The glycoproteins of the present invention may be produced by growing cells which express the desired glycoprotein under a variety of cell culture conditions. For instance, cell culture procedures for the large- or small-scale production of glycoproteins are potentially useful within the context of the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, in the later two systems, with or without microcarriers, and operated alternatively in a batch, fed-batch, or continuous mode.

In a particular embodiment the cell culture of the present invention is performed in a stirred tank bioreactor system and a fed-batch culture procedure is employed. In the preferred fed-batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed-batch culture can include, for example, a semi-continuous fed-batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium Fed-batch culture is distinguished from simple-batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed-batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers, etc., and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single-step or multiple-step culture procedure. In a single-step culture the host cells are inoculated into a culture environment and the processes of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a specific aspect of the invention, fed-batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (DO_2), and the like, are those used with the particular host and will be apparent to the ordinarily-skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 40° C. and preferably about 37° C. and a suitable DO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

Production of a target protein in mammalian, e.g., CHO, cells typically employs a semi-continuous process whereby cells are culture in a "seed-train" for various periods of time and are periodically transferred to inoculum fermentors to generate enough cell mass to inoculate a production fermentor at larger scale. Thus, cells used for the production of the desired protein are in culture for various periods of time up to a maximum predefined cell age. The parameters of the cell culture process, such as seed density, pH, DO_2 and temperature during culture, duration of the production culture, operating conditions of harvest, etc. are a function of the particular cell line and culture medium used, and can be determined empirically, without undue experimentation.

According to the present invention, the cell-culture environment during the production phase of the cell culture is controlled. In a preferred aspect, the production phase of the

cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged.

The desired polypeptide, such as antibody, preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the polypeptide is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g., Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When the polypeptide is produced in a recombinant cell other than one of human origin, it is free of proteins or polypeptides of human origin. However, it is usually necessary to recover or purify recombinant proteins from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the desired polypeptide. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. The heterologous polypeptide thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column such as SP-Sepharose™ or CM-Sepharose™; hydroxyapatite; hydrophobic interaction chromatography; ethanol precipitation;

chromatofocusing; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75™; and/or diafiltration.

Recombinant polypeptides can be isolated, e.g. by affinity chromatography.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for the purification and isolation of recombinant proteins, including antibodies, can be used herein, and modified if needed, using standard techniques.

Expression of the desired heterologous protein may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA* 1980, 77:5201-5205), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal

2. Antibodies

In a preferred embodiment, the methods of the present invention are used for the recombinant production of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_4\beta_7$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or Lympho-

Cide (Juweid et al., *Cancer Res* 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2): 435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab. sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-1 6, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2): 435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); W)02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171,586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677,180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224,866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albitar et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegall); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821 (Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332, 865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1) (part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" New Scientist (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): 5370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.* 30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): 5197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology;

October 24-29; New Orleans, La. 2002; Tuscano, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-CD20 antibodies. In certain embodiments, the humanized antibody composition of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized antibody compositions of the present invention include compositions of any of the preceding humanized antibodies having an Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to Fc(RIIIA (F158), which is not as effective as Fc(RIIIA (V158) in interacting with human IgG. Fc(RIIIA (F158) is more common than Fc(RIIIA (V 158) in normal, healthy African Americans and Caucasians. See Lehrnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*" in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the antigens are selected from the group consisting of CD-20, CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-HER2 antibodies. HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS* (*USA*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998) are FDA approved for the treatment of cancer. In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-VEGF antibodies.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for pharmaceutical compositions comprising anti-human CD11a antibodies.

Apomab Antibodies

Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference. Apomab is a fully human monoclonal antibody which is a DR5-targeted pro-apoptotic receptor agonist (PARA) specifically designed to induce apoptosis. Apoptosis is a natural process by which damaged or unwanted cells, including those that are cancerous, die and are cleared from the body. Pro-apoptotic receptor DR5 is expressed in a broad range of malignancies.

Anti-BR3 Antibodies and Immunoadhesins

Antibodies to the BR3 (anti-BR3) antibodies and BR3-Fc immunoadhesins can also be produced in accordance with the present invention. Such anti-BR3 antibodies and immunoadhesins specifically include all variants disclosed in U.S. Application Publication No. 20050070689. The entire content of U.S. Application Publication No. 20050070689 is hereby expressly incorporated by reference.

3. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically iden-

tified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕to ⅒the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by

using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J Mol. Biol.,* 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies,

BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L-AC_L;

AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

$(A\text{-}Y)_n$-(V_LC_L-V_HC_H)$_2$;

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown.

However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

Further details of the invention are provided in the following non-limiting Examples.

All patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties.

EXAMPLES

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC® accession numbers is the American Type Culture Collection, Manassas, Va.

Example 1

Production of Polypeptides in Glutamine-Free Production Medium

Materials and Methods:

Cell Lines.

In these studies, CHO host cells expressing an Apomab antibody, anti-VEGF antibody, and the fusion protein BR3-Fc, respectively were used. The host cells were adapted in suspension and serum free cultures. Frozen stocks were prepared as master or working cell banks in the media described below.

Cell line maintenance was carried out using a 250-mL or 1-Liter Corning® vented shake flasks maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37° C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform. Cell cultures were passed every 3 or 4 days with fresh media and seeded at 0.11% or 0.20% Packed Cell Volume (PCV). PCV was obtained using a glass 10-mL KIMAX® USA PCV tube.

Culture Media and Conditions.

Media studies were initiated using 250-mL Corning vented shake flask inoculated in singlet, duplicate, or triplicate at 100 mL working volume at 0.20% PCV for all cases using cell culture from a source1-Liter Corning® vented shake flask with a 500-mL working volume. PCV was obtained using a glass 10-mL KIMAX® USA PCV tube.

Prior to initiation of the study cell culture was centrifuged at 1000 rpm for 5-minutes in a Sorvall® RT 6000B centrifuge to complete a 100% media exchange of inoculum media containing glutamine with the respective test media. Different concentrations of Glutamine, Glutamate, Asparagine and Aspartate were evaluated in the different test media. The following concentrations were tested: Glutamine 0-10 mM, Glutamate 1-10 mM, Asparagine 0-15 mM, Aspartate 1-10 mM. Media conditions were evaluated in full factorial DOE studies.

The effect of Glutamine-free medium on was also tested in commercially available DMEM/F12 medium. The medium

was used at 5× concentration (7.05 g/L) with extra Asparagine (10 mM total), Aspartate (10 mM total), Glutamine (10 mM total for the Glutamine-containing medium), Glutamate (1 mM total), and glucose (8 g/L total). Glutamine-free and Glutamine-containing medium were compared using Apomab and anti-VEGF antibody expressing cells.

Shake flasks were maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37° C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform.

The medium used contained the following components:

Organic Salts and Trace Elements
Ammonium Paramolybdate, Tetrahydrate
Ammonium Vanadium Oxide
Calcium Chloride, Anhydrous
Cupric Sulfate, Pentahydrate
Ferrous Sulfate, Heptahydrate
Potassium Chloride
Magnesium Chloride, Anhydrous
Manganese Sulfate, Monohydrate
Nickel Chloride, Hexahydrate
Selenious Acid
Sodium Metasilicate, Nonahydrate
Sodium Phosphate, Monobasic, Monohydrate
Stannous Chloride, Dihydrate
Zinc Sulfate, Heptahydrate
Lipids
Linoleic Acid
Lipoic Acid (aka Thioctic Acid)
Putrescine, Dihydrochloride
Amino Acids
L-Alanine
L-Arginine, Monohydrochloride
L-Asparagine
L-Aspartic Acid
L-Cysteine, Monohydrochloride, Monohydrate
L-Glutamic Acid
L-Glutamine
L-Histidine, Monohydrochloride, Monohydrate
L-Isoleucine
L-Leucine
L-Lysine, Monohydrochloride
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine, Disodium Salt, Dihydrate
L-Valine
Vitamins
Biotin
D-Calcium Pantothenate
Choline Chloride
Folic Acid
I-Inositol
Niacinamide
Pyridoxine, Monohydrochloride
Riboflavin
Thiamine, Monohydrochloride
Vitamin B-12
Carbon Source, Growth Factors, and Miscellaneous
Fluronic F-68
D-Glucose
Sodium Bicarbonate
Sodium Pyruvate
Sodium Chloride
Sodium Hydroxide
Insulin
Galactose

The commercially-available DMEM/F-12 culture medium was also tested, having the following components;

	(mg/L)
VITAMINS	
Biotin	0.00365
D-calcium pantothenate	2.24
Choline chloride	8.98
Cyanocobalamin	0.68
Folic acid	2.65
i-inositol	12.6
Niacinamide	2.0185
Pyridoxal HCl	2
Pyridoxine HCl	0.031
Riboflavin	0.219
Thiamine HCl	2.17
AMINO ACIDS	
L-alanine	4.455
L-arginine HCl	147.5
L-asparagine monohydrate	7.5
L-aspartic acid	6.65
L-cysteine HCl monohydrate	17.56
L-cystine 2HCl	31.29
L-glutamic acid	7.35
L-glutamine	365
Glycine	18.75
L-histidine HCl monohydrate	31.48
L-isoleucine	54.47
L-leucine	59.05
L-lysine HCl	91.25
L-methionine	17.24
L-phenylalanine	35.48
L-proline	17.25
L-serine	26.25
L-threonine	53.45
L-tryptophan	9.02
L-tyrosine 2Na dihydrate	55.79
L-valine	52.85
OTHER	
Dextrose anhydrous	3151
HEPES	3575
Hypoxanthine sodium salt	2.39
Linoleic acid	0.042
DL-α-Lipoic acid	0.105
Phenol red sodium salt	8.602
Putrescine 2HCl	0.081
Sodium pyruvate	55
Thymidine	0.365
ADD: Sodium bicarbonate	1200
INORGANIC SALTS	
Calcium chloride anhydrous	116.61
Cupric sulfate pentahydrate	0.00125
Ferric nitrate nonahydrate	0.05
Ferrous sulfate heptahydrate	0.417
Magnesium chloride anhydrous	28.61
Magnesium sulfate anhydrous	48.84
Potassium chloride	311.8
Sodium chloride	6999.5
Sodium phosphate dibasic anhydrous	71.02
Sodium phosphate monobasic monohydrate	62.5
Zinc sulfate heptahydrate	0.4315

The medium for inoculum culture (as opposed for the production phase) was usually supplemented with 5 mM glutamine, 8 g/L glucose, and 75-2000 nM Methotroxate.

For studies pH adjustment was performed as needed to maintain pH value at 7.00±0.10 using 1M Sodium Carbonate.

Adjustment in pH value was made in by adding 1 mL/L of 1M Sodium Carbonate to raise pH units up 0.10.

Cell culture was analyzed up to 14-days by taking a 3.5-mL sample and analyzed for viable cell count, viability, and cell size using a Beckman Coulter ViCell™-1.0 cell counter. Nutrient analysis was performed using the Nova 400 Biomedical Bioprofile®. Osmolality was measured using an Advanced® Instrument multi-sample Osmometer (Model 3900). Recombinant product titer concentration was obtained using the Agilent 1100 Series HPLC.

Recombinant Proteins.

The recombinant proteins produced were Apomab (TRAIL), anti-VEGF, and the immunoadhesin BR3-Fc.

Data Analysis.

Statistical analyses of the data were carried out using a full factorial design of experiment, which is an experiment whose design consists of two or more factors, each with discrete possible values or "levels", and whose experimental units take on all possible combinations of these levels across all such factors. A full factorial design may also be called a fully-crossed design. Such an experiment allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable.

Results

As shown in FIGS. **1-5**, use of a glutamine-free production medium increased the final recombinant protein titer of Apomab antibody, BR3-Fc immunoadhesin and anti-VEGF antibody. In each case, cube plot analysis of titer results using Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate predict that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. (FIGS. **1-3**)

The effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer is shown in FIG. **4**. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free and low Glutamate conditions is illustrated in FIG. **5**. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

The effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer is demonstrated in FIGS. **6**A-C, wherein the final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

Similar results were obtained using the commercial DMEM/F-12 culture medium. As shown in FIGS. **7**A and B, the final titer for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

As shown in FIGS. **8** and **9**, use of a glutamine-free production medium also increased specific production measured as Qp (mg/mL-cell/day). FIGS. **8** A-C illustrate that cell specific productivity (Qp) for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was significantly higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing medium. FIGS. **9**A and B illustrate that cell specific productivity for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

As shown in FIGS. **10** and **11**, use of a glutamine-free production medium was shown to improve cell viability and extend culture longevity significantly. FIGS. **10**A-C. illustrate that cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing medium. FIGS. **11**A and B indicate that, in DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. Of note, viability was higher for Apomab antibody (FIG. **11**A), but lower for anti-VEGF antibody (FIG. **11**B) compared to Glutamine containing medium.

As shown in FIGS. **12** and **13**, use of a glutamine-free production medium reduced NH_4^+ accumulation significantly compared to glutamine-containing medium. FIGS. **12** A-C illustrate that ammonia levels were usually lower in Glutamine-free cultures supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing cultures. FIGS. **13** A and B illustrate that ammonia levels were significantly reduced in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention.

Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

What is claimed is:

1. A process for producing a polypeptide in a mammalian host cell expressing said polypeptide, comprising culturing the mammalian host cell in a production phase of the culture in a glutamine-free production culture medium containing asparagine, wherein the asparagine is added at a concentration in the range of 7.5 mM to 15 mM.

2. The process of claim **1** wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

3. The process of claim **1** wherein said recombinant host cell is an eukaryotic host cell.

4. The process of claim **3** wherein said eukaryotic host cell is a Chinese Hamster Ovary (CHO) cell.

5. The process of claim **4** wherein the mammalian host cell is a dhfr$^-$CHO cell.

6. The process of claim **1** wherein the production medium is serum-free.

7. The process of claim **1** wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

8. The process of claim **7** wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

9. The process of claim **1** wherein the production phase is a batch or fed batch culture phase.

10. The process of claim **1** further comprising the step of isolating said polypeptide.

11. The process of claim **10** further comprising determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

12. The process of claim **11** wherein at least one of the cell viability, culture longevity, specific productivity and final recombinant protein titer is increased relative to the same polypeptide produced in a glutamine-containing production medium of the same composition.

13. The process of claim **1** wherein the polypeptide is a mammalian glycoprotein.

14. The process of claim **1** wherein the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

15. The process of claim **14** wherein said antibody fragment is selected from the group consisting of Fab, Fab', F(ab')2, scFv, (scFv)2, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

16. The process of claim **14** wherein the antibody or antibody fragment is chimeric, humanized or human.

17. The process of claim **14** wherein said antibody or antibody fragment is a therapeutic antibody or a biologically functional fragment thereof.

18. The process of claim **17** wherein said therapeutic antibody is selected from the group consisting of anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha 4\beta 7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

19. The process of claim **17** wherein said therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, BR3 or DR5.

20. The process of claim **19** wherein said therapeutic antibody binding to DR5 is selected from the group consisting of Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3.

21. The process of claim **19** wherein said therapeutic antibody is an anti-BR3 antibody.

22. The process of claim **14** wherein said immunadhesin is a BR3-Fc immunoadhesin.

23. The process of claim **1** wherein said polypeptide is a therapeutic polypeptide.

24. The process of claim **23** wherein said therapeutic polypeptide is selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal

growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

25. A ready-to-use glutamine-free cell culture medium containing asparagine for the production of a polypeptide in a production phase, wherein the asparagine is at a concentration in the range of 7.5 mM to 15 mM.

* * * * *

EXHIBIT Q

US008574869B2

(12) **United States Patent**
Kao et al.

(10) **Patent No.: US 8,574,869 B2**
(45) **Date of Patent: Nov. 5, 2013**

(54) **PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES**

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(21) Appl. No.: **13/354,223**

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(60) Provisional application No. 60/948,677, filed on Jul. 9, 2007.

(51) **Int. Cl.**
C12P 1/00 (2006.01)
C12N 5/02 (2006.01)

(52) **U.S. Cl.**
USPC **435/41**; 435/325

(58) **Field of Classification Search**
None
See application file for complete search history.

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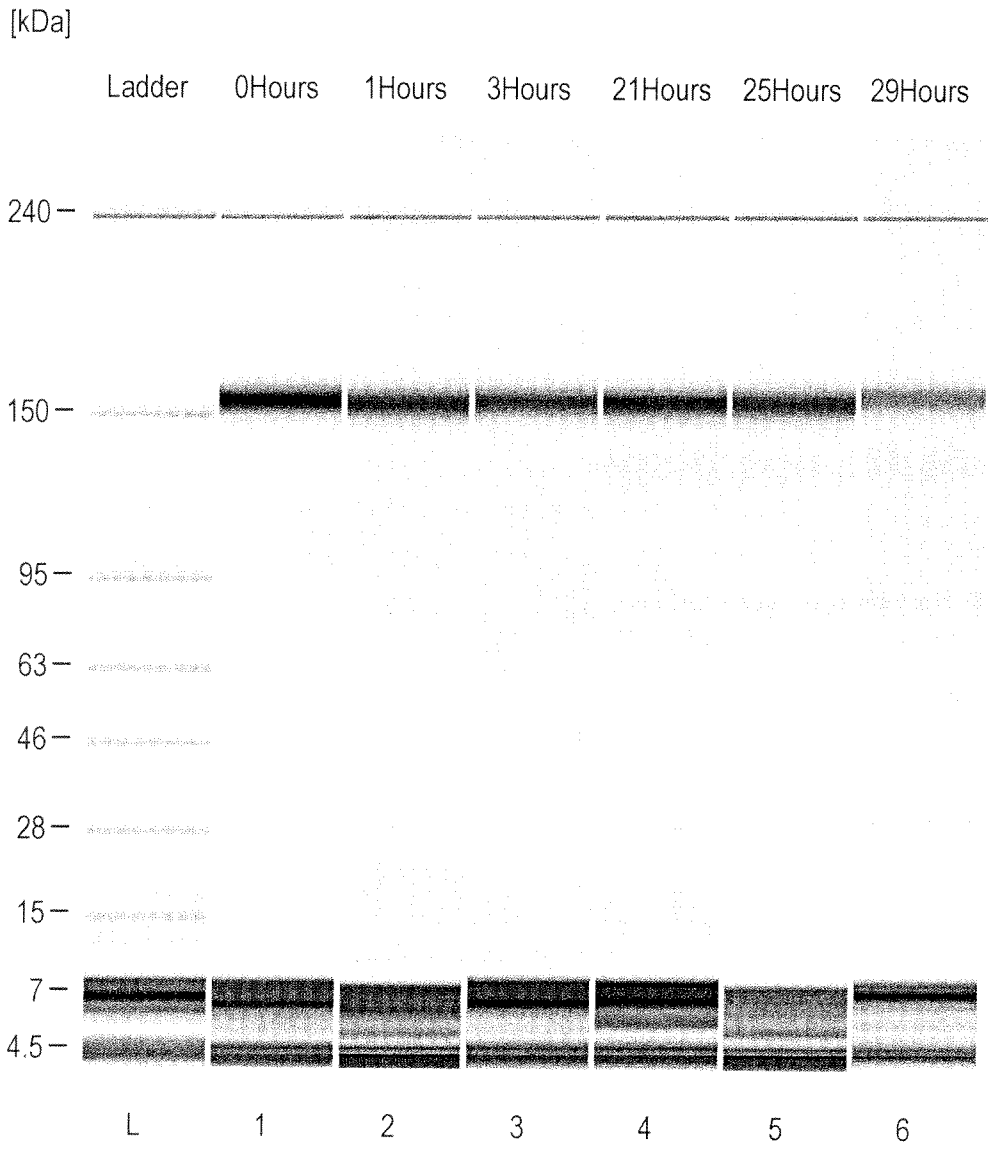
(57) **ABSTRACT**

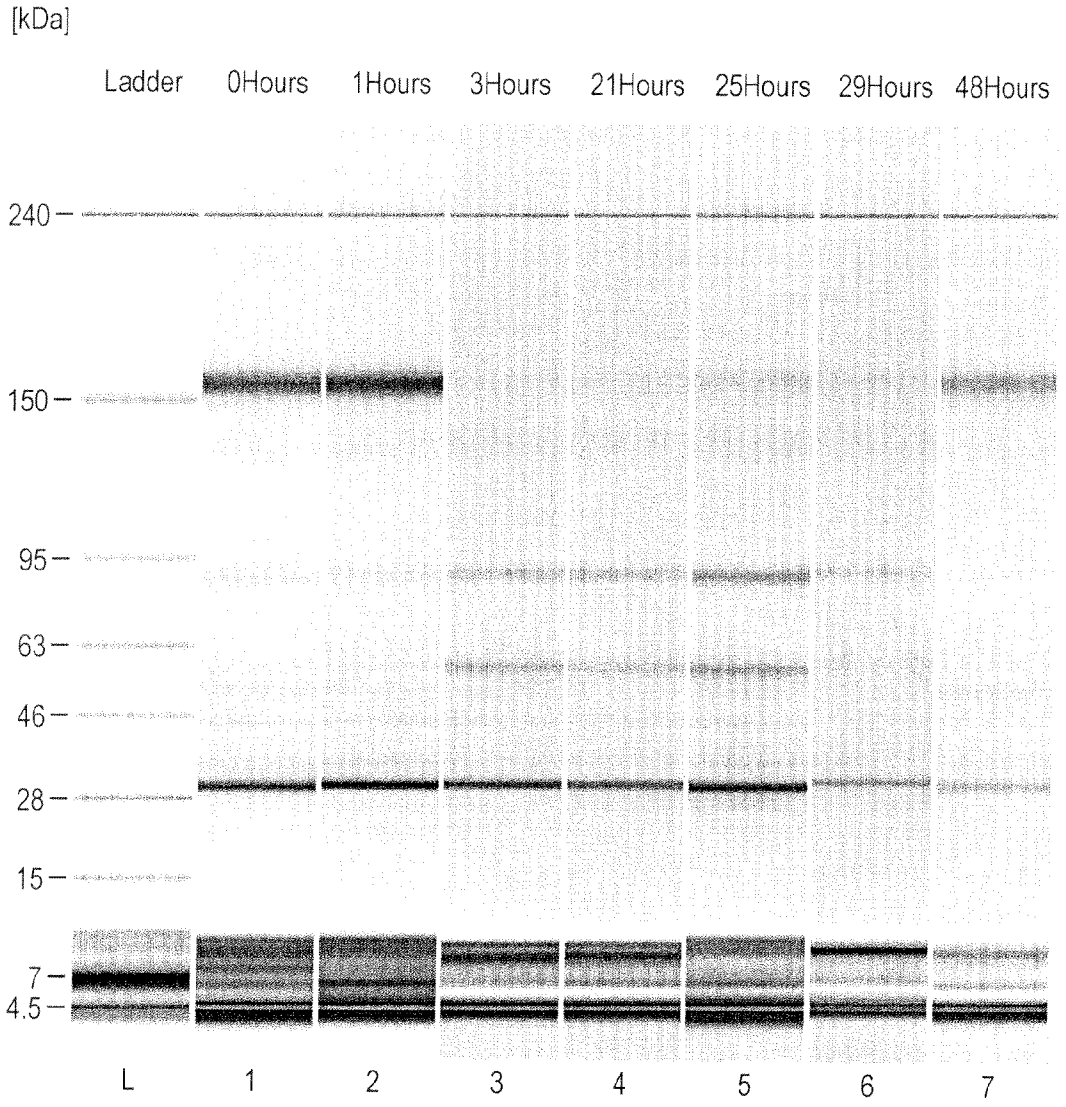
Provided herein are methods for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

10 Claims, 40 Drawing Sheets



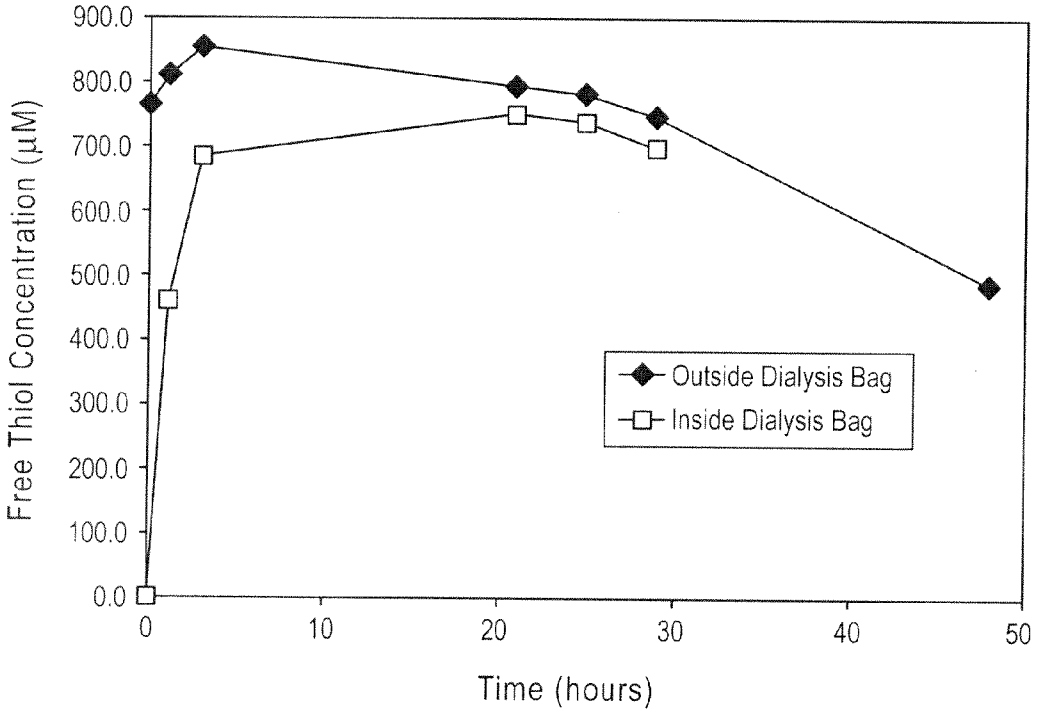
Dialysis Experiment

FIG. 1



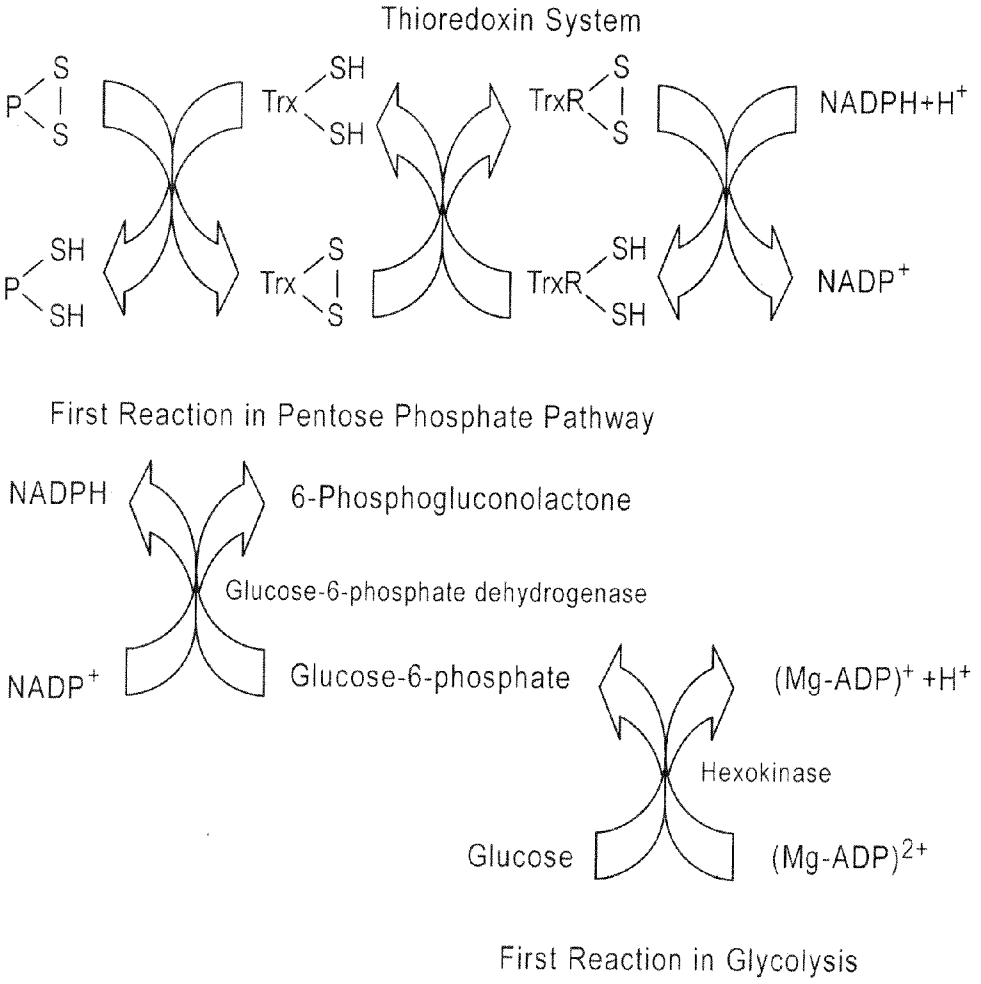
Dialysis Experiment

FIG. 2



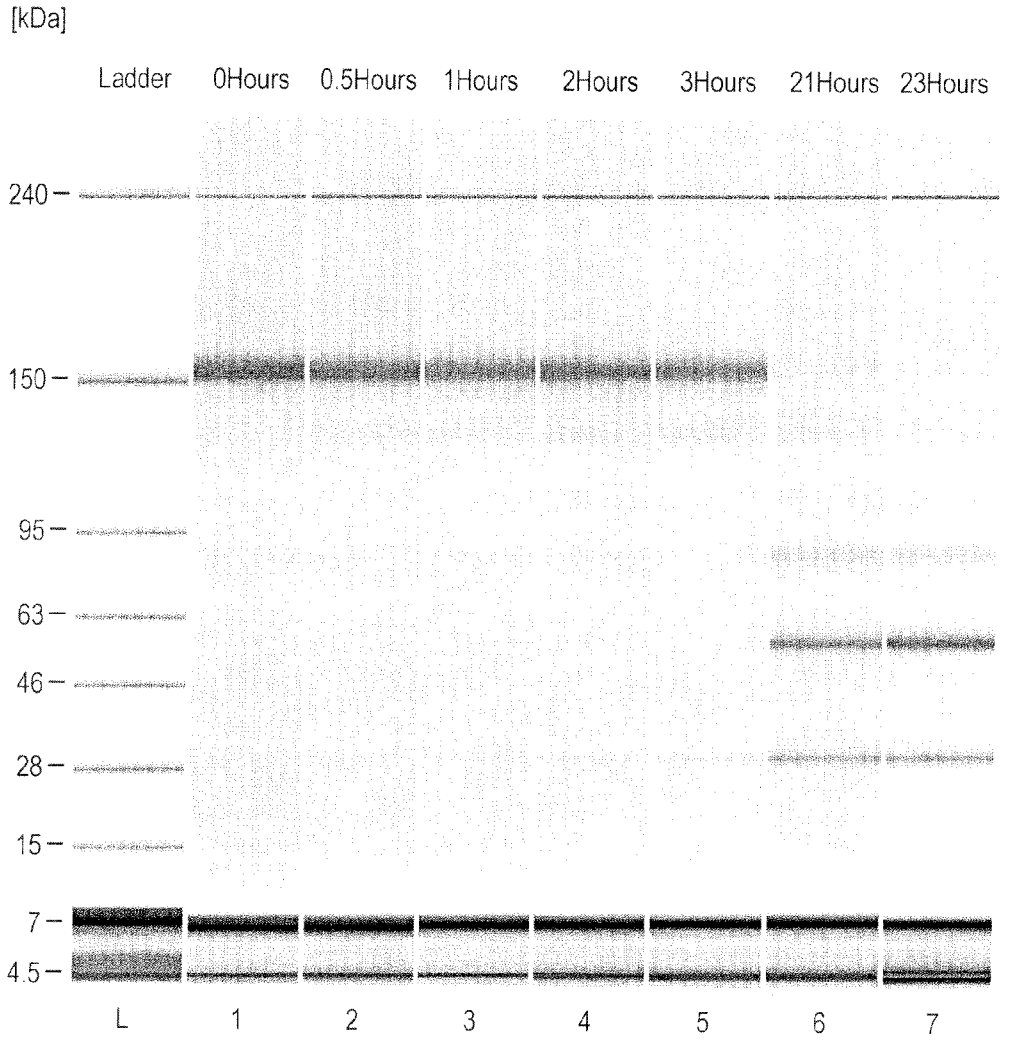
Free Thiol Levels from Dialysis Experiment

FIG. 3



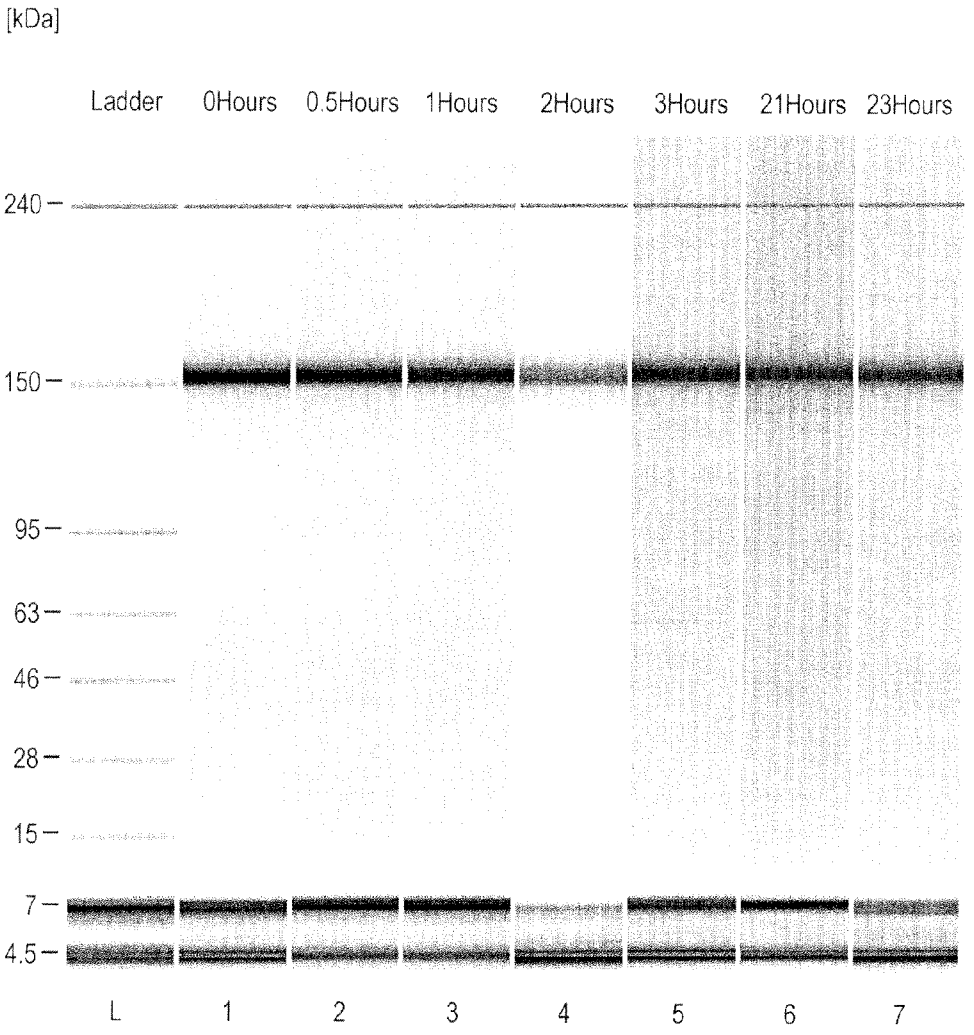
Thioredoxin System and Other Reactions Involved in Antibody Reduction

FIG. 4



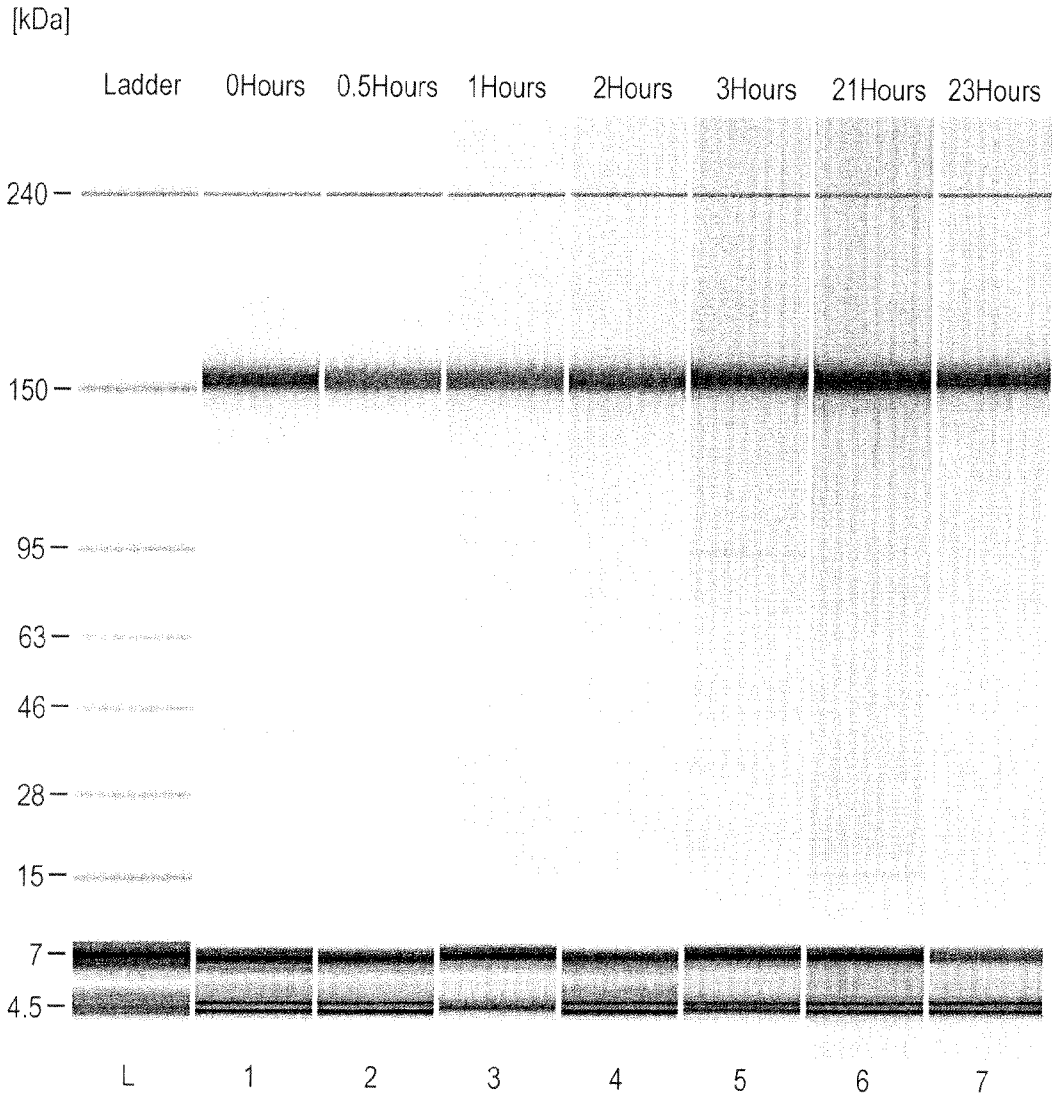
In *Vitro* Activity of Thioredoxin System

FIG. 5



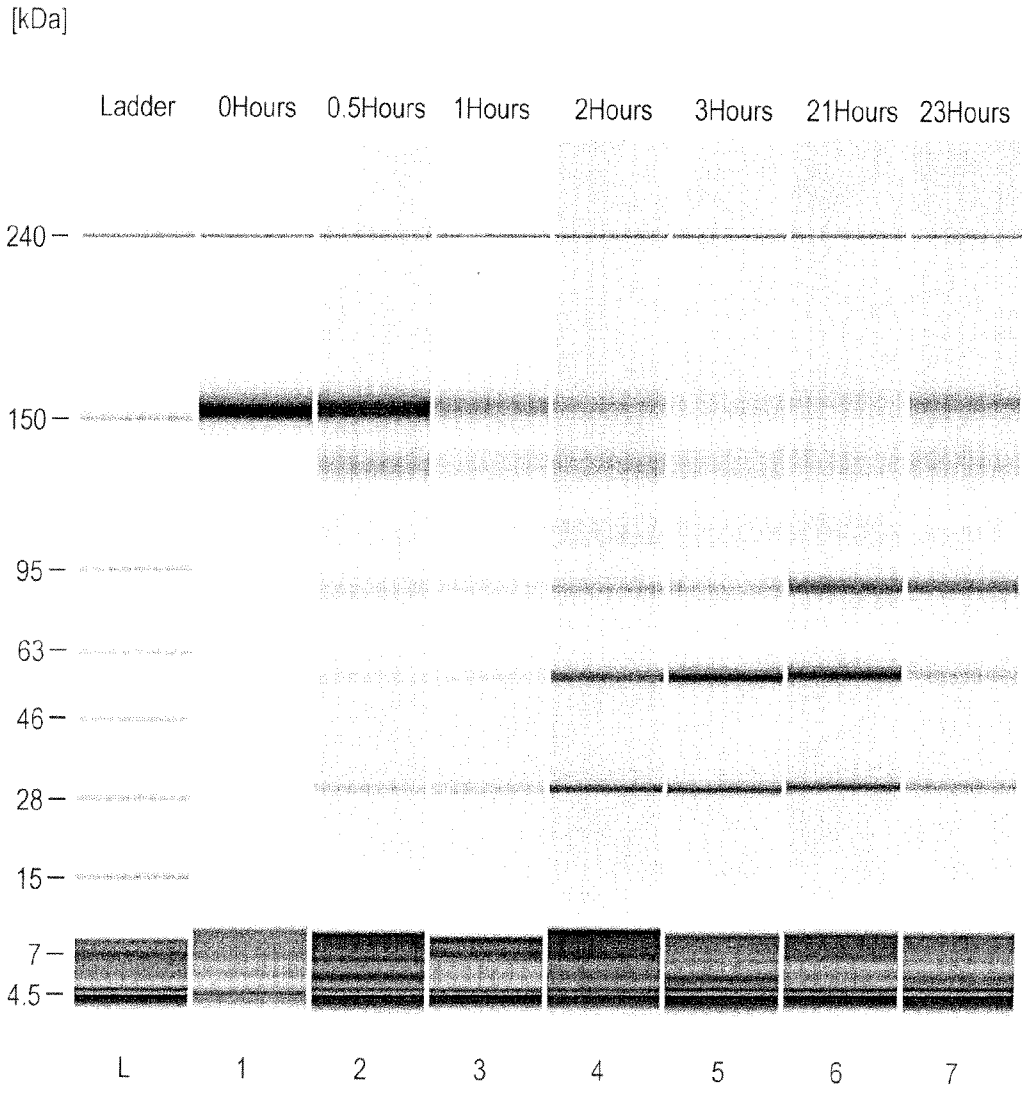
In *vitro* Activity of Thioredoxin System Inhibited by Aurothioglucose

FIG. 6



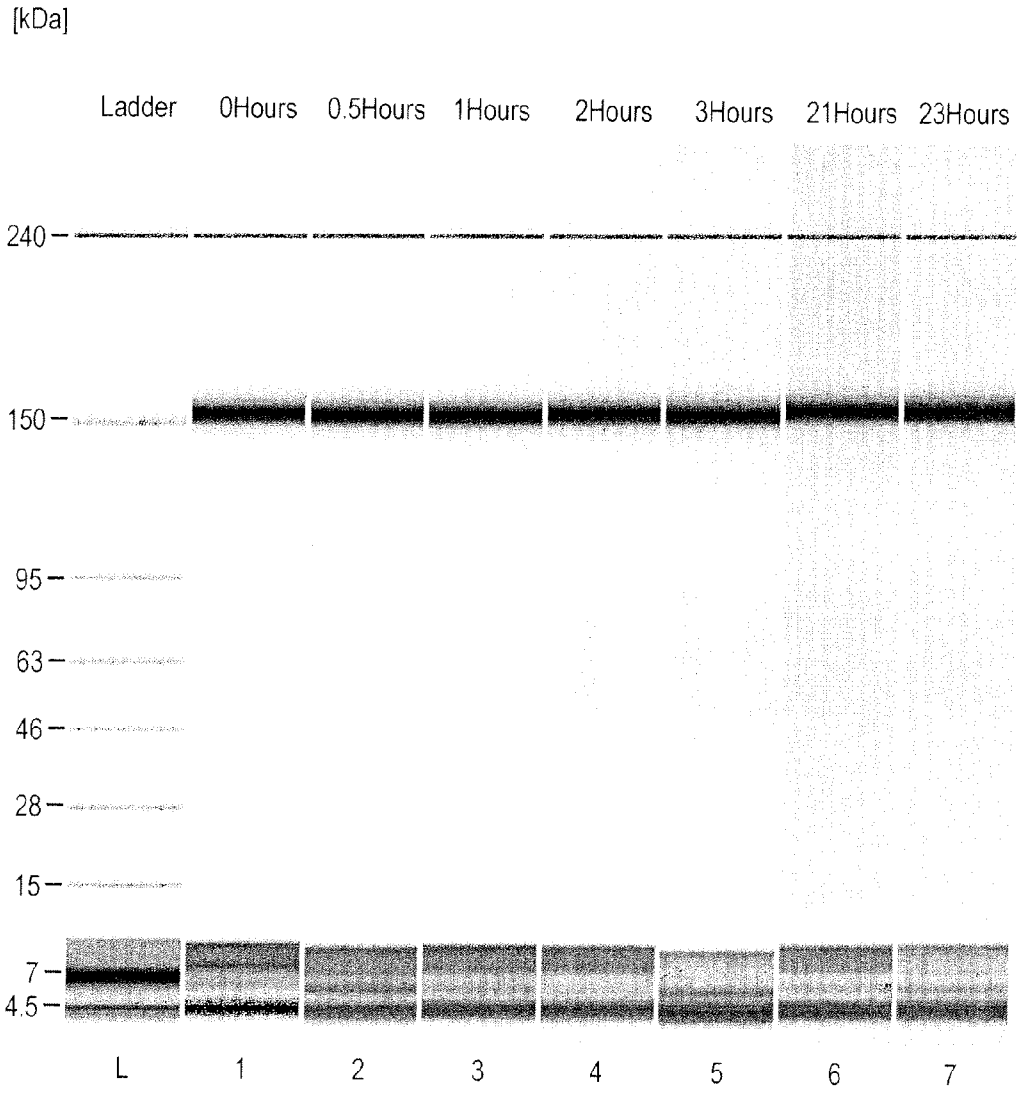
In *vitro* Activity of Thioredoxin System Inhibited by Aurothiomalate

FIG. 7



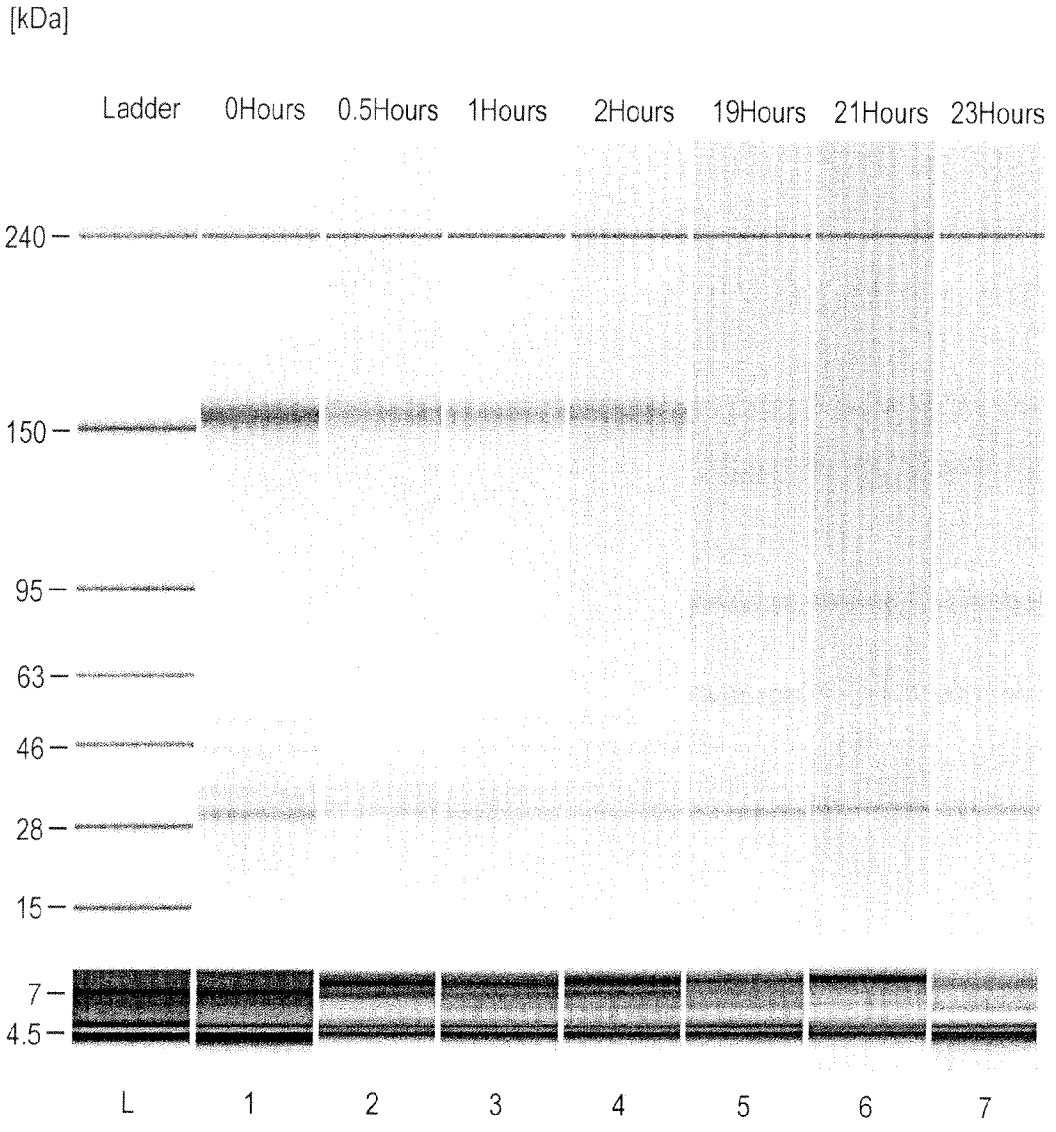
In *vitro* Activity of Thioredoxin System

FIG. 8



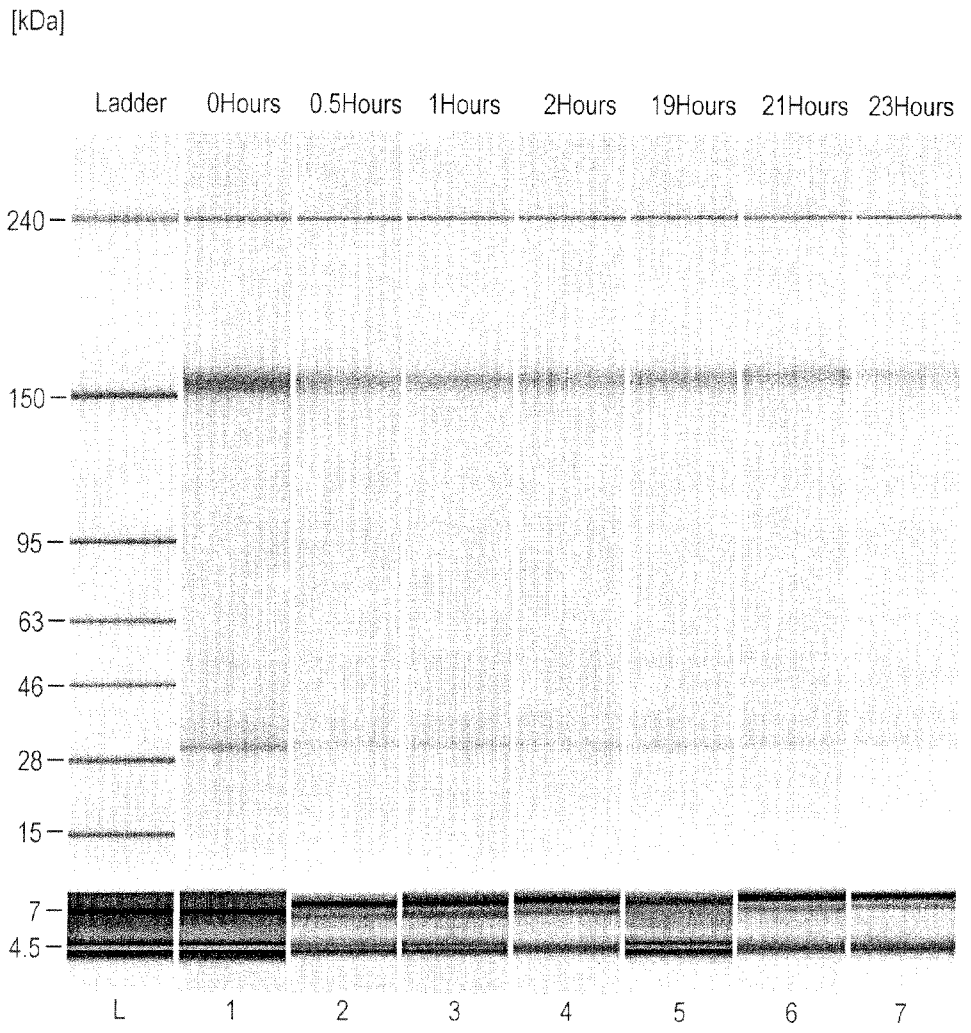
In *vitro* Activity of Thioredoxin System Inhibited by $CuSO_4$

FIG. 9



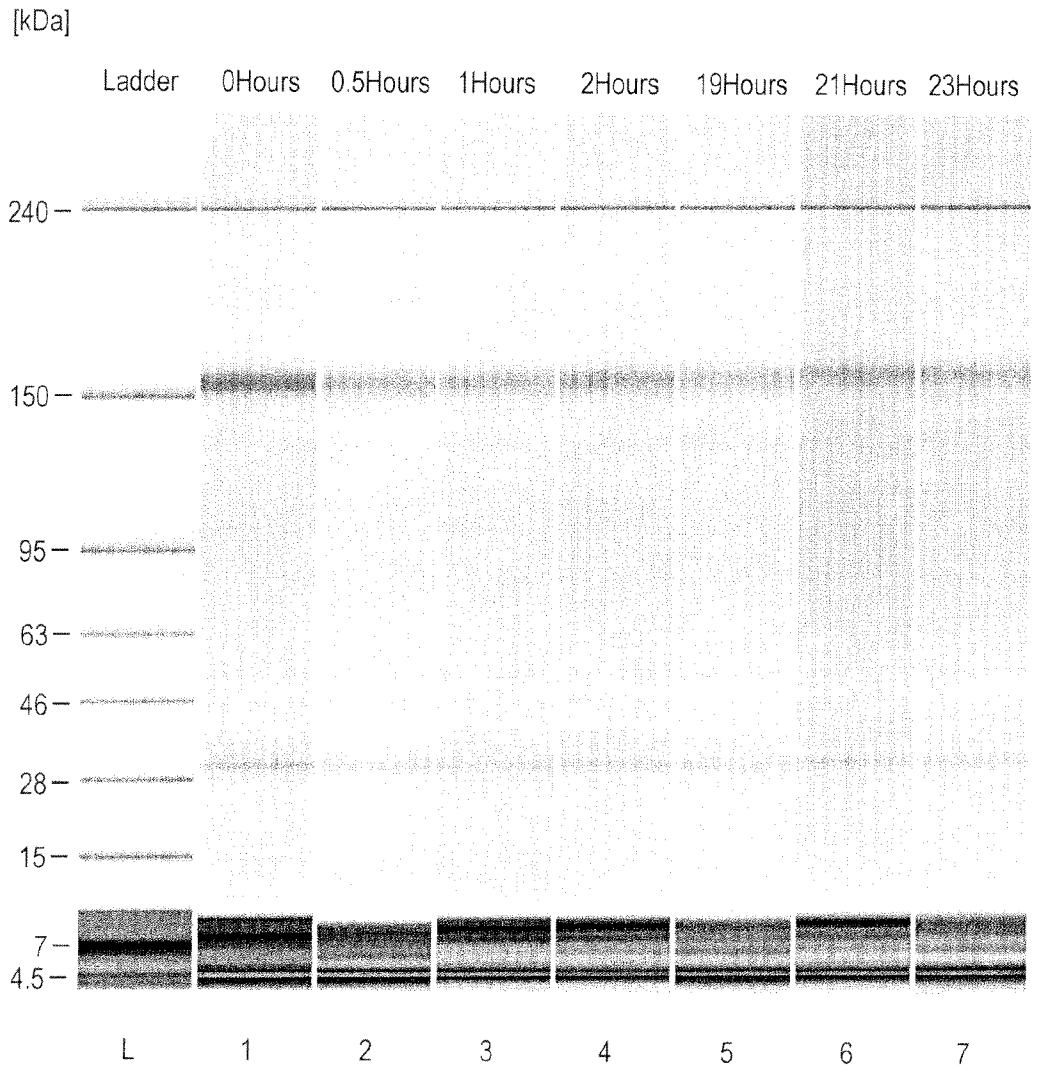
Ocrelizumab Reduction

FIG. 10



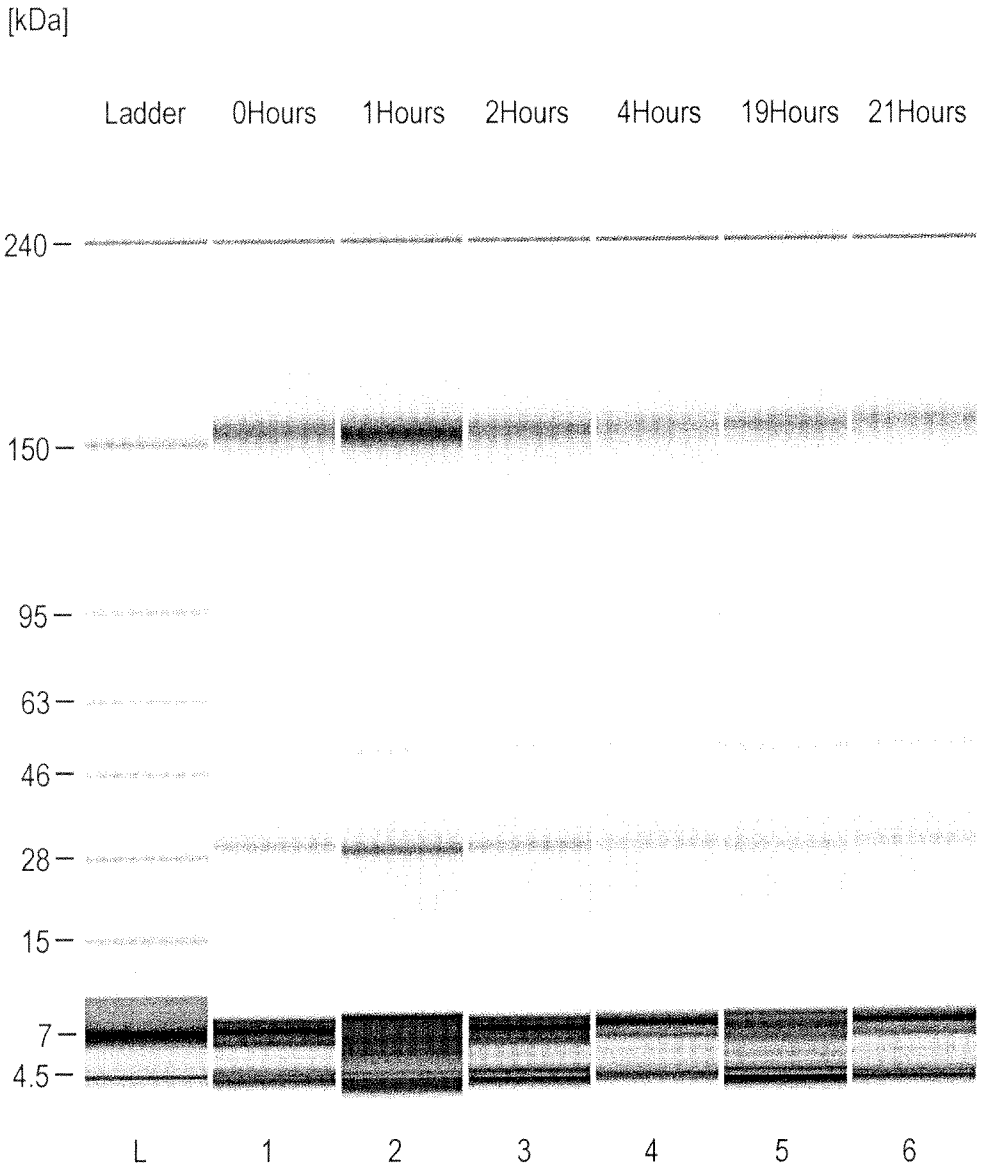
Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose

FIG. 11



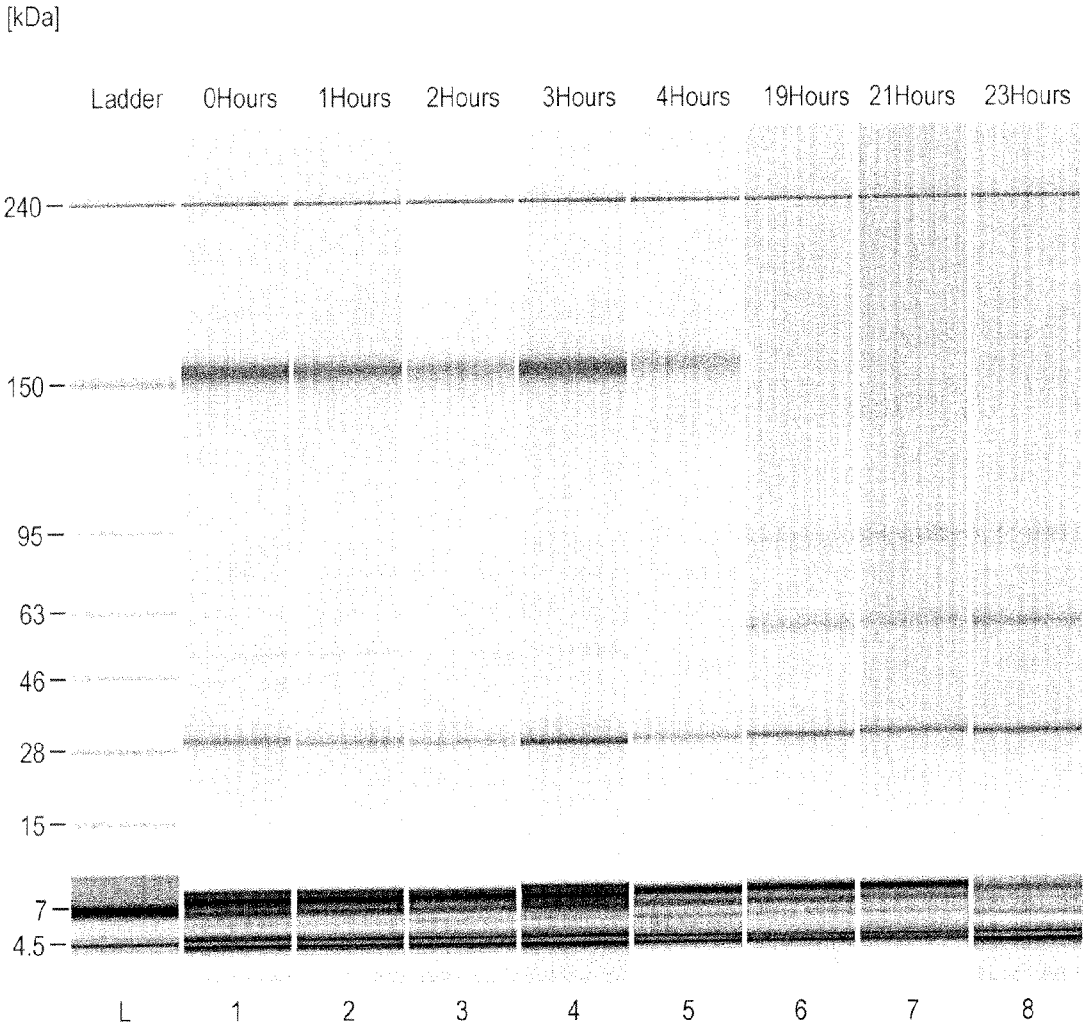
Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate

FIG. 12



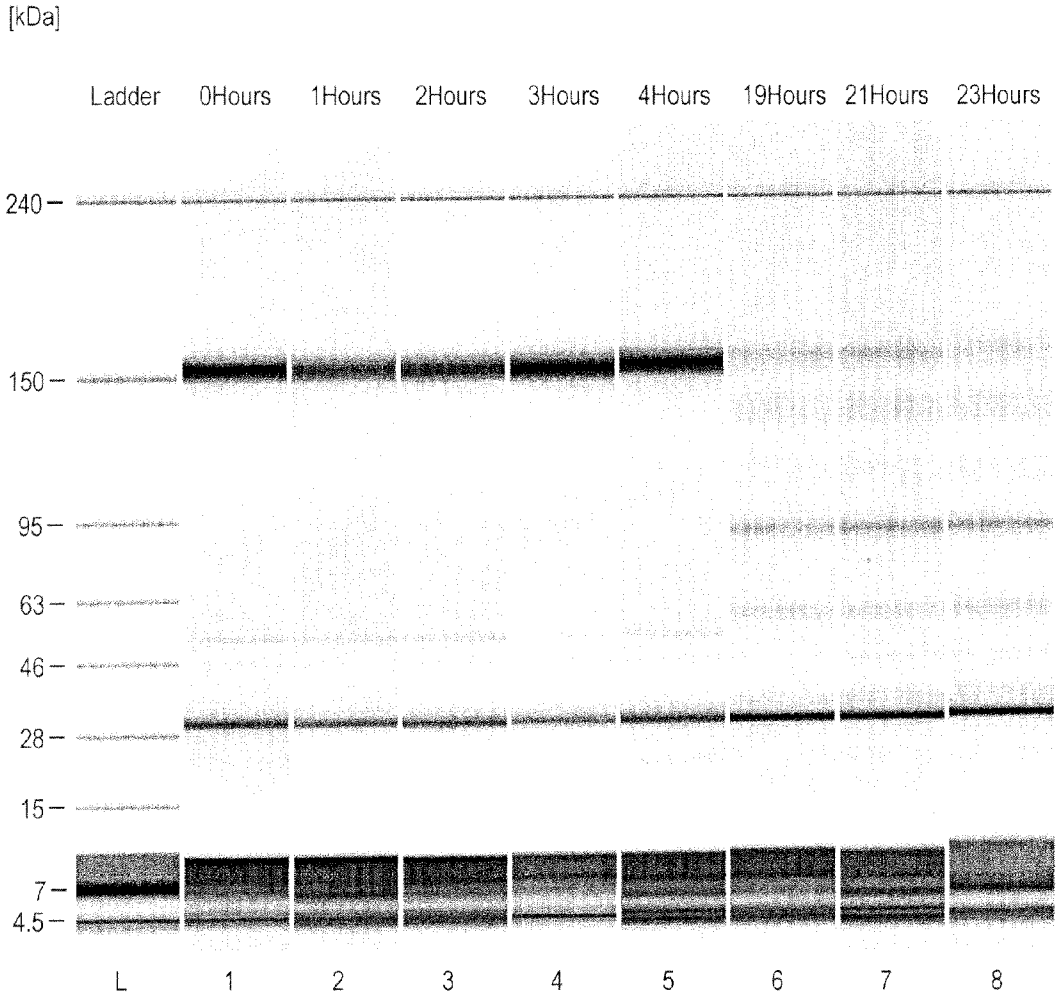
Losing Reduction Activity in HCCF

FIG. 13



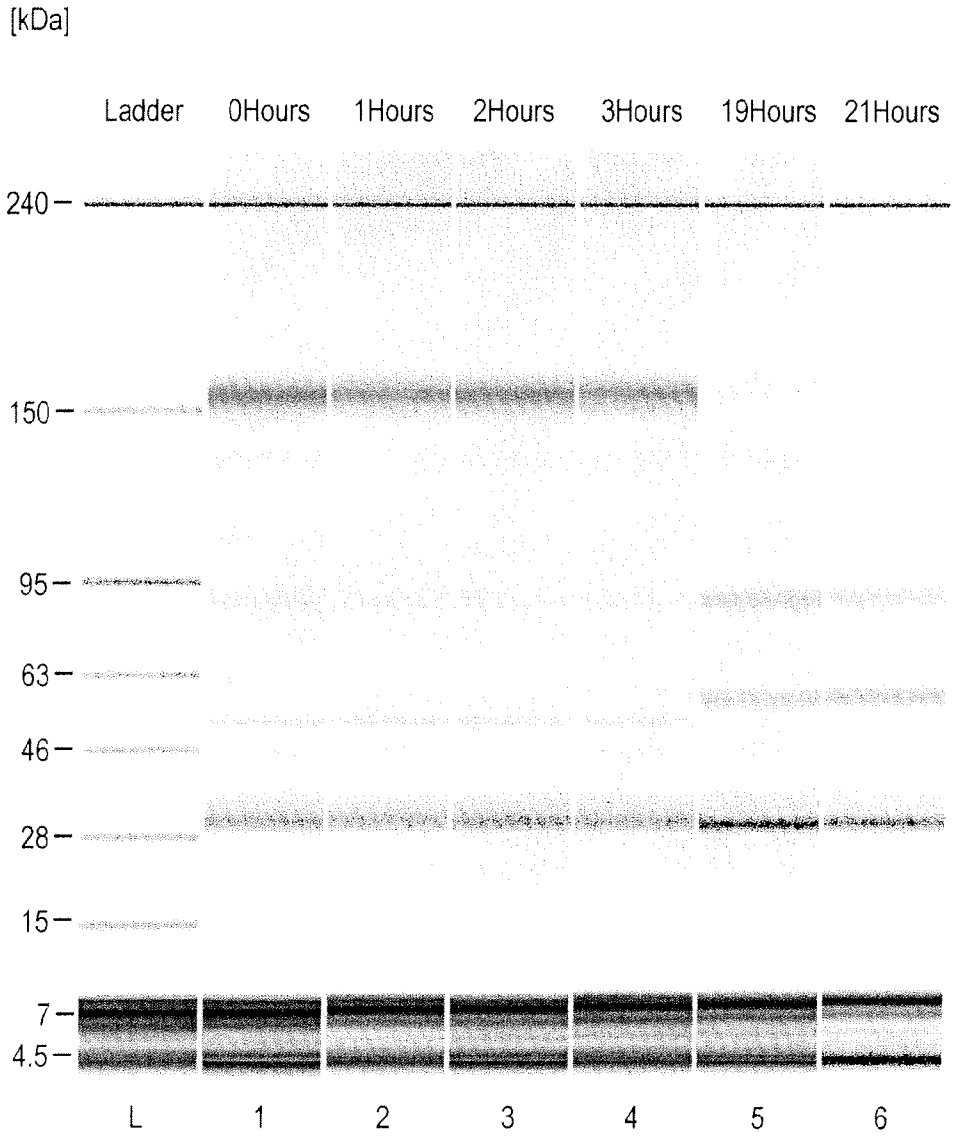
The Lost Reduction Activity in HCCF Restored by Addition of NADPH

FIG. 14



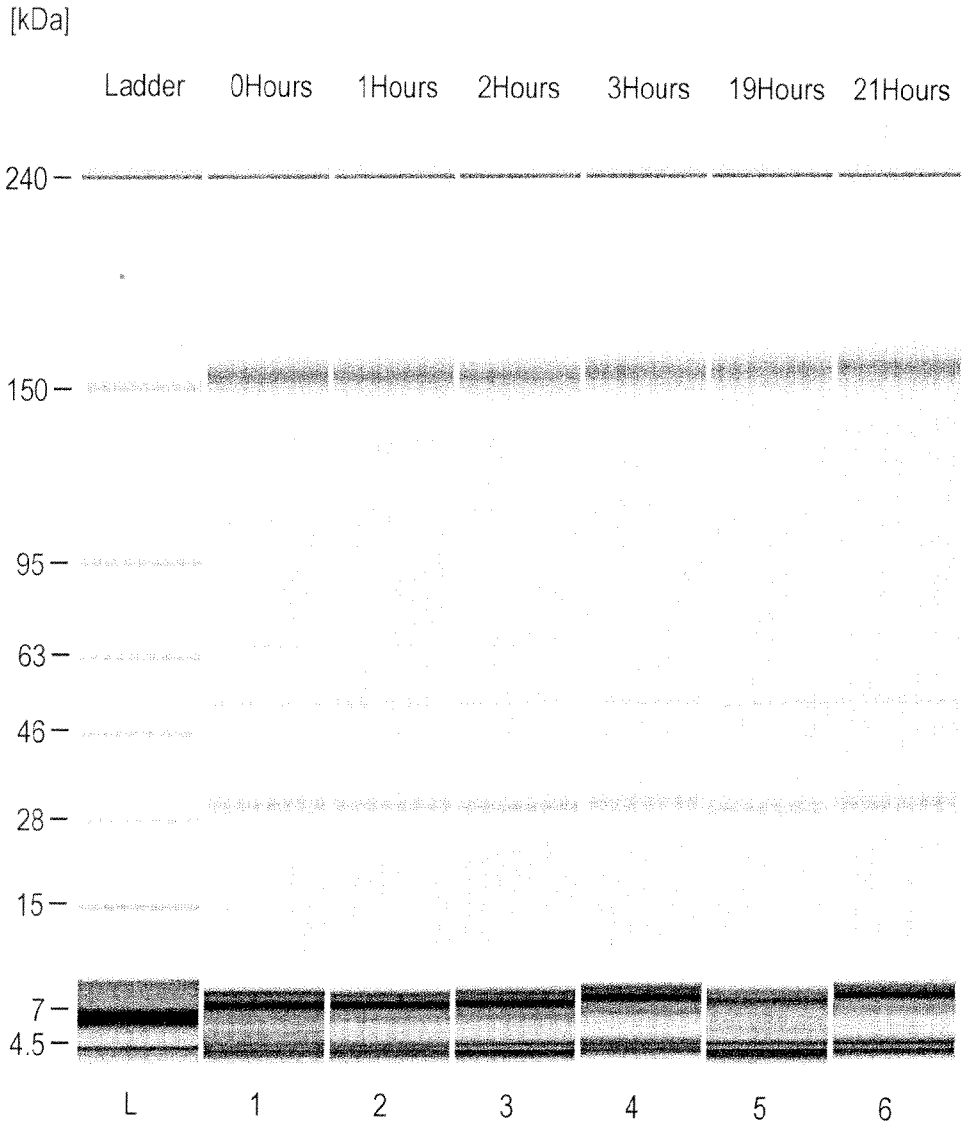
The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate

FIG. 15



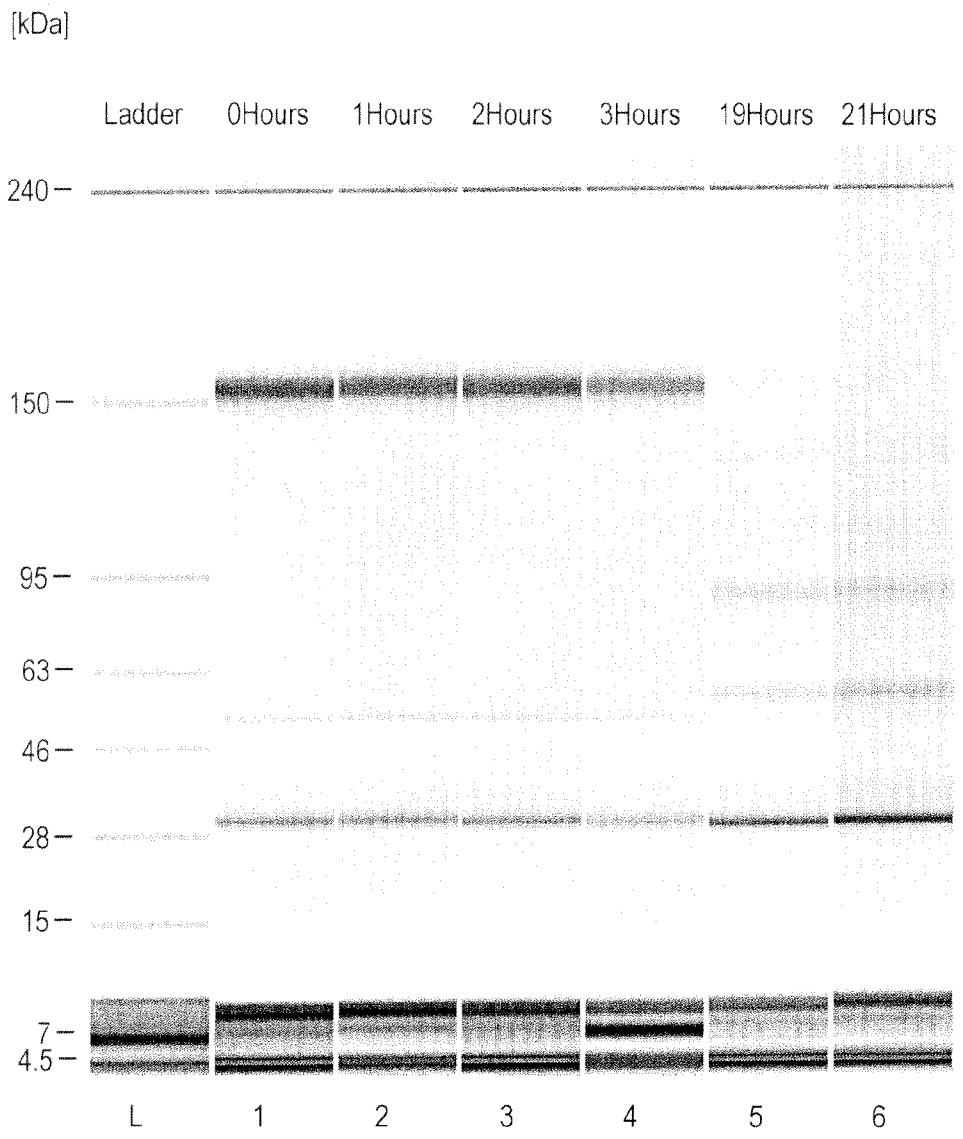
Ocrelizumab Reduction

FIG. 16



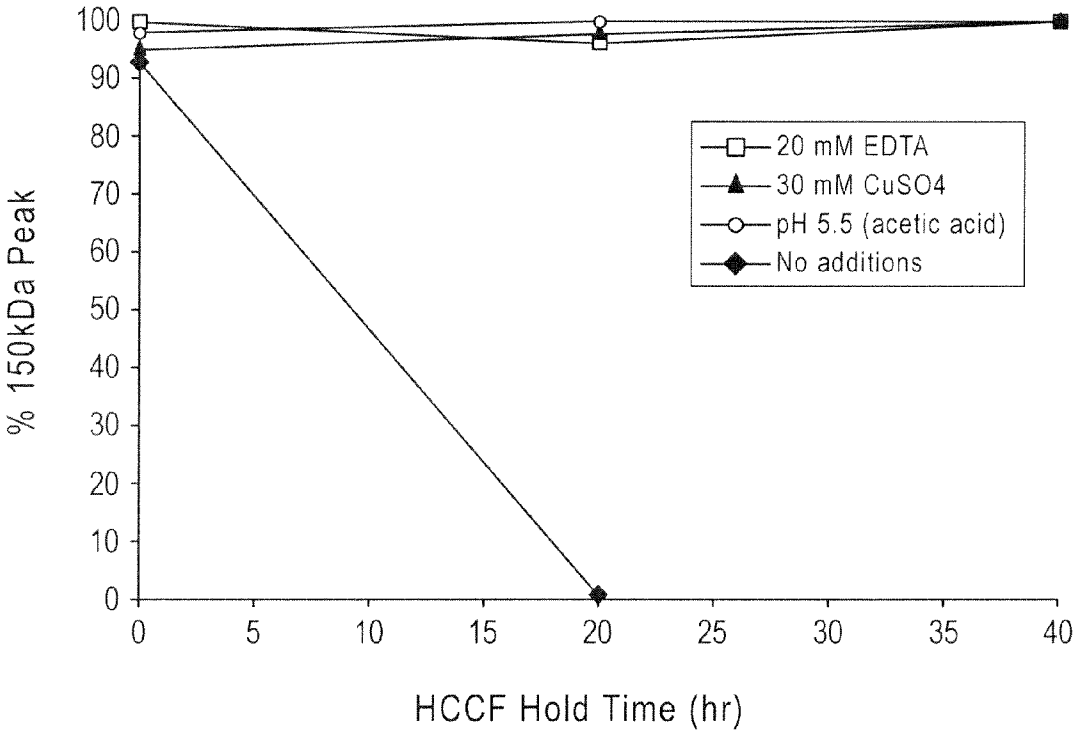
EDTA Inhibits Ocrelizumab Reduction

FIG. 17



The Lost Reduction Activity in Run 8 HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA

FIG. 18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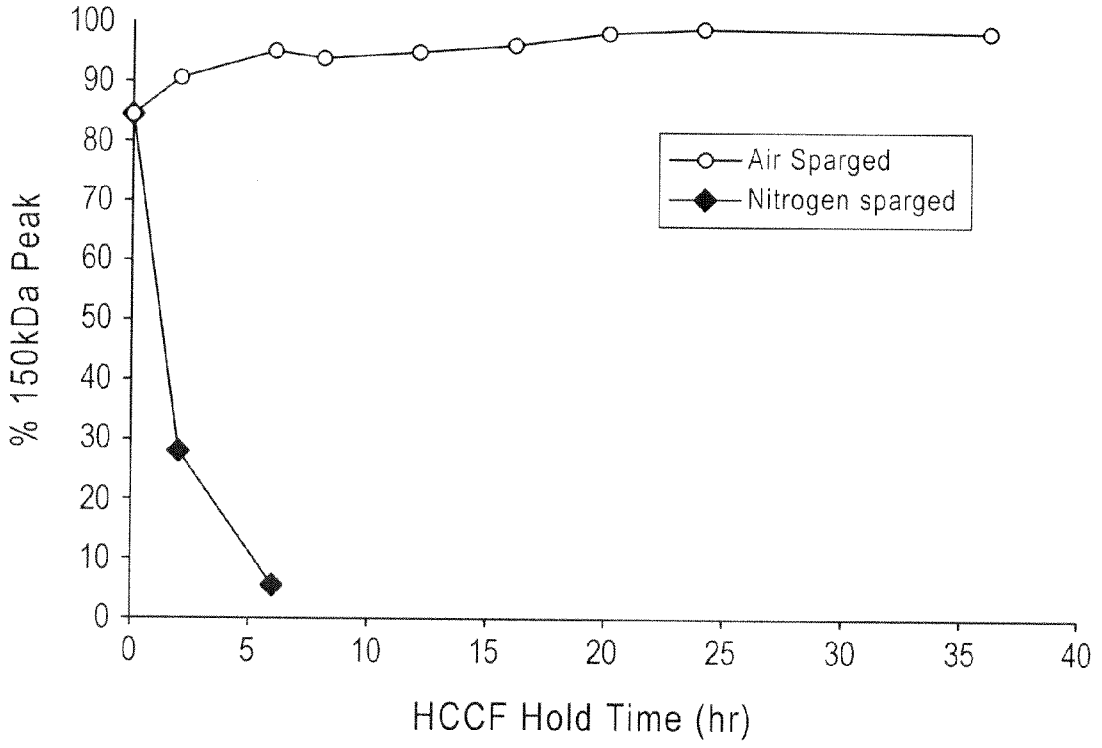
90
80
70
60
50
40
30
20
10
0
% 150kDa Peak
0
5
10
15
20
25
30
35
40
HCCF Hold Time (hr)
20 mM EDTA
30 mM CuSO4
pH 5.5 (acetic acid)
No additions

FIG. 19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90
80
70
60
50
40
30
20
10
0
% 150kDa Peak
0
5
10
15
20
25
30
35
40
HCCF Hold Time (hr)
Air Sparged
Nitrogen sparged

FIG. 20

Light Chain

```
1               15                  30                  45
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPK

46              60                  75                  90
LLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQ

91             105
HYTTPPTFGQGTKVEIK
```

FIG. 21

Heavy Chain

```
1               15                  30                  45
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGL

46              60                  75                  90
EWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAED

91             105                 120
TAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
```

FIG. 22

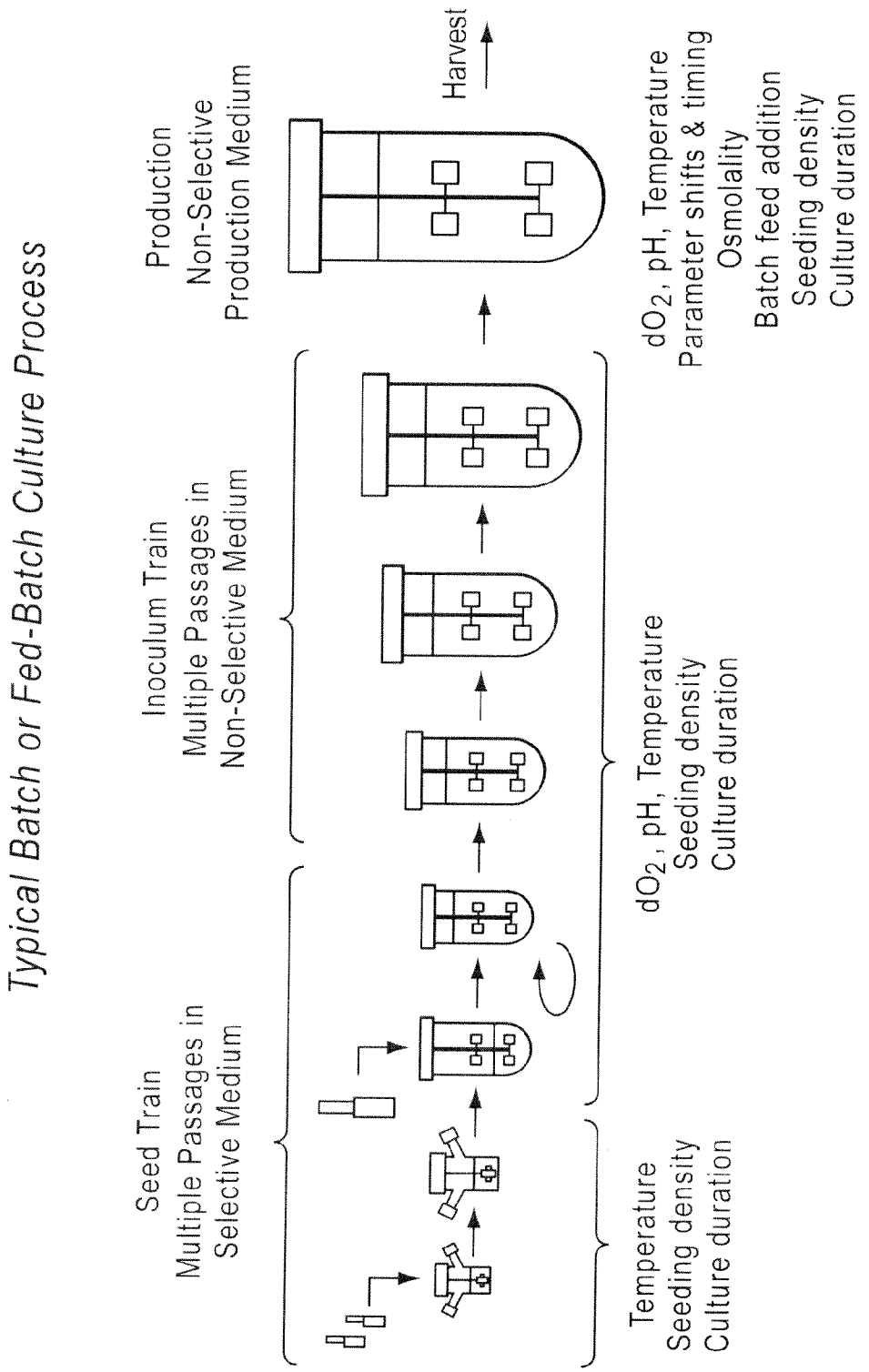
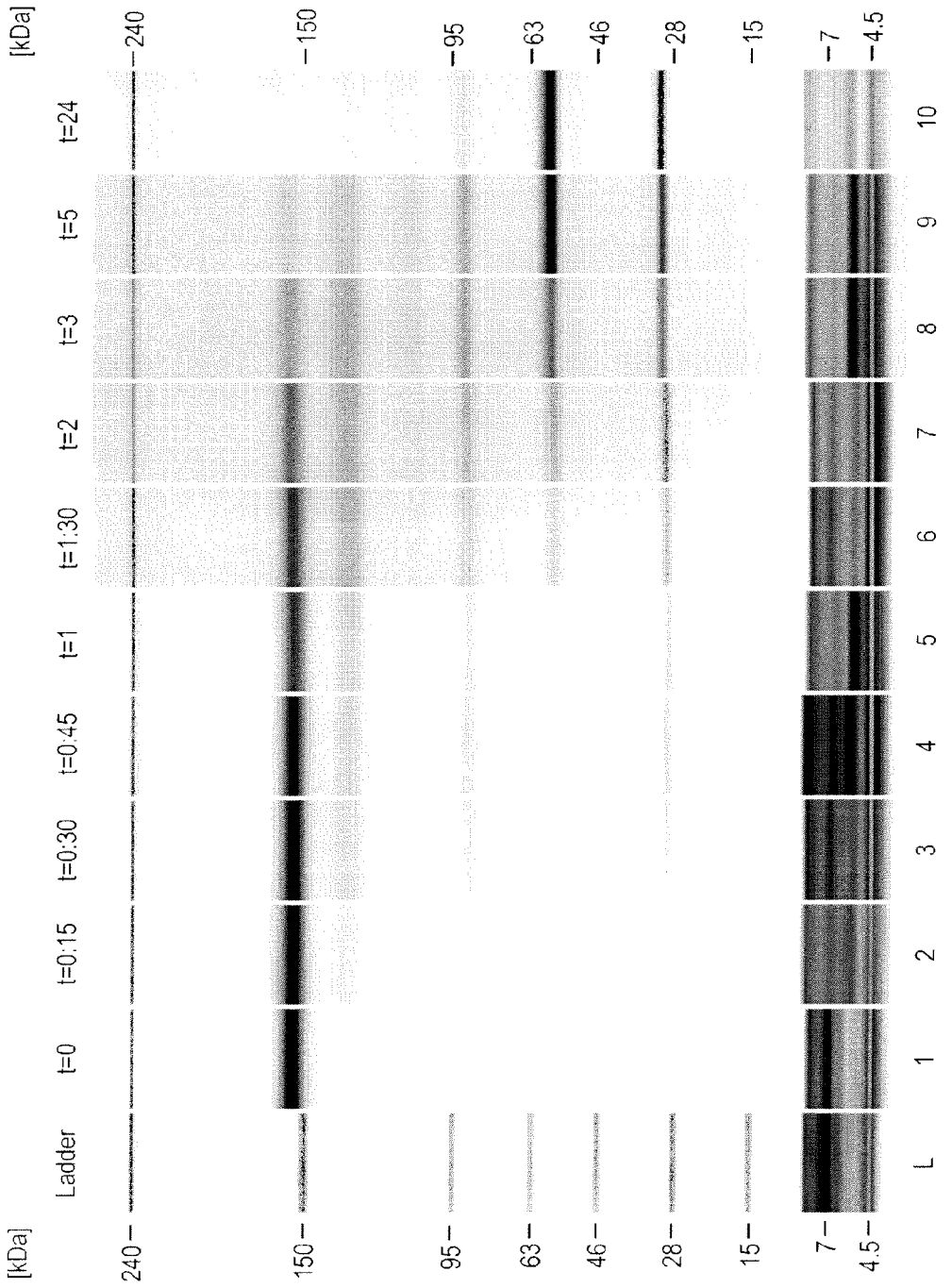

Typical Batch or Fed-Batch Culture Process
Seed Train
Multiple Passages in
Selective Medium
Inoculum Train
Multiple Passages in
Non-Selective Medium
Production
Non-Selective
Production Medium
Harvest
Temperature
Seeding density
Culture duration
dO2, pH, Temperature
Seeding density
Culture duration
dO2, pH, Temperature
Parameter shifts & timing
Osmolality
Batch feed addition
Seeding density
Culture duration

FIG. 23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46
28
15
7
4.5
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t=0:45
t=1
t=1:30
t=2
t=3
t=5
t=24
L
1
2
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4
5
6
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8
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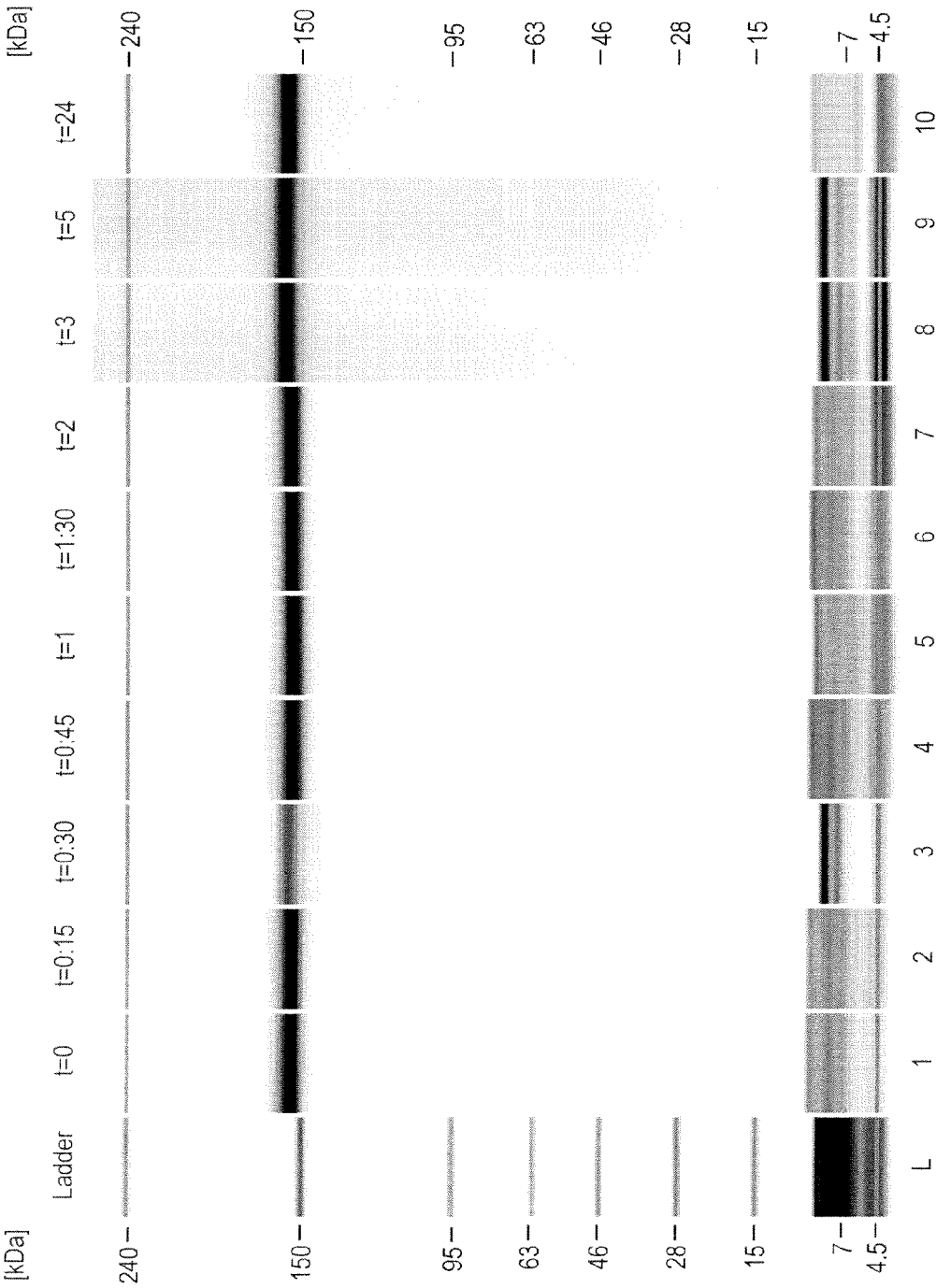
FIG. 24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t=24
L
1
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5
6
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9
10
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150
95
63
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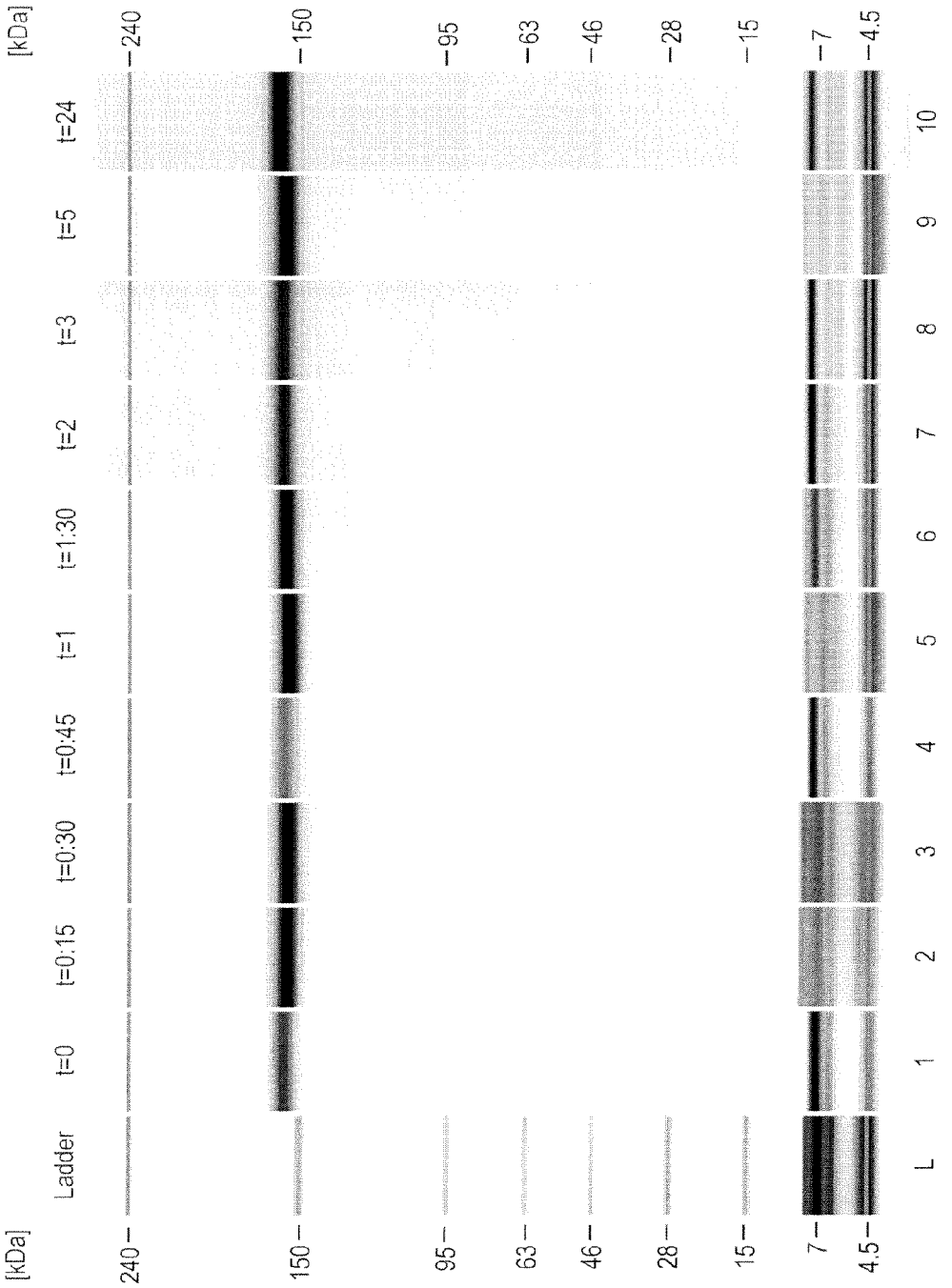
4.5

FIG. 25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t=1:30
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t=24
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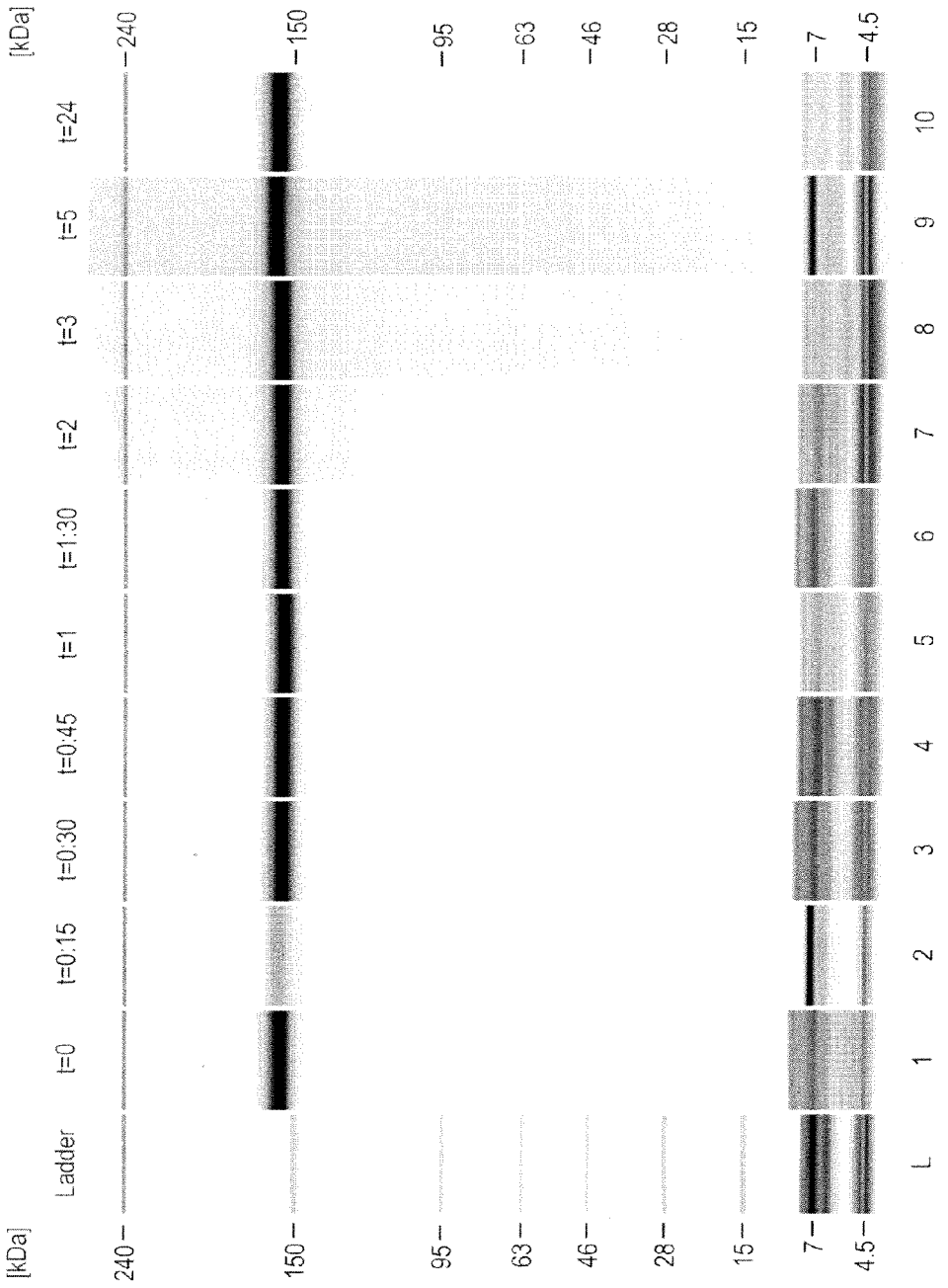
FIG. 26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t=3
t=5
t=24
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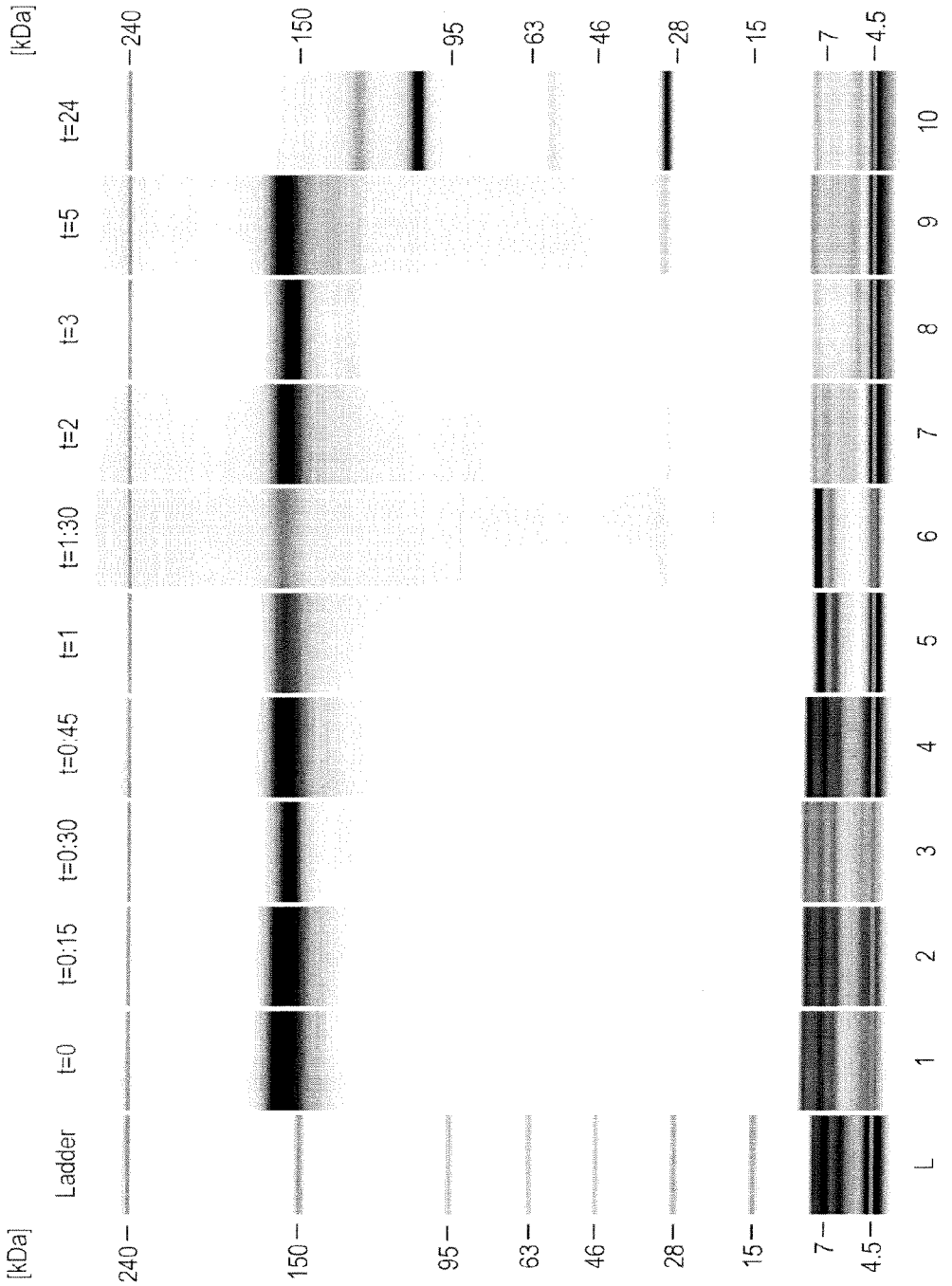
7
4.5

FIG. 27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t=24
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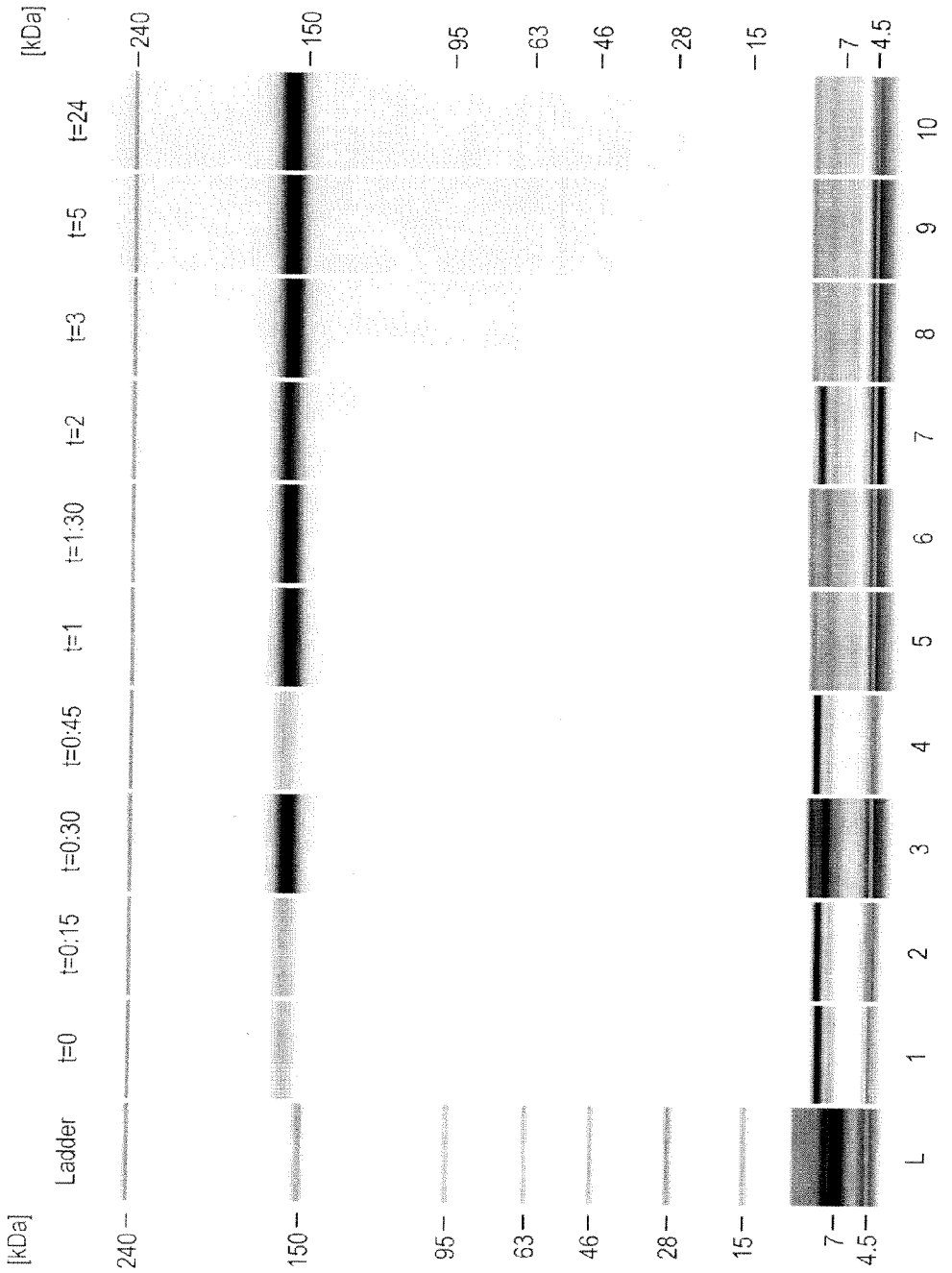
FIG. 28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t=5
t=24
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4.5

FIG. 29

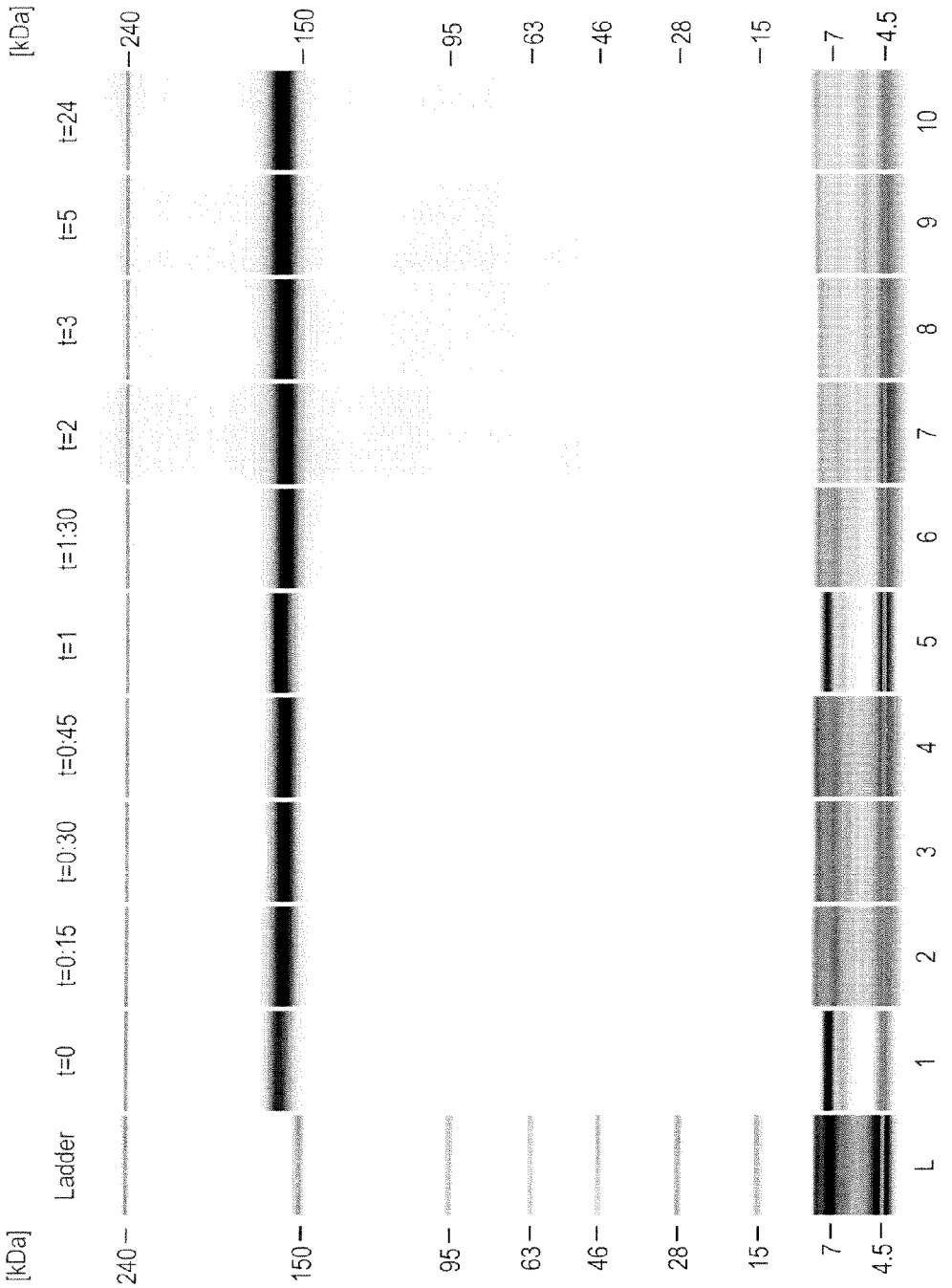
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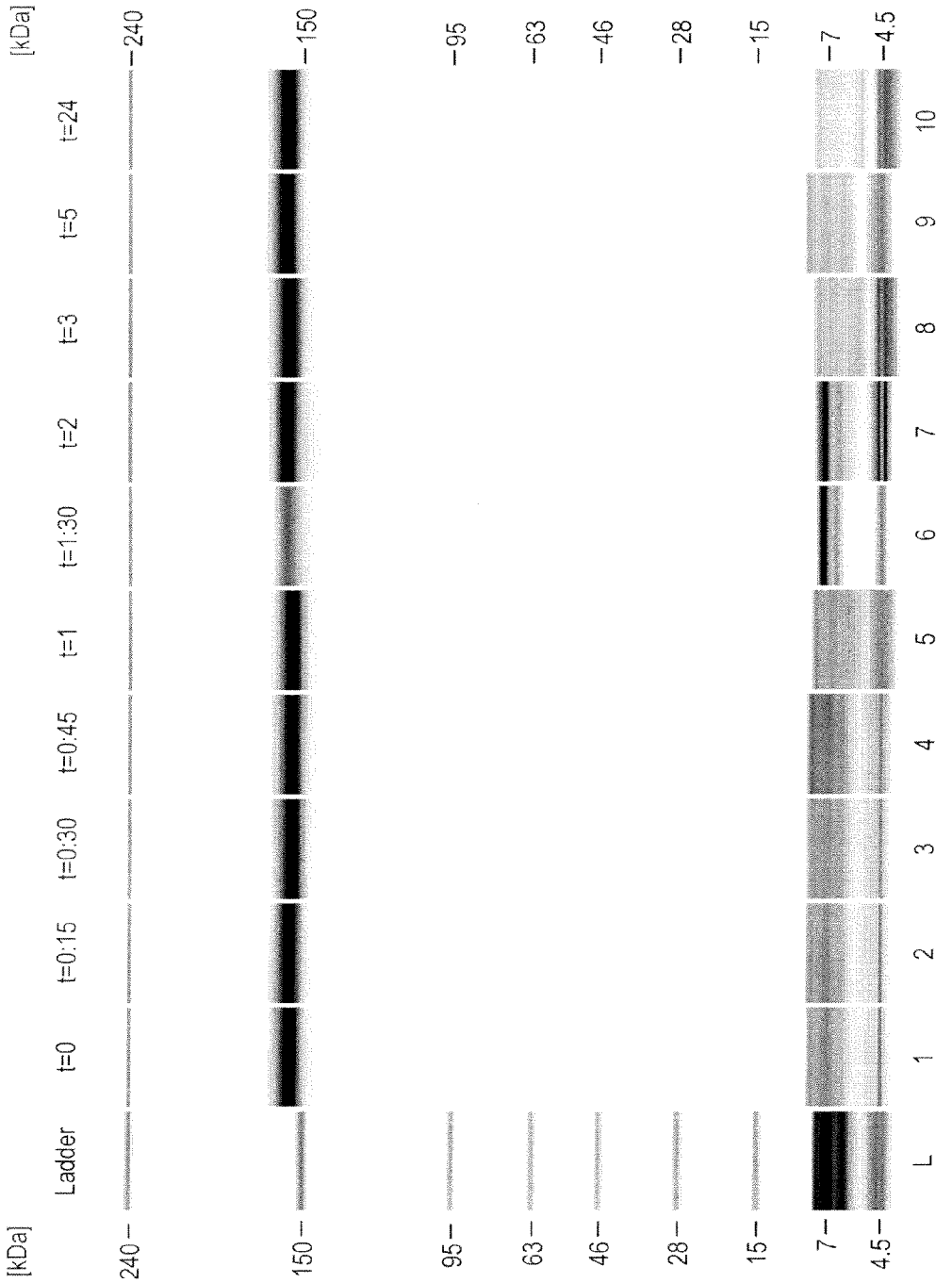
FIG. 30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4.5

FIG. 31

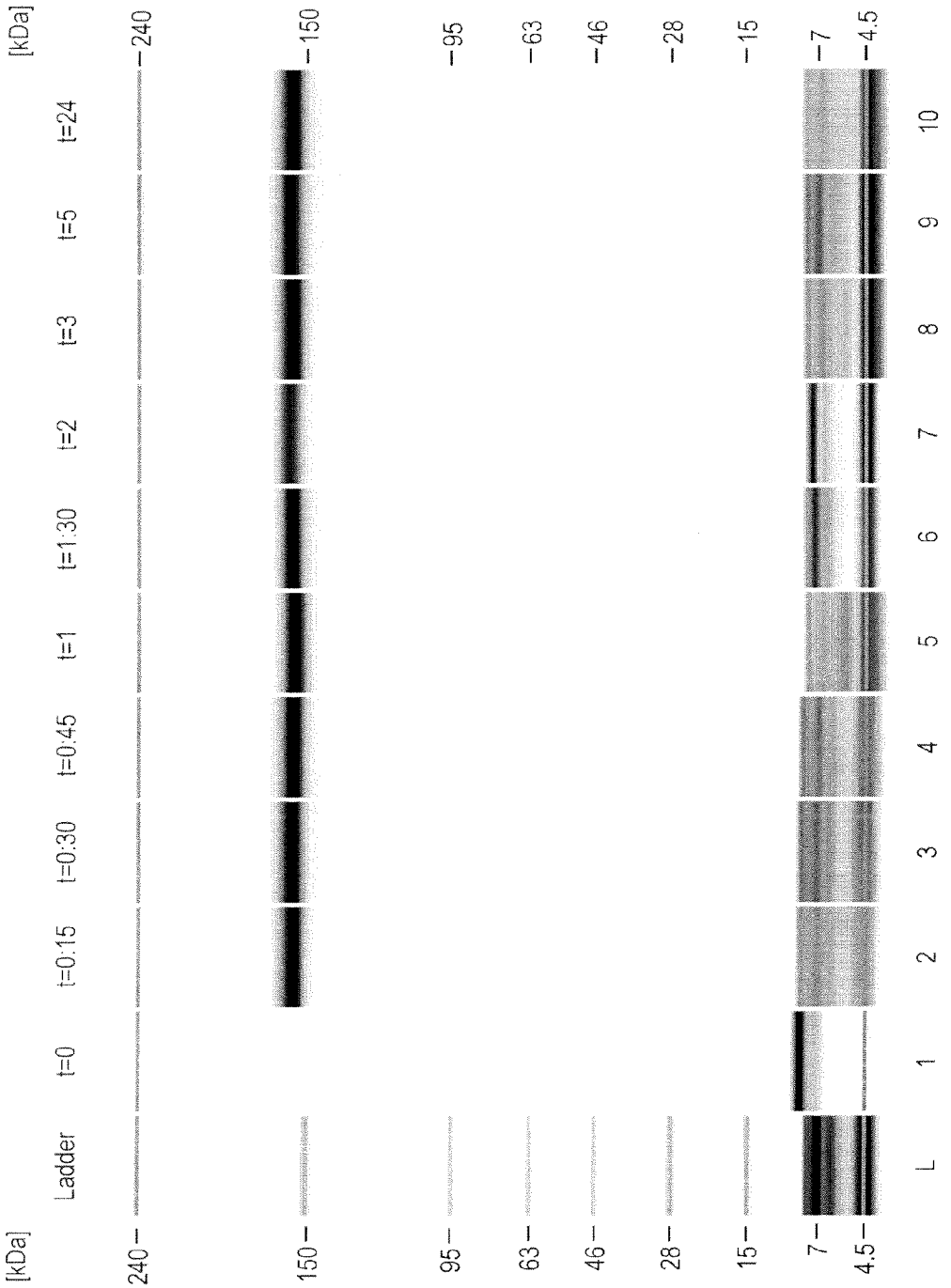
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FIG. 32

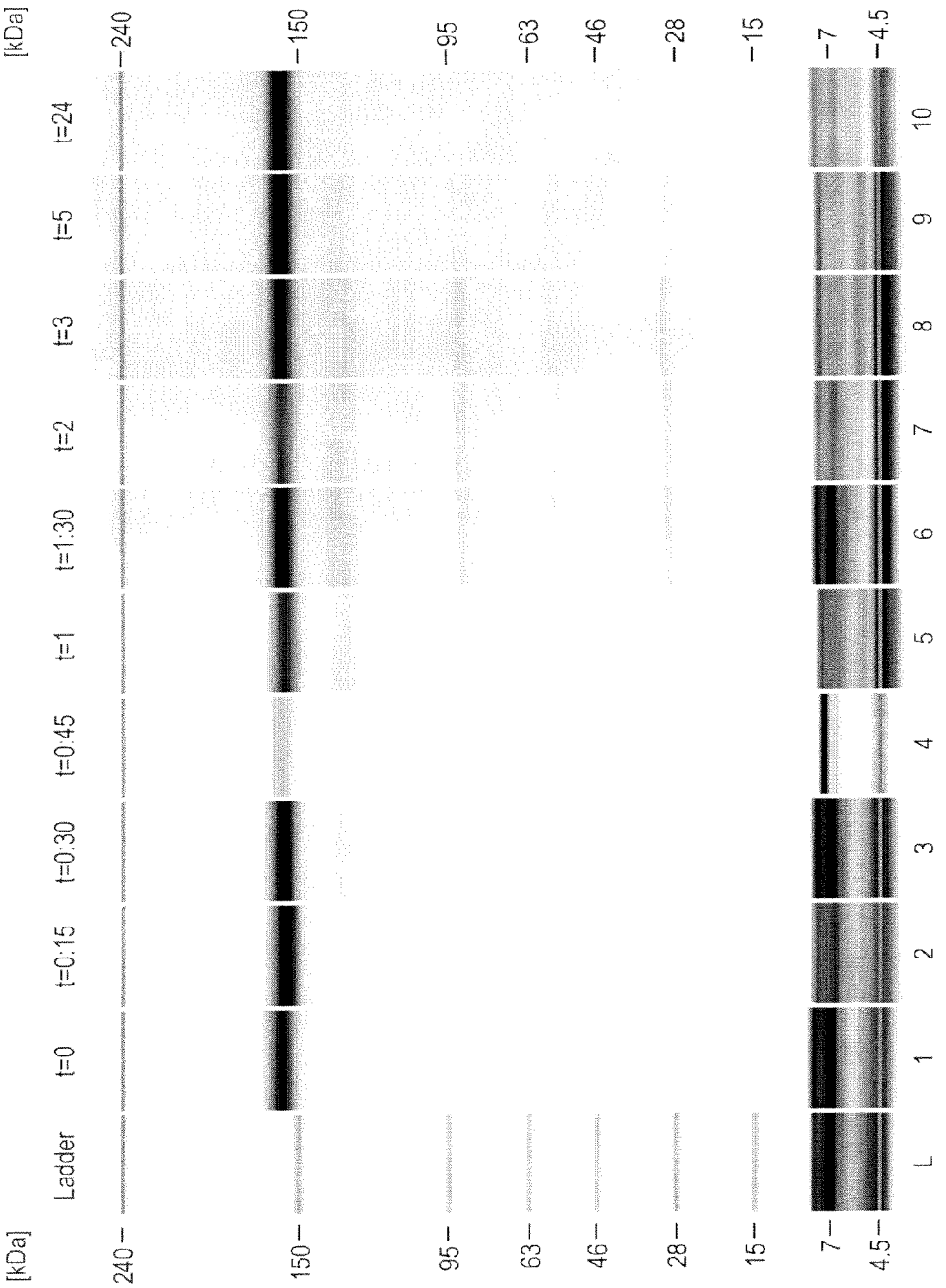
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FIG. 33

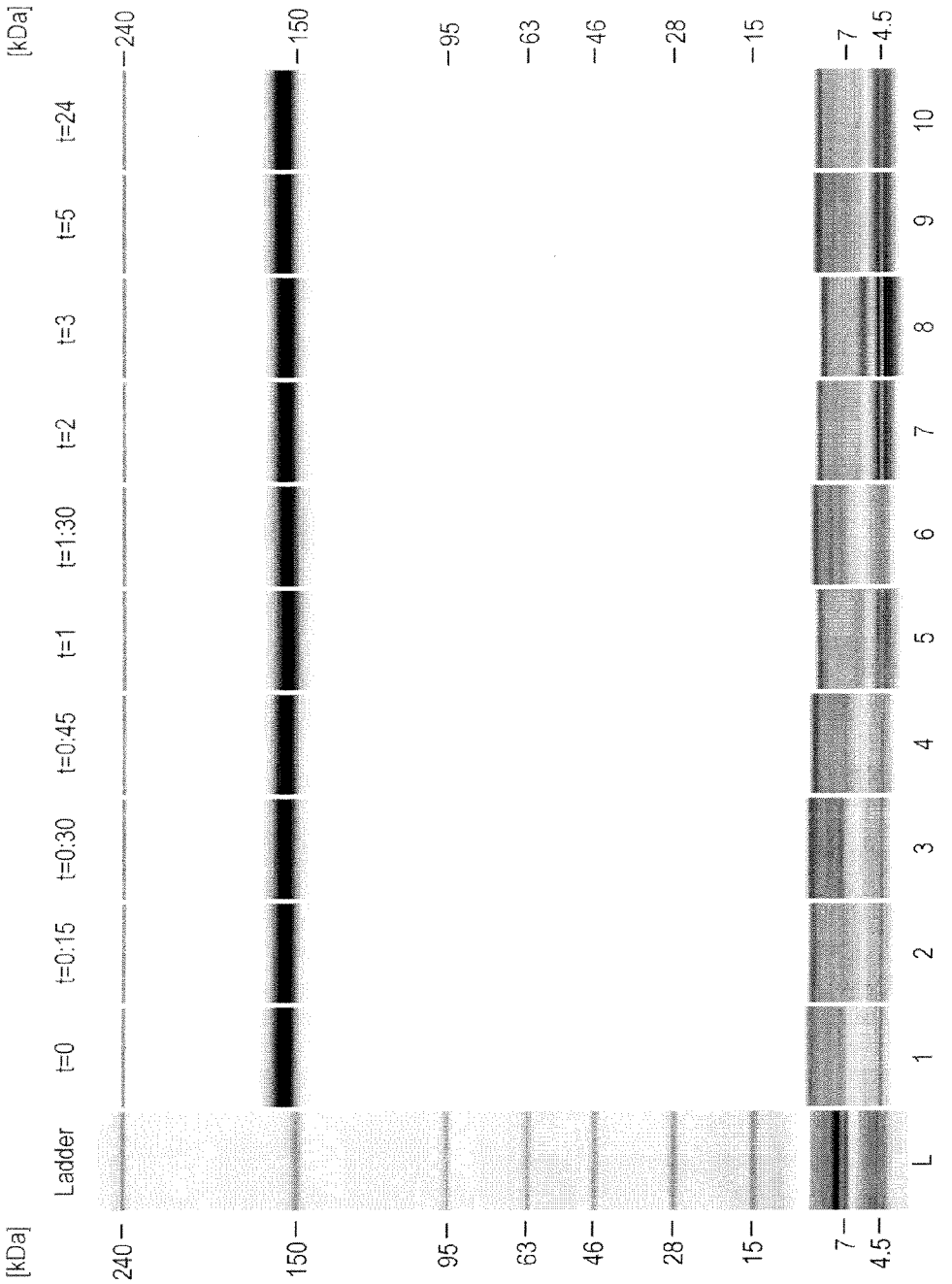
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FIG. 34

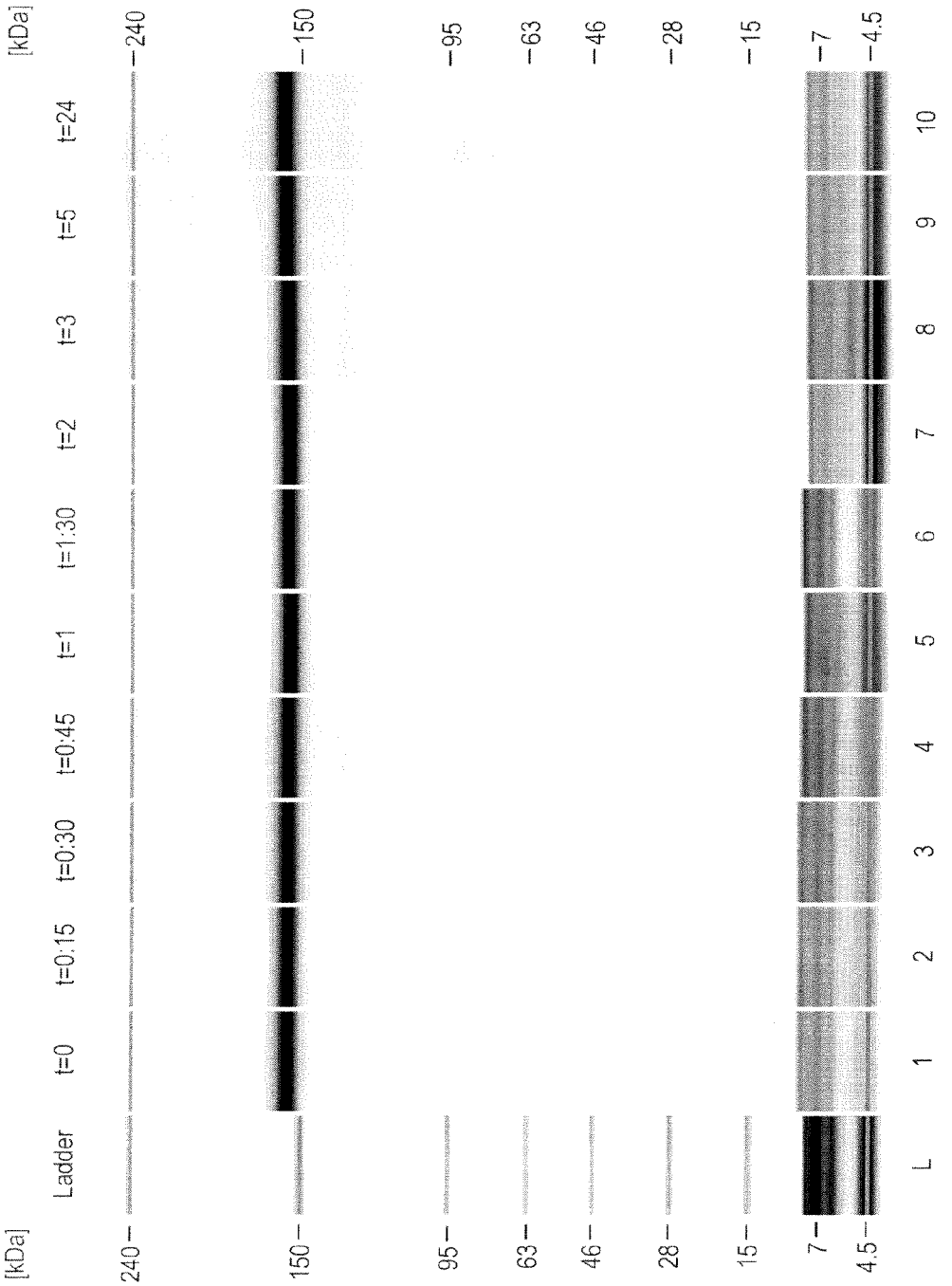
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FIG. 35

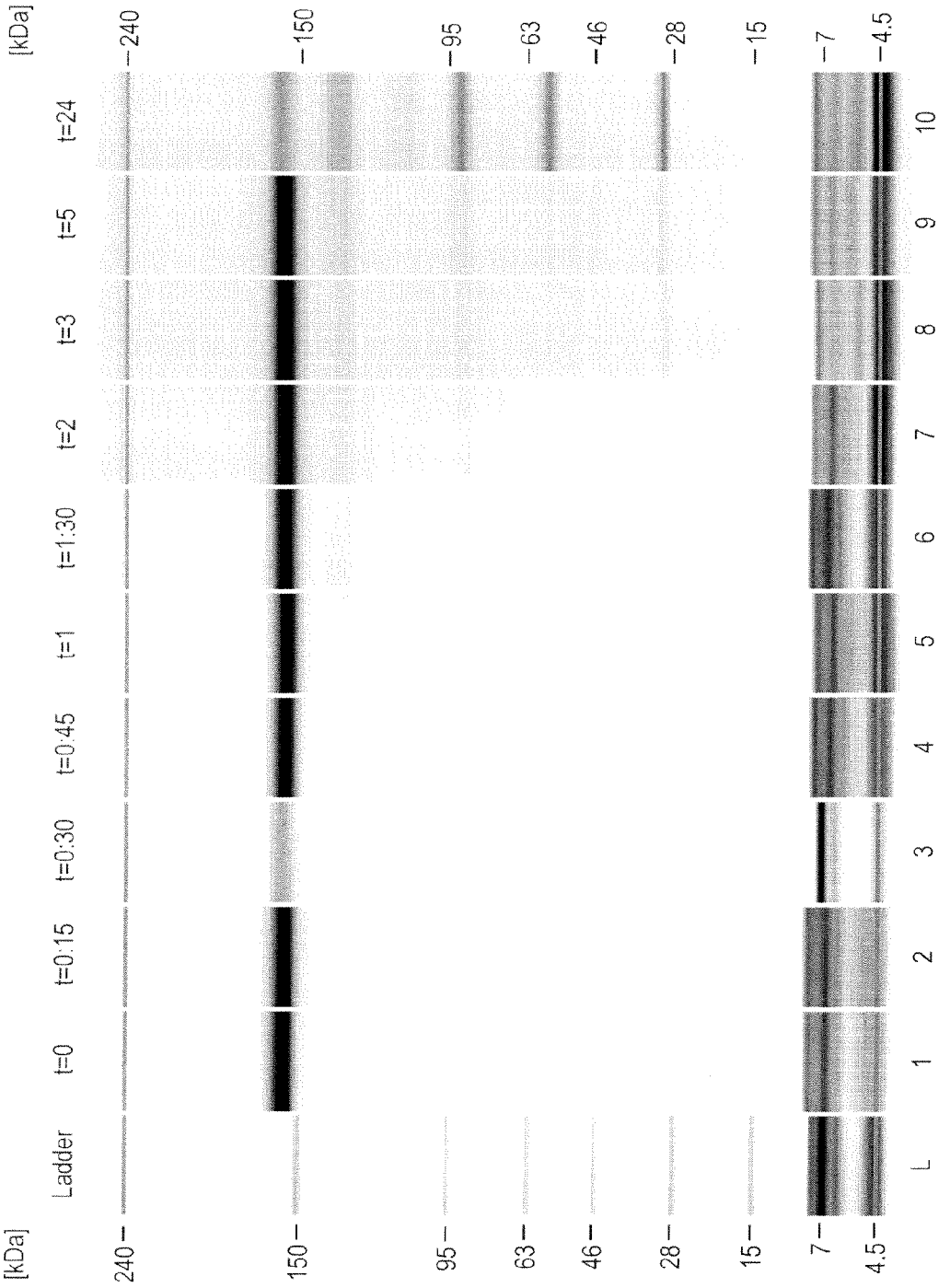
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FIG. 36

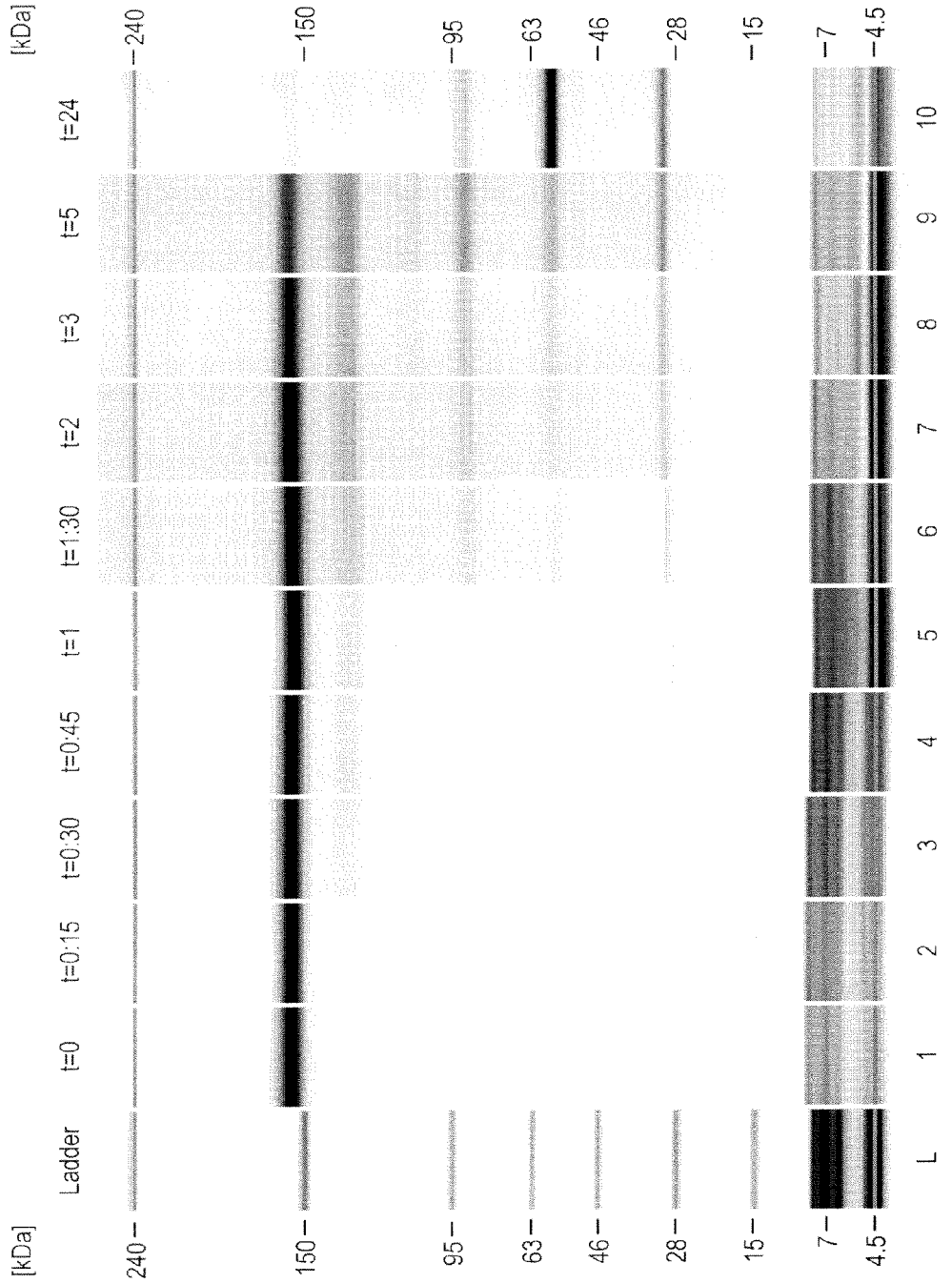
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FIG. 37

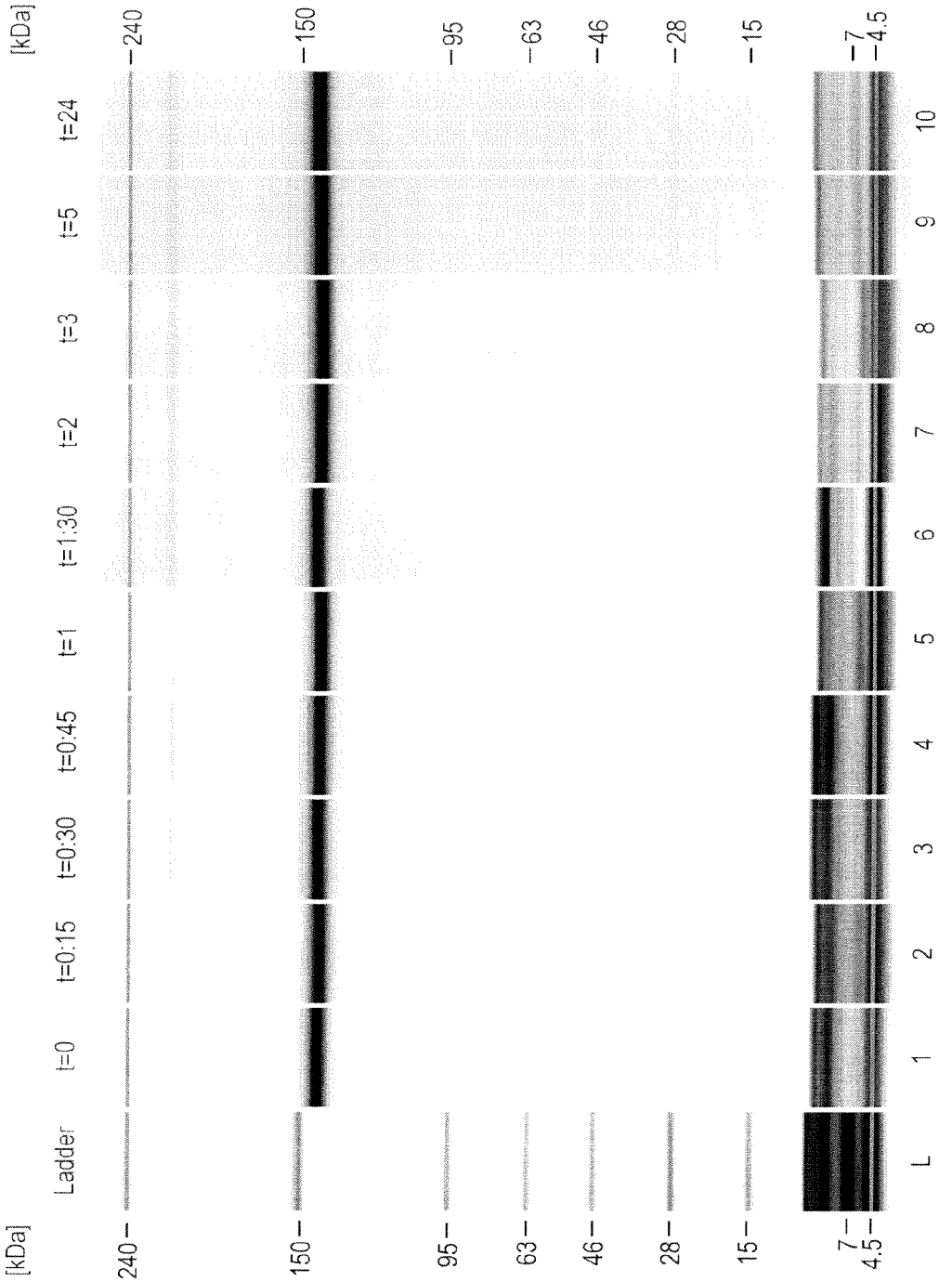
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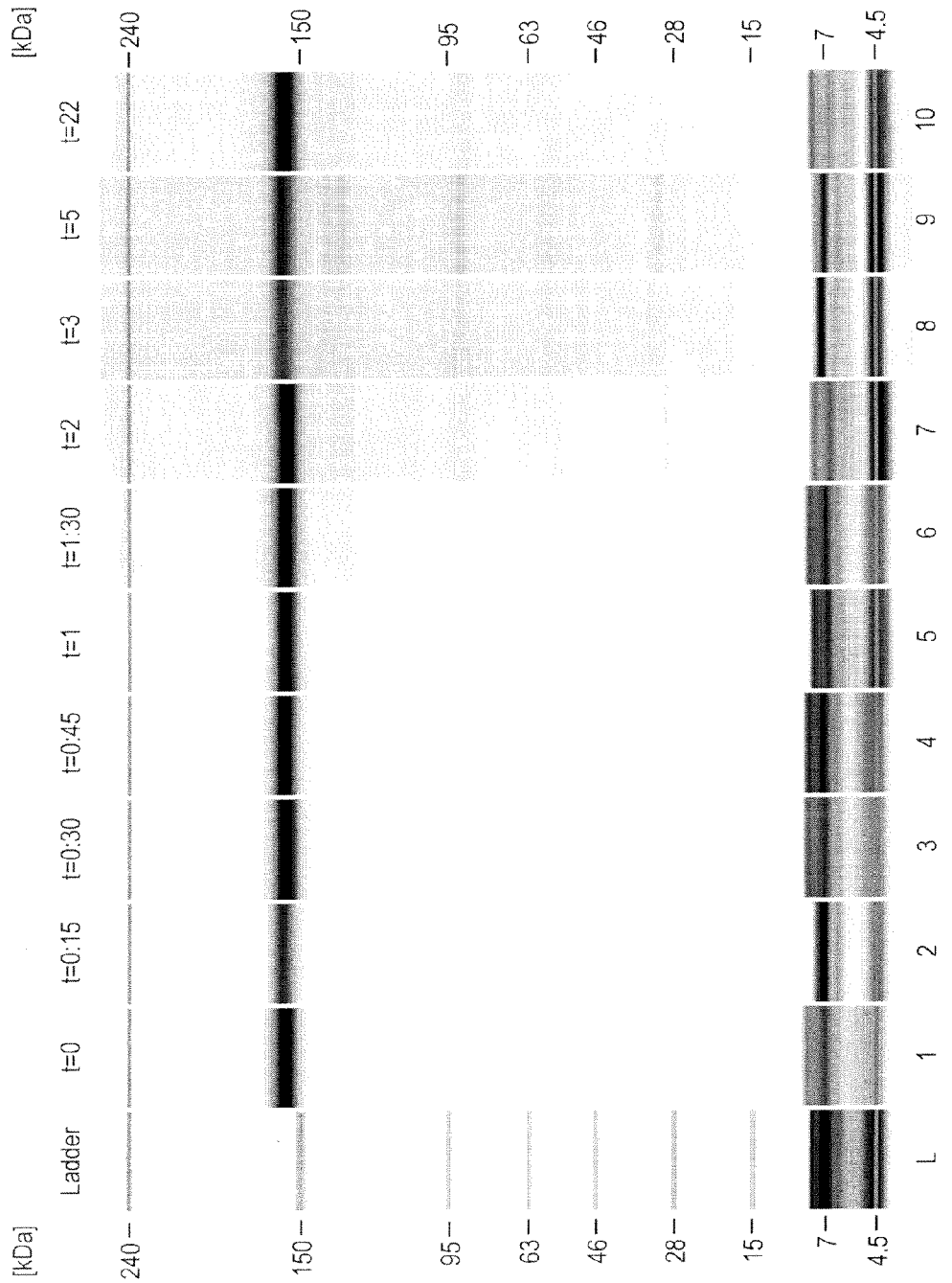
FIG. 38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t=22
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150
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7
4.5

FIG. 39

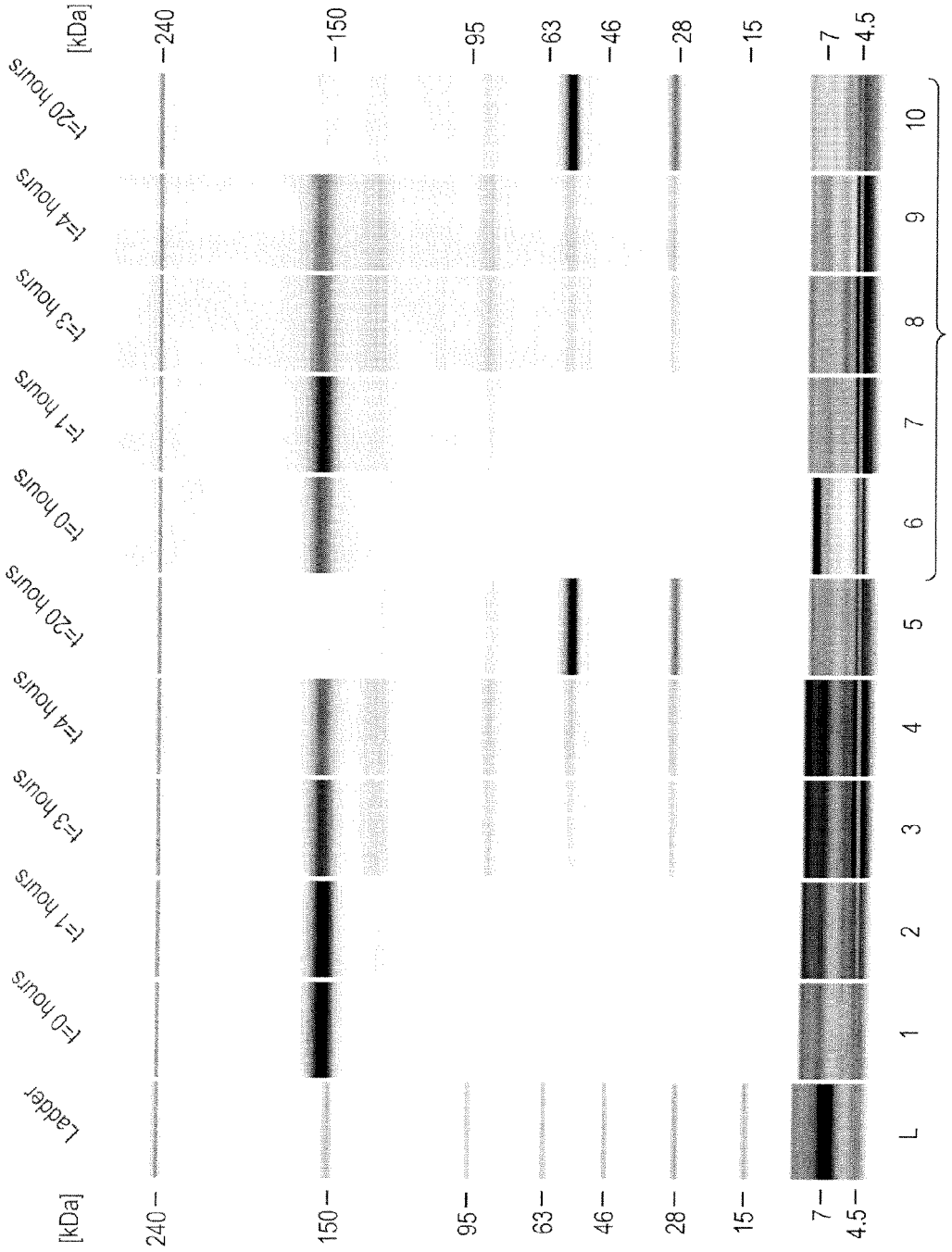
[kDa]
240
150
95
63
46
28
15
7
4.5
Ladder
t=0 hours
t=1 hours
t=3 hours
t=4 hours
t=20 hours
t=0 hours
t=1 hours
t=3 hours
t=4 hours
t=20 hours
L
1
2
3
4
5
6
7
8
9
10
+0.1 mM DHEA

FIG. 40

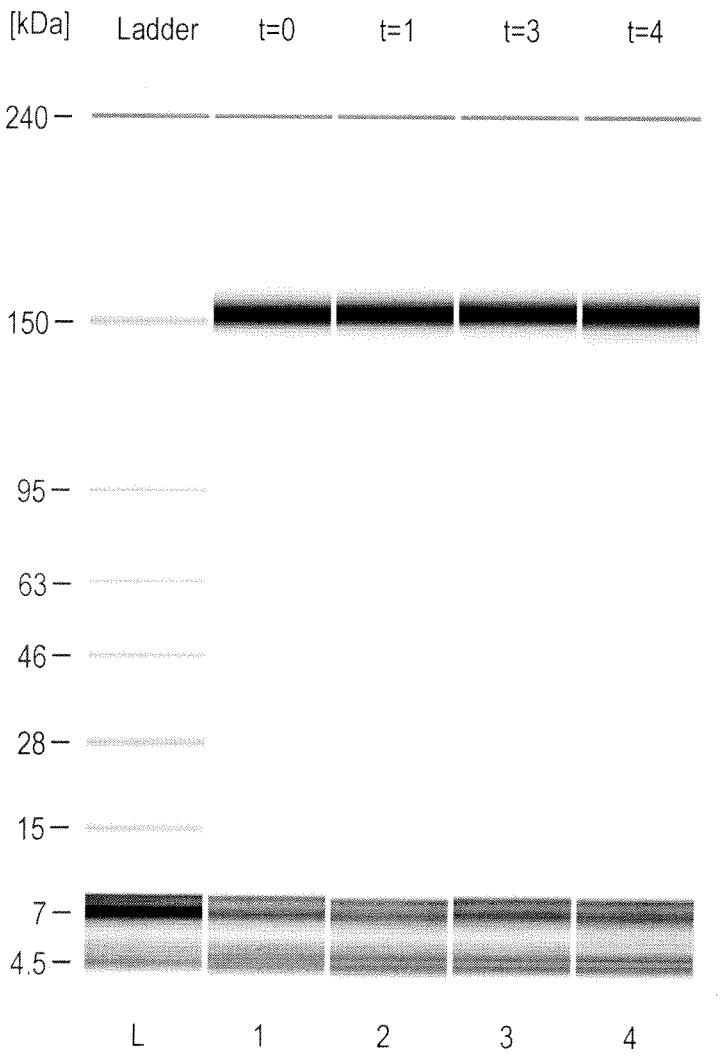

FIG. 41

PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/217,745, filed Jul. 8, 2008, which is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional Application No. 60/948,677 filed Jul. 9, 2007, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention concerns methods and means for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

BACKGROUND OF THE INVENTION

In the biotechnology industry, pharmaceutical applications require a variety of proteins produced using recombinant DNA techniques. Generally, recombinant proteins are produced by cell culture, using either eukaryotic cells, such as mammalian cells, or prokaryotic cells, such as bacterial cells, engineered to produce the protein of interest by insertion of a recombinant plasmid containing the nucleic acid encoding the desired protein. For a protein to remain biologically active, the conformation of the protein, including its tertiary structure, must be maintained during its purification and isolation, and the protein's multiple functional groups must be protected from degradation.

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells, such as the PER.C6® cell line isolated from a human retinal cell, which provides human glycosylation characteristics, and is able to naturally produce antibodies that match human physiology, have been approved by regulatory agencies for the production of biopharmaceutical products.

Usually, to begin the production cycle, a small number of transformed recombinant host cells are allowed to grow in culture for several days (see, e.g., FIG. **23**). Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Typically, harvesting includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, the production of recombinant proteins is still not without difficulties. Thus, for example, during the recombinant production of polypeptides comprising disulfide bonds, especially multi-chain polypeptides comprising inter-chain disulfide bonds such as antibodies, it is essential to protect and retain the disulfide bonds throughout the manufacturing, recovery and purification process, in order to produce properly folded polypeptides with the requisite biological activity.

SUMMARY OF THE INVENTION

The instant invention generally relates to a method for preventing reduction of a disulfide bond in a polypeptide expressed in a recombinant host cell, comprising supplementing the pre-harvest or harvested culture fluid of the recombinant host cell with an inhibitor of thioredoxin or a thioredoxin-like protein.

In one embodiment, the thioredoxin inhibitor is added to the pre-harvest culture fluid.

In another embodiment, the thioredoxin inhibitor is added to the harvested culture fluid.

In a further embodiment, the thioredoxin inhibitor is a direct inhibitor of thioredoxin.

In all embodiments, the thioredoxin inhibitor may, for example, be an alkyl-2-imidazolyl disulfide or a naphthoquinone spiroketal derivative.

In a further embodiment, the thioredoxin inhibitor is a specific inhibitor of thioredoxin reductase.

In a still further embodiment, the thioredoxin inhibitor is a gold complex, where the gold complex may, for example, be aurothioglucose (ATG) or aurothiomalate (ATM). While the effective inhibitory concentration may vary, it typically is between about 0.1 mM and 1 mM. Similarly, the minimum effective inhibitory concentration varies depending on the nature of the polypeptide and overall circumstances, and is typically reached when the ATG or ATG concentration is at least about four-times of thioreduxin concentration in the pre-harvest or harvested culture fluid.

In another embodiment of this aspect of the invention, the thioredoxin inhibitor is a metal ion, where the metal ion, without limitation, may be selected from the group consisting of Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}. When the metal ion is added in the form of cupric sulfate, the effective inhibitory concentration generally is between about 5 μM and about 100 μM, or between about 10 μM and about 80 μM, or between about 15 μM and about 50 μM. The minimum inhibitory concentration of cupric sulfate also varies, but typically is reached when cupric sulfate is added at a concentration at least about two-times of thioredoxin concentration in the pre-harves or harvested culture fluid.

In different embodiment, the thioredoxin inhibitor is an oxidizing agent, e.g., an inhibitor of G6PD, such as, for example, pyridoxal 5'-phosphate, 1 fluoro-2,4 dinitrobenzene, dehydroepiandrosterone (DHEA) or epiandrosterone (EA); cystine or cysteine. Typical effective inhibitor concen-

trations of DHEA are between about 0.05 mM and about 5 mM, or between about 0.1 mM and about 2.5 mM.

In a further embodiment, the thioredoxin inhibitor is an inhibitor of hexokinase activity, including, without limitation, chelators of metal ions, such as, for example, ethylenediamine tetraacetic acid (EDTA). EDTA is typically added and effective at a concentration between about 5 mM and about 60 mM, or about 10 mM and about 50 mM, or about 20 mM and about 40 mM.

In other preferred embodiments, the inhibitor of hexokinase activity is selected from the group consisting of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, and lyxose.

Other inhibitors include cystine, cysteine, and oxidized glutathione which are typically added at a concentration at least about 40-times of the concentration of the polypeptide in question in the pre-harvest or harvested culture fluid.

In a still further embodiment, the thioredoxin inhibitor is an siRNA, an antisense nucleotide, or an antibody specifically binding to a thioredoxin reductase.

In another embodiment, the thioredoxin inhibitor is a measure indirectly resulting in the inhibition of thioredoxin activity. This embodiment includes, for example, air sparging the harvested culture fluid of the recombinant host cell, and/or lowering the pH of the harvested culture fluid of the recombinant host cell.

In various embodiments, indirect means for inhibiting thioredoxin activity, such as air sparging and/or lowering of the pH, can be combined with the use of direct thioredoxin inhibitors, such as those listed above.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', $F(ab')_2$, scFv, $(scFv)_2$, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha_4\beta_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In a further embodiment, the HER receptor is HER1 and/or HER2, preferably HER2. The HER2 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NO: 16, 17, 18, and 19.

In another embodiment, the therapeutic antibody is an antibody that binds to CD20. The anti-CD20 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 1 through 15.

In yet another embodiment, the therapeutic antibody is an antibody that binds to VEGF. The anti-VEGF antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 20 through 25.

In an additional embodiment, the therapeutic antibody is an antibody that binds CD11a. The anti-CD11a antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 26 through 29.

In a further embodiment, the therapeutic antibody binds to a DR5 receptor. The anti-DR5 antibody may, for example, be selected from the group consisting of Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, and preferably is Apomab 8.3 or Apomab 7.3, and most preferably Apomab 7.3.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an

ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell. such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

In all embodiments, the recombinant host cell can also be a prokaryotic host cell, such as a bacterial cell, including, without limitation, *E. coli* cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) demonstrating that ocrelizumab (rhuMAb 2H7—Variant A) inside the dialysis bag remained intact during the incubation period.

FIG. **2**. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab outside the dialysis bag was reduced during the incubation period. This is evidenced by the loss of intact antibody (~150 kDa) and the formation of antibody fragments depicted in the Figure. At the 48-hour time point (Lane 7), the reduced antibody appeared to be reoxidized, presumably as a result of loosing reduction activity in the Harvested Cell Culture Fluid (HCCF). The band appearing just above the 28 kDa marker arose from the light chain of antibody. There was a significant amount of free light already present in the HCCF before the incubation began. The presence of excess free light chain and dimers of light chain in the HCCF is typical for the cell line producing ocrelizumab.

FIG. **3**. Free Thiol Levels from Dialysis Experiment: Purified ocrelizumab in phosphate buffered saline (PBS) was inside the dialysis bag and HCCF containing ocrelizumab was outside the bag. Free thiols inside (boxes) and outside (diamonds) the dialysis bag reached comparable levels within a few hours, indicating a good exchange of small molecule components in the HCCF between inside and outside the dialysis bag.

FIG. **4**. Thioredoxin System and Other Reactions Involved in Antibody Reduction: The thioredoxin system, comprising thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, functions as a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis.

FIG. **5**. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) demonstrating that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in PBS resulted in the complete reduction of ocrelizumab; the ocrelizumab was completely reduced in less than 21 hours.

FIG. **6**. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose: The addition of aurothioglucose (ATG) to the same reaction mixture as described in the caption for FIG. **5**, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **7**. In vitro Activity of Thioredoxin System Inhibited by Aurothiomalate: The addition of aurothiomalate (ATM) at a concentration of 1 mM to the same reaction mixture as described in the caption for FIG. **5**, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **8**. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in 10 mM histidine sulfate buffer resulted in the reduction of ocrelizumab in less than 1 hour.

FIG. **9**. In vitro Activity of Thioredoxin System Inhibited by $CuSO_4$: The addition of $CuSO_4$ at a concentration of 50 μM to the same reaction mixture as described in the caption for FIG. **8** effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **10**. Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab was reduced in an incubation experiment using HCCF from a homogenized CCF generated from a 3-L fermentor.

FIG. **11**. Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the addition of 1 mM aurothioglucose to the same HCCF as used for the incubation experiment as shown in FIG. **10** inhibited the reduction of ocrelizumab.

FIG. **12**. Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate: Digital gel-like image from Bioanalyzer (each lane representing a time point) analysis indicating that the addition of 1 mM aurothiomalate to the same HCCF as used for the incubation experiment shown in FIG. **10** inhibited the reduction of ocrelizumab.

FIG. **13**. Losing Reduction Activity in HCCF: The HCCF from one of the large scale manufacturing runs for ocrelizumab (the "beta" run) that was subject to several freeze/thaw cycles demonstrated no ocrelizumab reduction when used in an incubation experiment. This was shown by Bioanalyzer analysis (each lane representing a time point), and can be contrasted to the antibody reduction seen previously in the freshly thawed HCCF from the same fermentation batch.

FIG. **14**. The Lost Reduction Activity in HCCF Restored by Addition of NADPH: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of NADPH at a concentration of 5 mM into the HCCF where the reduction activity has been eliminated under the conditions described above in FIG. **13**.

FIG. **15**. The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 10 mM into the HCCF where the reduction activity has been eliminated due to the treatment described above in FIG. **13**.

FIG. **16**. Ocrelizumab Reduction: A digital gel-like image from Bioanalyzer analysis showing that ocrelizumab was reduced in an incubation experiment using a HCCF from a large scale manufacturing run (the "alpha" run).

FIG. **17**. EDTA Inhibits Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the reduction of ocrelizumab was inhibited in an incubation experiment using a HCCF from the alpha run with EDTA added at a concentration of 20 mM to the HCCF whose reducing activity is demonstrated in FIG. **16**.

FIG. **18**. The Lost Reduction Activity in "Beta Run" HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA: The reduction of ocrelizumab was observed in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 5 mM and 20 mM EDTA into the HCCF whose reduction activity had been lost (see FIG. **13**). In contrast to the results shown in FIG. **17**, the presence of EDTA did not block the reduction of ocreliumab.

FIG. **19**. Inhibition of Ocrelizumab Reduction: by (i) addition of EDTA, (ii) addition of $CuSO_4$, or (iii) adjustment of pH to 5.5. All three different methods, (1) addition of EDTA, (2) addition of $CuSO_4$, and (3) adjustment of pH to 5.5, used independently, were effective in inhibiting ocrelizumab reduction. This was demonstrated by the depicted quantitative Bioanalyzer results that showed that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was completely reduced in the control HCCF after 20 hours of HCCF hold time.

FIG. **20**. Inhibition of Ocrelizumab Reduction by Air Sparging: Sparging the HCCF with air was effective in inhibiting ocrelizumab disulfide bond reduction. This was demonstrated by the quantitative Bioanalyzer results showing that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was almost completely reduced in the control HCCF after 5 hours of sparging with nitrogen.

FIG. **21** shows the V_L (SEQ ID NO. 24) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. **22** shows the V_H (SEQ ID No. 25) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. **23** is a schematic showing some steps of a typical large scale manufacturing process.

FIG. **24** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate.

FIG. **25** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 1 mM histidine sulfate+1 mM ATG.

FIG. **26** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.6 μM ATG (6:1 ATG:TrxR).

FIG. **27** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.4 μM ATG (4:1 ATG:TrxR).

FIG. **28** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.2 μM ATG (2:1 ATG:TrxR).

FIG. **29** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.1 mM autothiomalate (ATM).

FIG. **30** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.01 mM autothiomalate (ATM).

FIG. **31** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+20 μM $CuSO_4$ (4:1 Cu^{2+}:Trx).

FIG. **32** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+10 μM $CuSO_4$ (2:1 Cu^{2+}:Trx).

FIG. **33** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+5 μM $CuSO_4$ (1:1 Cu^{2+}:Trx).

FIG. **34** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+532 μM cystamine (20:1 cystamine:2H7 disulfide).

FIG. **35** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+266 μM cystamine (10:1 cystamine:2H7 disulfide).

FIG. **36** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+133 μM cystamine (5:1 cystamine:2H7 disulfide).

FIG. **37** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+26.6 μM cystamine (1:1 cystamine:2H7 disulfide).

FIG. **38** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate (pH=7.6)+2.6 mM cystine.

FIG. **39** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+2.6 mM GSSG (oxidized glutathione).

FIG. **40** Reconstructed enzymatic reduction system. 1 mg/ml 2H7 (Variant A)+10 μg/mL hexokinase, 50 μg/mL glucose-6-phosphate dehydrogenase, 5 μM thioredoxin, 0.1 μM thioredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+}, and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

FIG. **41** The thioredoxin system requires NADPH. 1 mg/ml 2H7 (Variant A)+5 μM thioredoxin, 0.1 μM thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

In the present invention, in the context of proteins, including antibodies, in general, or with regard to any specific protein or antibody, the term "reduction" is used to refer to the reduction of one or more disulfide bonds of the protein or antibody. Thus, for example, the terms "ocrelizumab reduction" is used interchangeably with the term "ocrelizumab disulfide bond reduction" and the term "antibody (Ab) reduction" is used interchangeably with the term "antibody (Ab) disulfide bond reuction."

The terms "reduction" or "disulfide bond reduction" are used in the broadest sense, and include complete and partial reduction and reduction of some or all of the disulfide bonds, interchain or intrachain, present in a protein such as an antibody.

By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed

within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term "therapeutic protein" or "therapeutic polypeptide" refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. "Manufacturing scale" production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

The term "therapeutic antibody" refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell's death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A "biologically functional fragment" of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The term "diagnostic protein" refers to a protein that is used in the diagnosis of a disease.

The term "diagnostic antibody" refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A "biologically functional fragment" of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

"Purified" means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained.

The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An "essentially pure" protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An "essentially homogeneous" protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

As noted above, in certain embodiments, the protein is an antibody. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., *Nature,* 256: 495 (1975); Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (see, e.g., Clackson et al., *Nature,* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101 (34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes

encoding human immunoglobulin sequences (see, e.g., WO98/24893; WO96/34096; WO96/33735; WO91/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; Marks et al., *BioTechnology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996) and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., Bio/Technology 10:779-783 (1992) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., *Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier et al., *Gene* 169:147-155 (1995); Yelton et al., *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "V_H." The variable domain of the light chain may be referred to as "V_L." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called a, d, e, g, and m, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and *Mol. Immunology,* 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion retains at least one, and as many

as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO93/1161; Hudson et al., (2003) *Nat. Med.* 9:129-134; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) *Nat. Med.* 9:129-134.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150, 95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally asso-

ciated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The terms "thioredoxin inhibitor" and "Trx inhibitor" are used interchangeably, and include all agents and measures effective in inhibiting thioredoxin activity. Thus, thioredoxin (Trx) inhibitors include all agents and measures blocking any component of the Trx, G6PD and/or hexokinase enzyme systems. In this context, "inhibition" includes complete elimination (blocking) and reduction of thioredoxin activity, and, consequently, complete or partial elimination of disulfide bond reduction in a protein, such as an antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "Protein A" and "ProA" are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, GE Healthcare and Fermatech. Protein A is generally immobilized on a solid phase support material. The term "ProA" also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term "chromatography" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term "affinity chromatography" and "protein affinity chromatography" are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms "non-affinity chromatography" and "non-affinity purification" refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from GE Healthcare) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from GE Healthcare). A "mixed mode ion exchange resin" refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J. T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (GE Healthcare).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

The "intermediate buffer" is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and

where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter

sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

An "interfering RNA" or "small interfering RNA (siRNA)" is a double stranded RNA molecule less than about 30 nucleotides in length that reduces expression of a target gene. Interfering RNAs may be identified and synthesized using known methods (Shi Y., Trends in Genetics 19(1):9-12 (2003), WO/2003056012 and WO2003064621), and siRNA libraries are commercially available, for example from Dharmacon, Lafayette, Colo. Frequently, siRNAs can be successfully designed to target the 5' end of a gene.

II. Compositions and Methods of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); PCR: *A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells,* 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunochemistry in Practice,* 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering,* 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology.*

1. Prevention of Disulfide Bond Reduction

The present invention concerns methods for the prevention of the reduction of disulfide bonds of proteins during recombinant production. In particular, the invention concerns methods for preventing the reduction of disulfide bonds of recombinant proteins during processing following fermentation. The methods of the invention are particularly valuable for large scale production of disulfide bond containing proteins, such as at a manufacturing scale. In one embodiment, the methods of the invention are useful for large scale protein production at a scale of greater than 5,000 L.

It has been experimentally found that disulfide bond reduction occurs during processing of the Harvested Cell Culture Fluid (HCCF) produced during manufacturing of recombinant proteins that contain disulfide bonds. Typically, this reduction is observed after cell lysis, especially mechanical cell lysis during harvest operations, when it reaches a certain threshold, such as, for example, from about 30% to about 70%, or from about 40% to about 60%, or from about 50% to about 60% total cell lysis. This threshold will vary, depending on the nature of the protein (e.g. antibody) produced, the recombinant host, the production system, production parameters used, and the like, and can be readily determined experimentally.

Theoretically, such reduction might result from a variety of factors and conditions during the manufacturing process, and might be caused by a variety of reducing agents. The present invention is based, at least in part, on the recognition that the root cause of this reduction is an active thioredoxin (Trx) or thioredoxin-like system in the HCCF.

The Trx enzyme system, composed of Trx, thioredoxin reductase (TrxR) and NADPH, is a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis. The results presented herein demonstrate that NADPH, which is required for activity of the Trx system is provided by glucose-6-phosphate dehyrogenase (G6PD) activity, which generates NADPH from glucose and ATP by hexokinase (see FIG. **4**). These cellular enzymes (Trx system, G6PD, and hexokinase) along with their substrates are released into the CCF upon cell lysis, allowing reduction to occur. Accordingly, disulfide reduction can be prevented by inhibitors of the Trx enzyme system or upstream enzyme systems providing components for an active Trx system, such as G6PD and hexokinase activity.

For further details of these enzyme systems, or regarding other details of protein production, see, for example: Babson, A. L. and Babson, S. R. (1973) Kinetic Colorimetric Measurement of Serum Lactate Dehydrogenase Activity. *Clin. Chem.* 19: 766-769; Michael W. Laird et al., "Optimization of BLyS Production and Purification from *Eschericia coli*," *Protein Expression and Purification* 39:237-246 (2005); John C. Joly et al., "Overexpression of *Eschericia coli* Oxidoreductases Increases Recombinant Insulin-like Growth Factor-I Accumulation," Proc. Natl. Acad. Sci. USA 95:2773-2777 (March 1998); Dana C. Andersen et al., "Production Technologies for Monoclonal Antibodies and Their Fragments,"

Current Opinion in Biotechnology 15:456-462 (2004); Yariv Mazor et al., "Isolation of Engineered, Full-length Antibodies from Libraries Expressed in *Escherichia coli*," *Nature Biotech.* 25, 563-565 (1 Jun. 2007); Laura C. Simmons et al., "Expression of Full-length Immunoglobulins in *Escherichia coli*: Rapid and Efficient Production of Aglycosylated Antibodies," *Journal of Immunological Methods* 263:133-147 (2002); Paul H. Bessette et al., "Efficient Folding of Proteins with Multiple Disulfide Bonds in the *Escherichia coli* cytoplasm," Proc. Natl. Acad. Sci. 96(24):13703-08 (1999); Chaderjian, W. B., Chin, E. T., Harris, R. J., and Etcheverry, T. M., (2005) "Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed," *Biotechnol. Prog.* 21: 550-553; Gordon G., Mackow M. C., and Levy H. R., (1995) "On the mechanism of interaction of steroids with human glucose 6-phosphate dehydrogenase," Arch. Biochem. Biophys. 318: 25-29; Gromer S., Urig S., and Becker K., (2004) "TheTrx System—From Science to Clinic," Medicinal Research Reviews, 24: 40-89; Hammes G. G. and Kochavi D., (1962a) "Studies of the Enzyme Hexokinase. I. Steady State Kinetics at pH 8," J. Am. Chem. Soc. 84:2069-2073; Hammes G. G. and Kochavi D., (1962b) "Studies of the Enzyme Hexokinase. III. The Role of the Metal Ion," J. Am. Chem. Soc. 84:2076-2079; Johansson C., Lillig C. H., and Holmgren A., (2004) "Human Mitochondrial Glutaredoxin Reduces S-Glutathionylated Proteins with High Affinity Accepting Electrons from Either Glutathione or Thioredoxin Reductase," J. Biol. Chem. 279:7537-7543; Legrand, C., Bour, J. M., Jacob, C., Capiaumont J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G., Demangel, C., Duval, D., and Hache J., (1992) "Lactate Dehydrogenase (LDH) Activity of the Number of Dead Cells in the Medium of Cultured Eukaryotic Cells as Marker," J. Biotechnol., 25: 231-243; McDonald, M. R., (1955) "Yeast Hexokinase: ATP+ Hexose→Hexose-6-phosphate+ADP," Methods in Enzymology, 1: 269-276, Academic Press, NY; Sols, A., DelaFuente, G., Villar-Palasi, C., and Asensio, C., (1958) "Substrate Specificity and Some Other Properties of Bakers' Yeast Hexokinase," Biochim Biophys Acta 30: 92-101; Kirkpatrick D. L., Kuperus M., Dowdeswell M., Potier N., Donald L. J., Kunkel M., Berggren M., Angulo M., and Powis G., (1998) "Mechanisms of inhibition of the Trx growth factor system by antitumor 2-imidazolyl disulfides," Biochem. Pharmacol. 55: 987-994; Kirkpatrick D. L., Watson S., Kunkel M., Fletcher S., Ulhaq S., and Powis G., (1999) "Parallel syntheses of disulfide inhibitors of the Trx redox system as potential antitumor agents," Anticancer Drug Des. 14: 421-432; Milhausen, M., and Levy, H. R., (1975) "Evidence for an Essential Lysine in G6PD from *Leuconostoc mesenteroides*," Eur. J. Biochem. 50: 453-461; Pleasants, J. C., Guo, W., and Rabenstein, D. L., (1989) "A comparative study of the kinetics of selenol/diselenide and thiol/disulfide exchange reactions," J. Am. Chem. Soc. 111: 6553-6558; Whitesides, G. M., Lilburn, J. E., and Szajewski, R. P., (1977) "Rates of thioldisulfide interchange reactions between mono- and dithiols and Ellman's reagent," J. Org. Chem. 42: 332-338; and Wipf P., Hopkins T. D., Jung J. K., Rodriguez S., Birmingham A., Southwick E. C., Lazo J. S., and Powis G, (2001) "New inhibitors of the Trx-TrxR system based on a naphthoquinone spiroketal natural product lead," *Bioorg. Med. Chem. Lett.* 11: 2637-2641.

According to one aspect of the present invention, disulfide bond reduction can be prevented by blocking any component of the Trx, G6PD and hexokinase enzyme systems. Inhibitors of these enzyme systems are collectively referred to herein as "thioredoxin inhibitors," or "Trx inhibitors." The Trx inhibitors are typically added to the cell culture fluid (CCF), which contains the recombinant host cells and the culture media, and/or to the harvested cell culture fluid (HCCF), which is obtained after harvesting by centrifugation, filtration, or similar separation methods. The HCCF lacks intact host cells but typically contains host cell proteins and other contaminants, including DNA, which are removed in subsequent purification steps. Thus, the Trx inhibitors may be added before harvest and/or during harvest, preferably before harvest.

Alternatively or in addition other, non-specific methods can also be used to prevent the reduction of disulfide bond reduction following fermentation during the recombinant production of recombinant proteins, such as air sparging or pH adjustment. Certain reduction inhibition methods contemplated herein are listed in the following Table 1.

TABLE 1

Reduction Inhibition Methods

Method[1]	Purpose
Addition of EDTA, EGTA, or citrate	To inhibit hexokinase
Addition of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, or lyxose	To inhibit hexokinase
Addition of epiandrosterone or dehydroepiandrosterone (DHEA)	To inhibit G6PD
Addition of pyridoxal 5'-phosphate or 1-fluoro-2,4-dinitrobenzene	To inhibit G6PD
Addition of metal ions such as Cu^{2+}, Zn^{2+} Hg^{2+}, Co^{2+}, or Mn^{2+}	To inhibit Trx system
Addition of alkyl-2-imidazolyl disulfides and related compounds (e.g., 1 methylpropyl-2-imidazolyl disulfide[2]) or naphthoquinone spiroketal derivatives (e.g. palmarumycin CP_1[2])	To inhibit Trx
Addition of aurothioglucose (ATG) or aurothiomalate (ATM)	To inhibit TrxR
Air sparging	To deplete G6P and NADPH; oxidizing agent
Cystine	Oxidizing agent
Oxidized glutathione	Oxidizing agents
pH Adjustment to below 6.0	To reduce thiol-disulfide exchange rate and Trx system activity

[1]Applied to CCF prior to harvest or in HCCF immediately after harvest.
[2]Currently not available commercially.

"Trx inhibitors" for use in the methods of the present invention include, without limitation, (1) direct inhibitors of Trx, such as alkyl-2-imidazolyl disulfides and related compounds (e.g., 1 methylpropyl-2-imidazolyl disulfide) (Kirkpatrick et al., 1998 and 1999, supra) and naphthoquinone spiroketal derivatives (e.g., palmarumycin CP_1) (Wipf et al., 2001, supra); (2) specific inhibitors of TrxR, including gold complexes, such as aurothioglucose (ATG) and aurothiomalate (ATM) (see, e.g., the review by Gromer et al., 2004), which are examples of irreversible inhibitors of TrxR; (3) metal ions, such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}, which can form readily complexes with thiols and selenols, and thus can be used in embodiments of the instant invention as inhibitors of TrxR or Trx; (4) inhibitors of G6PD, such as, for example, pyridoxal 5'-phosphate and 1 fluoro-2,4 dinitrobenzene (Milhausen and Levy 1975, supra), certain steroids, such as dehydroepiandrosterone (DHEA) and epiandrosterone (EA) (Gordon et al., 1995, supra); and (4) inhibitors of hexokinase activity (and thereby production of G6P for the G6PD), including chelators of metal ions, e.g. Mg^{2+}, such as EDTA, and compounds that react with SH groups, sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose,

2-C-hydroxy-methylglucose, xylose and lyxose (Sols et al., 1958, supra; McDonald, 1955, supra); further hexokinase inhibitors are disclosed in U.S. Pat. No. 5,854,067 entitled "Hexokinase Inhibitors." It will be understood that these inhibitors are listed for illustration only. Other Trx inhibitors exists and can be used, alone or in various combinations, in the methods of the present invention.

"Trx inhibitors" for use in the methods of the present invention also include reagents whereby the reduction of recombinantly produced antibodies or proteins may be reduced or prevented by decreasing the levels of enzymes of the Trx system, the pentose phosphate pathway or hexokinase at various points during the production campaign. In some embodiments, this reduction of enzyme levels may be accomplished by the use of targeted siRNAs, antisense nucleotides, or antibodies. To design targeted siRNAs or antisense nucleotides to the genes as found in CHO cells, these gene sequences are available from public databases to select sequences for targeting enzymes in different organisms. See Example 9 below for examples of the genes of the *E. coli* and mouse Trx system.

In addition to using inhibitors discussed above, it is also possible in certain embodiments of the instant invention to prevent the reduction of a recombinant protein to be purified by sparging the HCCF with air to maintain an oxidizing redox potential in the HCCF. This is a non-directed measure that can deplete glucose, G6P and NADPH by continuously oxidizing the reduced forms of Trx and TrxR. Air sparging of the HCCF tank can be performed, for example, with an air flow of about 100 liters to about 200 liters, such as, for example, 150 liters per minutes. Air sparging can be performed to reach an endpoint percentage of saturation; for example, air sparging can be continued until the HCCF is about 100% saturated with air, or it can be continued until the HCCR is about 30% saturated with air, or until it is between about 100% saturated to about 30% saturated with air. The minimum amount of dissolved oxygen (dO_2) required for the desired inhibitory effect also depends on the antibody or other recombinant protein produced. Thus, for example, about 10% dO_2 (or about 10 sccm for continuous stream) will have the desired effect during the production of antibody 2H7 (Variant A), while Apomab might require a higher (about 30%) dO_2.

In further embodiments of the instant invention, another non-directed method usable to block the reduction of the recombinant protein is lowering the pH of the HCCF. This embodiment takes advantage of particularly slow thiol-disulfide exchange at lower pH values (Whitesides et al., 1977, supra; Pleasants et al., 1989, supra). Therefore, the activity of the Trx system is significantly lower at pH values below 6, and thus the reduction of the recombinant protein, such as ocrelizumab, can be inhibited.

The non-directed approaches can also be combined with each other and/or with the use of one or more Trx inhibitors.

Disulfide bond reduction can be inhibited (i.e., partially or fully blocked) by using one or more Trx inhibitors and/or applying non-directed approaches following completion of the cell culture process, preferably to CCF prior to harvest or in the HCCF immediately after harvest. The optimal time and mode of application and effective amounts depend on the nature of the protein to be purified, the recombinant host cells, and the specific production method used. Determination of the optimal parameters is well within the skill of those of ordinary skill in the art.

For example, in a mammalian cell culture process, such as the CHO antibody production process described in the Examples herein, if cupric sulfate ($CuSO_4$ in the form of pentahydrate or the anhydrous form) is used as a Trx inhibitor, it can be added to supplement the CCF or HCCF in the concentration range of from about 5 μM to about 100 μM, such as from about 10 μM to about 80 μM, preferably from about 15 μM to about 50 μM. Since some cell cultures already contain copper (e.g. about 0.04 μM $CuSO_4$ for the CHO cell cultures used in the Examples herein), this amount is in addition to the copper, if any, already present in the cell culture. Any copper (II) salt can be used instead of $CuSO_4$ as long as solubility is not an issue. For example, copper acetate and copper chloride, which are both soluble in water, can be used instead of $CuSO_4$. The minimum effective concentration may also depend on the antibody produced and the stage where the inhibitor is used. Thus, for example, when cupric sulfate is added pre-lysis, for antibody 2H7 (Variant A) the minimum effective concentration is about 30 μM, for Apomab is about 75 μM, and for antibody Variant C (see Table 2) is about 50 μM. When cupric sulfate is added in CC medium, for antibody 2H7 (Variant A) the minimum effective concentration is about 15 μM, for Apomab is about 25 μM, and for antibody Variant C is about 20 μM. One typical minimal $CuSO_4$ inhibitor concentration of 2×Trx concentration (or Trx equivalence).

EDTA can be used in a wide concentration range, depending on the extent of cell lysis, the recombinant host cell used, and other parameters of the production process. For example, when using CHO or other mammalian host cells, EDTA can be typically added in a concentration of between about 5 mM to about 60 mM, such as from about 10 mM to about 50 mM, or from about 20 mM to about 40 mM, depending on the extent of cell lysis. For lower degree of cell lysis, lower concentrations of EDTA will suffice, while for a cell lysis of about 75%-100%, the required EDTA concentration is higher, such as, for example, from about 20 mM to about 40 mM. The minimum effective concentration may also depend on the antibody produced. Thus, for example, for antibody 2H7 (Variant A) the minimum effective EDTA concentration is about 10 mM.

DHEA as a Trx inhibitor is typically effective at a lower concentration, such as for example, in the concentration range from about 0.05 mM to about 5 mM, preferably from about 0.1 mM to about 2.5 mM.

Other Trx inhibitors, such as aurothioglucose (ATG) and aurothiomalate (ATM) inhibit reduction of disulfide bonds in the μM concentration range. Thus, for example, ATG or ATM may be added in a concentration between about 0.1 mM to about 1 mM. While the minimum inhibitory concentration varies depending on the actual conditions, for ATG and ATM typically it is around 4×TrxR concentration.

It is noted that all inhibitors can be used in an excess amount, therefore, it is not always necessary to know the amount of Trx or TrxR in the system.

In a preferred embodiment, the mammalian host cell used in the manufacturing process is a chinese hamster ovary (CHO) cell (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)). Other mammalian host cells include, without limitation, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture), Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary

tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; a human hepatoma line (Hep G2); and myeloma or lymphoma cells (e.g. Y0, J558L, P3 and NS0 cells) (see U.S. Pat. No. 5,807,715).

A preferred host cell for the production of the polypeptides herein is the CHO cell line DP12 (CHO K1 dhfr$^-$). This is one of the best known CHO cell lines, widely used in laboratory practice (see, for example, EP 0,307,247, published Mar. 15, 1989). In addition, other CHO-K1 (dhfr$^-$) cell lines are known and can be used in the methods of the present invention.

The mammalian host cells used to produce peptides, polypeptides and proteins can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM, Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace (1979), Meth. in Enz. 58:44, Barnes and Sato (1980), Anal. Biochem. 102:255, U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985; or U.S. Pat. No. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

A protocol for the production, recovery and purification of recombinant antibodies in mammalian, such as CHO, cells may include the following steps:

Cells may be cultured in a stirred tank bioreactor system and a fed batch culture, procedure is employed. In a preferred fed batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, a single step or multiple step culture procedure may be employed. In a single step culture the host cells are inoculated into a culture environment and the processes are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture can be used. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

In certain embodiments, fed batch or continuous cell culture conditions may be devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., $Na2CO_3$ or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30° C. to 38° C., and a suitable dO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

The cell culture environment during the production phase of the cell culture is typically controlled. Thus, if a glycoprotein is produced, factors affecting cell specific productivity of the mammalian host cell may be manipulated such that the desired sialic acid content is achieved in the resulting glycoprotein. In a preferred aspect, the production phase of the cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged. Further details of this process are found in U.S. Pat. No. 5,721,121, and Chaderjian et al., *Biotechnol. Prog.* 21(2):550-3 (2005), the entire disclosures of which are expressly incorporated by reference herein.

Following fermentation proteins are purified. Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins and components in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a

physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through." Thus, purification of recombinant proteins from the cell culture of mammalian host cells may include one or more affinity (e.g. protein A) and/or ion exchange chomarographic steps.

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

For further details of the industrial purification of therapeutic antibodies see, for example, Fahrner et al., *Biotechnol. Genet. Eng. Rev.* 18:301-27 (2001), the entire disclosure of which is expressly incorporated by reference herein.

In addition to mammalian host cells, other eukaryotic organisms can be used as host cells for expression of the recombinant protein. For expression in yeast host cells, such as common baker's yeast or *Saccharomyces cerevisiae*, suitable vectors include episomally-replicating vectors based on the 2-micron plasmid, integration vectors, and yeast artificial chromosome (YAC) vectors. Other yeast suitable for recombinant production of heterologous proteins include *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290: 140 (1981); EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529; Fleer et al., Bio/Technology, 2: 968 975 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., J. Bacteriol., 737 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; Van den Berg et al., Bio/Technology, 8: 135 (1990)), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., J. Basic Microbiol., 28: 265 278 (1988)); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case et al., Proc. Natl. Acad. Sci. USA, 76: 5259 5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 Oct. 1990); and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium* (WO 91/00357 published 10 Jan. 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., Biochem. Biophys. Res. Commun., 112: 284 289 (1983); Tilburn et al., Gene, 26: 205 221 (1983); Yelton et al., Proc. Natl. Acad. Sci. USA, 81: 1470 1474 (1984)) and *A. niger* (Kelly and Hynes, EMBO J., 4: 475 479 (1985)). Methylotropic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula, Candida, Kloeckera, Pichia, Saccharomyces, Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982). Expression systems for the listed and other yeasts are well known in the art and/or are commercially available.

For expression in insect host cells, such as Sf9 cells, suitable vectors include baculoviral vectors. For expression in plant host cells, particularly dicotyledonous plant hosts, such as tobacco, suitable expression vectors include vectors derived from the Ti plasmid of *Agrobacterium tumefaciens.*

The methods of the present invention also extend to cultures of prokaryotic host cells. Prokaryotic host cells suitable for expressing antibodies and other proteins to be protected by means of the instant invention include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include *Escherichia* (e.g., *E. coli*), *Bacilli* (e.g., *B. subtilis*), *Enterobacteria, Pseudomonas* species (e.g., *P. aeruginosa*), *Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla*, or *Paracoccus*. In one embodiment, gram-negative cells are used. Examples of *E. coli* strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) and *E. coli* RV308 (ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli, Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Methods for the production, recovery and purification of recombinant proteins from non-mammalian host cell cultures are also well known in the art. If the polypeptide is produced in a non-mammalian cell, e.g., a microorganism such as fungi or *E. coli*, the polypeptide will be recovered inside the cell or in the periplasmic space (Kipriyanov and Little, *Molecular Biotechnology,* 12: 173 201 (1999); Skerra and Pluckthun, *Science,* 240: 1038 1040 (1988)). Hence, it is necessary to release the protein from the cells to the extracellular medium by extraction such as cell lysis. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration.

Cell lysis is typically accomplished using mechanical disruption techniques such as homogenization or head milling. While the protein of interest is generally effectively liberated, such techniques have several disadvantages (Engler, *Protein Purification Process Engineering*, Harrison eds., 37 55 (1994)). Temperature increases, which often occur during processing, may result in inactivation of the protein. Moreover, the resulting suspension contains a broad spectrum of contaminating proteins, nucleic acids, and polysaccharides. Nucleic acids and polysaccharides increase solution viscosity, potentially complicating subsequent processing by centrifugation, cross-flow filtration, or chromatography. Complex associations of these contaminants with the protein of interest can complicate the purification process and result in unacceptably low yields. Improved methods for purification of heterologous polypeptides from microbial fermentation

broth or homogenate are described, for example, in U.S. Pat. No. 7,169,908, the entire disclosure of which is expressly incorporated herein by reference.

It is emphasized that the fermentation, recovery and purification methods described herein are only for illustration purposes. The methods of the present invention can be combined with any manufacturing process developed for the production, recovery and purification of recombinant proteins.

2. Antibodies

In a preferred embodiment, the methods of the present invention are used to prevent the reduction of inter- and/or intrachain disulfide bonds of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_4\beta_7$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23 Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl): 5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al., *Cancer Res* 55(23 Suppl): 5899s-5907s (1995)); anti-Ep-CAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2):435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab. sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-1 6, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2):435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application

No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); W) 02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171,586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677,180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224,866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albitar et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegall); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821 (Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332,865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1) (part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" New Scientist (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): 5370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.* 30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): S197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002; Tuscan, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized 2H7 anti-CD20 antibodies. In specific embodiments, the humanized 2H7 antibody is an antibody listed in Table 2.

TABLE 2

Humanized anti-CD20 Antibody and Variants Thereof

2H7 variant	V_L SEQ ID NO.	V_H SEQ ID NO.	Full L chain SEQ ID NO.	Full H chain SEQ ID NO.
A	1	2	6	7
B	1	2	6	8
C	3	4	9	10
D	3	4	9	11
F	3	4	9	12
G	3	4	9	13
H	3	5	9	14
I	1	2	6	15

Each of the antibody variants A, B and I of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 1)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYA

PSNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSFNPPTFG

QGTKVEIKR;
and

the heavy chain variable sequence (V_H):

(SEQ ID NO: 2)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA

IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV

YYSNSYWYFDVWGQGTLVTVSS.

Each of the antibody variants C, D, F and G of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 3)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYLHWYQQKPGKAPKPLIYAP

SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWAFNPPTFGQG

TKVEIKR,
and

the heavy chain variable sequence (V_H):

(SEQ ID NO: 4)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSS.

The antibody variant H of Table 2 comprises the light chain variable sequence (V_L) of SEQ ID NO:3 (above) and the heavy chain variable sequence (V_H):

(SEQ ID NO: 5)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSYRYWYFDVWGQGTLVTVSS

Each of the antibody variants A, B and I of Table 2 comprises the full length light chain sequence:

(SEQ ID NO: 6)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAP
SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSFNPPTFGQG
TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC.

Variant A of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 7)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant B of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 8)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

-continued

Variant I of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 15)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Each of the antibody variants C. D, F, G and H of Table 2 comprises the full length light chain sequence:

(SEQ ID NO: 9)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYLHWYQQKPGKAPKPLIYAP
SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWAFNPPTFGQG
TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC.

Variant C of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 10)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant D of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 11)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant F of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 12)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant G of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 13)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHWHYTQKSLSLSP
GK.

Variant H of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 14)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSYRYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

In certain embodiments, the humanized 2H7 antibody of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized 2H7 antibody compositions of the present invention include compositions of any of the preceding humanized 2H7 antibodies having a Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to FcγRIIIA(F158), which is not as effective as FcγRIIIA (V158) in interacting with human IgG. FcγRIIIA (F158) is more common than FcγRIIIA (V158) in normal, healthy African Americans and Caucasians. See Lehrnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*" in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized 2H7 antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized 2H7 in antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the second antigen is selected from the group consisting of CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In one embodiment, the anti-HER2 antibody comprises the following V_L and V_H domain sequences:

humanized 2C4 version 574 antibody V_L (SEQ ID NO: 16)

DIQMTQSPSSLSASVGDRVTITC**KASQDVSIGVA**WYQQKPGKAPKLLIYS

ASYRYTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC**QQYYIYPYT**FGQ

GTKVEIK.

and humanized 2C4 version 574 antibody V_H (SEQ ID NO: 17)

EVQLVESGGGLVQPGGSLRLSCAAS**GFTFTDYTMD**WVRQAPGKGLEWVA**D**

VNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARN**L**

GPSFYFDYWGQGTLVTVSS.

In another embodiment, the anti-HER2 antibody comprises the V_L (SEQ ID NO:18) and V_H (SEQ ID NO:19) domain sequences of trastuzumab as shown in FIG. **21** and FIG. **22**, respectively.

Other HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS (USA)* 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

The anti-VEGF antibodies may, for example, comprise the following sequences:

In one embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:20):

DIQMTQTTSS LSASLGDRVI ISCSASQDIS NYLNWYQQKP

DGTVKVLIYF TSSLHSGVPS RFSGSGSGTD YSLTISNLEP

EDIATYYCQQ YSTVPWTFGG GTKLEIKR;
and

the following V_H sequence (SEQ ID NO:21):

EIQLVQSGPE LKQPGETVRI SCKASGYTFT NYGMNWVKQA

PGKGLKWMGW INTYTGEPTY AADFKRRFTF SLETSASTAY

LQISNLKNDD TATYFCAKYP HYYGSSHWYF DVWGAGTTVT VSS.

In another embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:22):

DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP

GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP

EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

the following V_H sequence (SEQ ID NO:23):

EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA

PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY

LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTLVT VSS.

In a third embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:24):

DIQLTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP

GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP

EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

the following V_H sequence (SEQ ID NO:25):

EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA

PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY

LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTLVT VSS.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva® (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for an anti-human CD11a antibody comprising the V_L and V_H sequences of HuMHM24 below:

V_L (SEQ ID NO: 26):
DIQMTQSPSSLSASVGDRVTITCRASKTISKYLAWYQQKPGKAPKLLIYS

GSTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGQ

GTKVEIKR;
and

V_H (SEQ ID NO: 27):
EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWVRQAPGKGLEWVGM

IHPSDSETRYNQKFKDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARGI

YFYGTTYFDYWGQGTLVTVSS.

The anti-human CD11a antibody may comprise the V_H of SEQ ID NO:27 and the full length L chain of HuMHM24 having the sequence of:

(SEQ ID NO: 28)
DIQMTQSPSSLSASVGDRVTITCRASKTISKYLAWYQQKPGKAPKLLIYS

GSTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGQ

GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV

DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG

LSSPVTKSFNRGEC,
or

the L chain above with the H chain having the sequence of:

(SEQ ID NO: 29)
EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWVRQAPGKGLEWVGM

IHPSDSETRYNQKFKDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARGI

YFYGTTYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV

KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ

TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK

PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY

NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP

QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP

VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

K.

Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference.

3. Other Disulfide-Containing Proteins

In addition to antibodies, the methods of the present invention find utility in the manufacturing of other polypeptides including disulfide bonds. Representative examples of such polypeptides include, without limitation, the following therapeutic proteins: tissue plasminogen activators (t-PAs), such as human tissue plasminogen activator (htPA, alteplase, ACTIVASE®), a thrombolytic agent for the treatment of myocardial infarction; a TNKase™, a ht-PA variant with extended half-life and fibrin specificity for single-bolus administration; recombinant human growth hormone (rhGH, somatropin, NUTROPIN®, PROTROPIN®) for the treatment of growth hormone deficiency in children and adults; and recombinant human deoxyribonuclease I (DNase I) for the treatment of cystic fibrosis (CF).

Examples of disulfide-containing biologically important proteins include growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

4. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically identified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150, 95, VLA-4, ICAM-1, VCAM and αv/133 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group

antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production.

Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first

antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L-AC_L;

AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

$(A\text{-}Y)_n$-(V_LC_L-V_HC_H)$_2$,

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;
C_H is an immunoglobulin heavy chain constant domain;
n is an integer greater than 1;
Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., Cell 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Va.

Example 1

Description of Materials and Methods

The following materials and methods were used in Examples 2-8 below.

Materials

Materials and devices used in the experiments described in the experimental examples include: stainless steel vials (mini-tanks, Flow Components, Dublin, Calif.; short (50 cc) and tall (55 cc)); dialysis tubing (Spectra/Por, 6-8000 MWCO, cat. #132645), 0.22 μm filter (Millipore Millipak Gamma Gold cat. # MPGL04GH2); phosphate buffered saline (PBS, EMD, cat. #6506); ethylenediaminetetraacetic acid (EDTA, Sigma, cat. # E4884); α-nicotinamide adenine dinucleotide phosphate (NADPH, Calbiochem, cat. #481973); dehydroepiandrosterone (DHEA, TCI, cat. # D0044); cupric sulfate (Sigma, cat. # C8027), glucose-6-phosphate (G6P, Calbiochem, cat. #346764); aurothioglucose (ATG, USP, cat. #1045508); aurothiomalate (ATM, Alfa Aesar, cat. #39740); reduced glutathione (GSH, J. T. Baker, cat. # M770-01); monobromobimane (mBB, Fluka, cat. #69898); histidine (J. T. Baker, cat. #2080-05); sodium sulfate (J. T. Baker, cat. #3897-05); Trx (Sigma, cat. # T8690); TrxR (Sigma, cat. # T9698). All chemicals and reagents were used as received with no further purification. Stock solutions of EDTA (250 mM, pH 7.5), $CuSO_4$ (10 mM), ATG (30 mM), ATM (30 mM), NADPH (75 mM), G6P (300 mM) were prepared for use in the mini-tank time course studies.

Generation of Cell Culture Fluid (CCF)

In order to generate ocrelizumab CCF for the various reduction studies, a representative small-scale fermentation process was utilized similar to the methods described previously (Chaderjian et al., 2005). Briefly, 3 liter glass stirred-tank Applikon® bioreactors fitted with pitched blade impellers were used for the inoculum-train and production cultures with the ocrelizumab media components. The bioreactors were outfitted with calibrated dissolved oxygen (DO), pH and temperature probes. DO, pH, temperature, and agitation rate were controlled using digital control units to the defined parameters of the ocrelizumab manufacturing process. The working volume for both the inoculum-train and production cultures was 1.5 L. Daily samples were analyzed on a NOVA Bioprofile blood gas analyzer to ensure the accuracy of the on-line value for pH and dissolved oxygen as well as to monitor the glucose, lactate, ammonium, glutamine, glutamate, and sodium concentrations in the cultures. Daily samples were also taken to monitor cell growth, viability, and titer. Cell growth was measured both by viable cell counts using a ViCell as well as on a packed cell volume (PCV) basis. Culture viability was determined by trypan blue exclusion on a ViCell instrument. Supernatant samples were assayed by an HPLC-based method to measure ocrelizumab titer values.

Harvested Cell Culture Fluid (HCCF) Preparation

Complete lysis of CCF was achieved by high pressure homogenization using a Microfluidics HC-8000 homogenizer. The pressure regulator of the instrument was set to 4,000-8,000 psi, and the CCF was pulled in through the homogenizer to obtain complete cell lysis (membrane breakage) after a single pass. The CCF homogenate was collected once water was purged through the system. The homogenate was transferred to centrifuge bottles and centrifuged in a Sorval RC-3B rotor centrifuge at 4,500 rpm for 30 minutes at 20° C. The centrate was decanted and then depth filtered followed by 0.22 μm sterile filtration using a peristaltic pump with silicon tubing to generate the final HCCF from the homogenized CCF (100% cell lysis). Alternatively, the CCF

was centrifuged straight from the fermentor without any homogenization and then the centrate was filtered with a sterile 0.22 μm filter to generate the HCCF.

Mini-Tank Handling

A laminar flow hood was used in handling all mini-tanks and all materials used in the HCCF incubation experiments were either autoclaved or rinsed using 70% isopropanol to minimize bacterial contamination.

Lactate Dehydrogenase Assay

For lactate dehydrogenase assay, see Babson & Babson (1973) and Legrand et al., (1992), which are hereby incorporated by reference.

Dialysis Experiment

A dialysis experiment was carried out in order to determine whether the components causing reduction of ocrelizumab were small molecules or macromolecules (i.e. enzymes). A sample of 3 mL of purified and formulated ocrelizumab (30.2 mg/mL) was dialyzed against 1 L of phosphate buffered saline (PBS, 10 mM pH 7.2) for 24 hours and the PBS was changed after 8 hours. The concentration of the ocrelizumab sample was then adjusted to 1 mg/mL using the absorbance at 280 nm. Aliquots were stored at −70° C. prior to use. Dialysis tubing was hydrated overnight in a 0.05% azide solution and rinsed with sterile water prior to use. The HCCF obtained from homogenization of CCF from a 3-L fermentor was thawed and filtered through a 0.22 μm Millipak filter using a peristaltic pump. Six short mini-tanks were filled with 30 mL of HCCF each. To each mini-tank, 500 μL of ocrelizumab sample in sealed dialysis tubing was added. The mini-tanks were sealed and loaded into a bench top mixer (Barnstead Lab-Line MAX Q 4000) operating at 35 rpm and ambient temperature. For each time-point, one mini-tank was removed from the mixer, and aliquots of the HCCF (in the mini-tank) and ocrelizumab sample (in the dialysis bag) were taken and stored at −70° C. until analyzed with the free thiol assay and the Bioanalyzer assay (described below).

Test Inhibitors for Reduction in a Small-Scale In Vitro System

A tall mini-tank was filled with 27 mL of HCCF. Depending on the experiment design, various reagents (NADPH, G6P, inhibitors of G6PD or TrxR) were added to the desired concentration, and the final volume in the mini-tank was brought to 30 mL with PBS (10 mM pH 7.2). The mini-tanks were sealed and loaded into a bench top mixer running at 35 rpm and ambient temperature. At each-time point for sampling, the exteriors of the mini-tanks were sterilized with 70% IPA and opened in a laminar flow hood for the removal of an aliquot. The mini-tanks were then re-sealed and loaded back into the bench top mixer. All aliquots were stored at −70° C. until analyzed with the free thiol assay and Bioanalyzer assay (described below).

In Vitro Trx/TrxRreductase Studies

A commercial TrxR (rat liver) solution (4 μM) was diluted with water to yield a 2.86 μM solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μM solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μL PBS, 25 μL NADPH, 16 μL formulated ocrelizumab solution (30.2 mg/mL) and 5 μL Trx were gently mixed. The reaction was initiated by the addition of 17.5 μL TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μL were taken at each sampling time-point and stored at −70° C. until analyzed by the Bioanalyzer assay (see below). Controls were performed to determine if the enzymatic pathway was active when an enzyme was omitted by substituting an equal volume of PBS for either Trx and/or TrxR in the reaction mixture.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of 5 μL ATG or ATM. To demonstrate the inhibition of Trx system by Cu^{2+}, 2.5 μL of $CuSO_4$ (10 mM) was added to reaction mixture using the same enzymes but a different buffer (10 mM histidine, 10 mM Na_2SO_4, 137 mM NaCl, 2.5 mM KCl, pH 7.0) to prevent formation of insoluble $Cu_3(PO_4)_2$.

Free Thiol Assay

A standard curve using GSH was generated in PBS (10 mM, pH 6.0±0.05). From a 110 mM GSH solution, standards were prepared at concentrations of 0, 5.5, 11, 22, 44, 55, 110 and 550 μM through serial dilution. From an acetonitrile stock solution of mBB (10 mM stored at −20° C.), a 100 μM solution of mBB was prepared in PBS (10 mM, pH 10.0±0.05) and stored away from light.

In a black, flat bottomed 96 well plate, 100 μL of mBB was dispensed into each well. For the standard curve, 10 μL of standard GSH solution was added yielding a working pH of 8.0±0.2. For samples, 10 μL of sample was added to the wells. All wells were prepared in triplicate. The plate was incubated at room temperature for 1 hour in the dark then read using a fluorescence plate reader (Molecular Devices SpectraMax® Gemini XS) with an excitation wavelength of 390 nm and an emission wavelength of 490 nm. A linear standard curve was generated using the average result of the three standard wells plotted versus GSH concentration. Free thiol levels in samples were calculated from the linear equation of the standard curve using the average value of the three sample wells.

Bioanalyzer Assay

Capillary electrophoresis measurements were acquired using the Agilent 2100 Bioanalyzer. Sample preparation was carried out as described in the Agilent Protein 230 Assay Protocol (manual part number G2938-90052) with minor changes. HCCF samples were diluted, 1:4 and Protein A samples were diluted to 1.0 g/L with water prior to preparation. For HCCF samples at the denaturing step, 24 μL of a 50 mM iodoacetamide (IAM), 0.5% SDS solution was added in addition to the 2 μL of denaturing solution provided. For Protein A samples, 0.5% SDS with no JAM and 2 μL of denaturing solution were used. Digital gel-like images were generated using Agilent 2100 Expert software.

Stock Solutions for HCCF Hold Time Studies

Three separate stock solutions were used in the lab scale HCCF hold time studies: (1) 250 mM stock solution of EDTA (pH 7.4) prepared using EDTA, disodium dihydrate (Mallinckrodt, cat. #7727-06 or Sigma, cat. # E-5134) and EDTA, tetrasodium dihydrate (Sigma, cat. #E-6511), (2) 50 mM stock solution of cupric sulfate pentahydrate ($CuSO_4$, Sigma, cat. # C-8027), and (3) 1 M acetic acid solution (Mallinckrodt, cat. # V193).

Inhibitor Additions and Cell Culture Fluid (CCF) Blending

A stock solution of either 250 mM EDTA or 50 mM $CuSO_4$ was added to the CCF prior to homogenization to evaluate a range of final concentrations to prevent antibody disulfide reduction. Once the final HCCF was generated from the homogenized CCF, these solutions were then mixed with the HCCF generated from the non-homogenized CCF (also containing EDTA or $CuSO_4$) in order to dilute and decrease the total level of cell lysis to below the 100% maximum. Alternatively, a stock solution of 1 M acetic acid was added to a final blended HCCF solution (homogenized CCF and non-homogenized CCF) to decrease the pH of the solution to prevent antibody disulfide reduction.

Approximately 30-50 mL of each HCCF solution (containing EDTA, $CuSO_4$, acetic acid, or no addition for the control) was held in a 50 mL 316L stainless steel vial. The vial was sealed with a clamp, and the solution was not aerated or agitated. The vial was stored at room temperature (18-22° C.). At pre-determined time points, the solution was removed and purified over a lab scale protein A affinity resin.

Similar results can be obtained with other oxidizing agents, such as, for example, cystine and oxidized glutathione.

Air Sparging

To evaluate air sparging of the HCCF generated from homogenized CCF to prevent antibody disulfide reduction, 3-L glass or 15-L stainless steel vessels were utilized. Approximately 1-5 L of HCCF was 0.22 μm sterile filtered into each sterilized vessel. Experimental conditions were maintained at 18-22° C. and 50 (15-L fermentor) or 275 rpm (3-L fermentor) agitation either with or without pH control by the addition of carbon dioxide. Solutions were either sparged with air to increase the dissolved oxygen level to air saturation or with nitrogen (control) to remove any dissolved oxygen in solution. Gas flow to each vessel was variable dependent upon whether a constant aeration rate was used or a minimum level of dissolved oxygen was maintained. At pre-determined time points, 25-50 mL samples were removed from both vessels and purified over a lab scale protein A affinity resin prior to analysis.

Protein A Processing

Antibody in harvested cell culture fluid samples can be captured and purified using a specific affinity chromatography resin. Protein A resin (Millipore, Prosep-vA High Capacity) was selected as the affinity resin for antibody purification. The resin was packed in a 0.66 cm inner diameter glass column (Omnifit®) with a 14 cm bed height resulting in a 4.8 mL final column volume. Chromatography was performed using an AKTA Explorer 100 chromatography system (GE Healthcare).

The resin was exposed to buffers and HCCF at a linear flow rate between 350-560 cm/hr. The resin was equilibrated with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1. For each purification, the resin was loaded between 5-15 mg antibody per mL of resin. The antibody concentration in the HCCF was determined using an immobilized protein A HPLC column (Applied Biosystems, POROS A). After loading, the resin was washed with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M TMAC, pH 7.1, and then the antibody was eluted using 0.1M acetic acid, pH 2.9. Elution pooling was based on UV absorbance at 280 nm measured inline after the column. The purified elution pools were pH-adjusted using 1 M Sodium HEPES to pH 5.0-5.5. After regeneration of the resin with 0.1M phosphoric acid, the same or similar packed resins were used for subsequent purification of other HCCF solutions.

The antibody concentration in the purified protein A pool was measured using UV spectrometry at 280 nm. The purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa molecular weight.

Example 2

Dialysis Experiment

A dialysis experiment was designed and carried out to determine if the reduction of ocrelizumab was caused by small reducing molecules or macromolecules (e.g., enzymes). In this dialysis experiment, purified intact ocrelizumab was placed in a dialysis bag with a molecular weight cut off (MWCO) of 7000 and incubated the dialysis bag in HCCF containing ocrelizumab in a stainless steel mini-tank. As shown in FIGS. **1** and **2**, the ocrelizumab inside the bag was not reduced after the incubation period (FIG. **1**), whereas the ocrelizumab outside the bag in the HCCF was significantly reduced soon after the incubation started. This was evidenced by the loss of intact ocrelizumab (~150 kDa) and the formation of ocrelizumab fragments (various combinations of heavy and light chains) (FIG. **2**). The mass spectrometry analysis of the ocrelizumab in the protein A elution pools from the reduced manufacturing runs indicated that those observed fragments were formed by reduction of only the inter-chain disulfide bonds.

The free thiol measurement showed that no free thiols were present inside the dialysis bag at the beginning of the incubation; however the levels of free thiols inside and outside the dialysis bag become comparable in less than five hours after the incubation started, indicating that the small molecule components in the HCCF are fully equilibrated inside and outside the dialysis bag (FIG. **3**). Since the reduction was observed only outside but not inside the dialysis bag with a MWCO of 7000 Da, the molecular weight of the reducing molecule(s) must be greater than 7000 Da. Thus, an enzymatic reaction is responsible for the reduction of ocrelizumab.

Example 3

Reduction of Ocrelizumab (rhuMAb 2H7, Variant A) by Trx/TrxR In Vitro

The Trx system was tested for its ability to reduce ocrelizumab in vitro by incubating intact ocrelizumab with Trx, TrxR, and NADPH. The Bioanalyzer results indicate that ocrelizumab was reduced in vitro by the Trx system (FIG. **5**). The rate of reduction in this in vitro system appears to be slower than that in the HCCF (for example when compared to the reduction shown in FIG. **2**). This is likely due to lower concentrations of the enzymes (Trx and Trx-R) and/or the buffer system used in the in vitro reaction because reaction rate of Trx system is dependent on both the enzyme concentrations and buffer systems.

Example 4

Inhibitors of the Trx System

(i) Inhibition of Reduction of Recombinant Antibody by Cupric Sulfate

Cupric sulfate is known for its ability to provide oxidizing redox potential and has been used in the cell culture processes to minimize free thiol (i.e., minimize unpaired cysteine) levels in recombinant antibody molecules (Chaderjian et al., 2005, supra). Cupric sulfate was tested for efficacy in inhibiting the Trx system in vitro and the subsequent reduction of ocrelizumab. In this in vitro reduction experiment, the buffer system was changed from PBS to histidine sulfate to avoid the formation of insoluble $Cu_3(PO_4)_2$. FIG. **8** shows that ocrelizumab was readily reduced by the Trx system in the histidine sulfate buffer (even faster than in PBS buffer). The addition of $CuSO_4$ to this reaction clearly inhibits the ocrelizumab reduction (FIG. **9**).

(ii) Inhibition of Reduction of Recombinant Antibody in HCCF by ATG and ATM

Two commercially available specific inhibitors of TrxR, aurothioglucose (ATG) and aurothiomalate (ATM), were tested for their ability to inhibit the Trx system in vitro and the reduction of ocrelizumab. Both ATG and ATM can effectively

inhibit the reduction of ocrelizumab in the assay described above (see FIGS. **6** and **7**). The addition of aurothioglucose or aurothiomalate, at a concentration of 1 mM to the same reaction mixture as described in the caption for FIG. **5** effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis.

If the Trx system was active in the HCCF and reduced ocrelizumab as observed in the manufacturing runs resulting in reduced antibody molecules or in the lab scale experiments, both gold compounds (ATG and ATM) should be able to inhibit the reduction of ocrelizumab in HCCF. FIG. **10** shows that ocrelizumab was readily reduced in an HCCF from homogenized CCT generated from a 3-L fermentor after a period of incubation. However, the ocrelizumab reduction event was completely inhibited when either 1 mM ATG or ATM was added to the HCCF (FIGS. **11** and **12**). These results demonstrated that the Trx system is active in the HCCF and is directly responsible for the reduction of ocrelizumab.

Example 5

The Source of NADPH for Trx System Activity and the Roles of G6P and Glucose in Reduction Mechanism

The reduction of disulfides by the Trx system requires the reducing equivalents from NADPH (FIG. **4**). The main cellular metabolic pathway that provides NADPH for all reductive biosynthesis reactions is the pentose phosphate pathway. For the antibody reduction event to occur, the enzymes in this pathway must be still active in the HCCF in order to keep the Trx system active. At a minimum, the first step in the pentose phosphate pathway (catalyzed by G6PD) must be active to reduce $NADP^+$ to NADPH while converting G6P to 6-phosphogluconolactone. In addition, G6P is most likely produced from glucose and adenosine 5'-triphosphate (ATP) by the hexokinase activity in HCCF. The overall mechanism of ocrelizumab reduction is summarized in FIG. **4**.

The reducing activity in the HCCF appeared to be transitory in some cases and may be inhibited over time under certain storage conditions or after multiple freeze/thaw cycles. HCCF that has fully lost reducing activity provided an opportunity to explore the role of NADPH and G6P in the reduction of ocrelizumab by Trx system.

An HCCF from a large scale manufacturing run (the "beta" run) was subjected to several freeze/thaw cycles and used in an experiment designed to measure reduction; no ocrelizumab reduction was observed (FIG. **13**) despite its ability to bring about antibody reduction seen previously in freshly-thawed HCCF from this same fermentation. NADPH was added to this non-reducing HCCF at a concentration of 5 mM and the reduction event returned (FIG. **14**). Therefore, the Trx system is still intact and active in the HCCF where reduction no longer occurs, and capable of reducing protein and/or antibody if supplied with cofactors. Additionally, the reducing activity was lost over time as the NADPH source was depleted (presumably due to the oxidation of NADPH by all of the reductive reactions that compete for NADPH), and not because the Trx system was degraded or inactivated.

This was verified by another experiment. 10 mM G6P was added to a HCCF that had been repeatedly freeze-thawed from the beta run. This G6P addition reactivated the Trx system which subsequently reduced ocrelizumab in the HCCF incubation experiment (FIG. **15**). This demonstrated that the reduction of ocrelizumab in the HCCF was caused by the activities of both the Trx system and G6PD. Furthermore, G6PD is still active in a repeatedly freeze/thawed HCCF of the beta run; the loss of reduction activity in this a repeatedly freeze/thawed HCCF beta run appears to be due to the depletion of G6P, which thus eliminated the conversion of $NADP^+$ to NADPH.

In our studies, we have observed that EDTA can effectively inhibit the ocrelizumab reduction in the HCCF incubation experiment. As shown in FIG. **16**, the ocrelizumab was reduced after incubating the HCCF from a 12,000 L scale ocrelizumab manufacturing run (not repeatedly freeze/thawed and no loss of reducing activity) at ambient temperature for more than 19 hours. However, the reduction was completely inhibited when 20 mM EDTA was added to the 12 kL HCCF and held in a separate stainless steel minitank (FIG. **17**). In the first step of glycolysis, the hexokinase catalyzes the transfer of phosphate group from Mg2+-ATP to glucose, a reaction that requires the complexation of Mg2+ with ATP (Hammes & Kochavi, 1962a & 1962b, supra). Since EDTA is a metal ion chelator, especially for Mg2+, it can be an effective inhibitor of hexokinase. The observation that an excess amount of EDTA can effectively block the reduction indicates the involvement of hexokinase (i.e. providing G6P) in the mechanism of ocrelizumab reduction. Without being bound by this, or any other theory, EDTA blocks the reduction of ocrelizumab by eliminating the hexokinase activity and thereby reducing the G6P level available for G6PD, and subsequently the NADPH level available for the Trx system.

Although EDTA is every effective in blocking the reduction of ocrelizumab in fresh HCCF, it was unable to prevent the reduction of ocerlizumab in the beta run HCCF in which the Trx system activity was lost then reactivated by the addition of G6P. For example, the reduction of ocrelizumab was observed in an HCCF incubation experiment in which 5 mM G6P and 20 mM EDTA (final concentrations) were added to the beta run HCCF that had fully lost reducing activity (FIG. **18**). However, no reduction was seen in the control incubation experiment in which no G6P and EDTA were added. Without being bound by this or any other theory, the EDTA used in this manner may therefore inhibit neither the Trx system nor the G6PD, and may function as an inhibitor for hexokinase, which produces the G6P for the G6PD. Without G6P, the Trx system would not be supplied with the necessary NADPH for activity.

Example 6

Inhibition of Reduction of Recombinant Antibody by DHEA

Dehydroepiandrosterone (DHEA), as well as other similar G6PD inhibitors, effectively blocks G6PD activity (Gordon et al., 1995, supra). G6PD inhibitors also prevent the reduction of an antibody in HCCF, for example, ocrelizumab, by blocking the generation of NADPH. The ability of DHEA to inhibit the reduction of orcelizumab is demonstrated in an HCCF incubation experiment. Adding DHEA to a HCCF prevents antibody reduction.

DHEA is typically used in the concentration range from about 0.05 mM to about 5 mM. DHEA is also typically used in the concentration range from about 0.1 mM to about 2.5 mM.

Example 7

Inhibition of Reduction of Recombinant Antibody by (i) EDTA, (ii) Cupric Sulfate, and (iii) Acetic Acid Additions

Four different HCCFs were stored and held in the stainless steel vials. The solutions were similar in the amount of cell

lysis, which were generated by diluting HCCF from homogenized CCF with HCCF from non-homogenized CCF. For example, 150 mL of the first lysed solution was mixed with 50 mL of the second solution, respectively. The four HCCF mixtures evaluated in this study contained either: (1) 20 mM EDTA, (2) 30 μM $CuSO_4$, (3) 15 mM acetic acid (pH 5.5), and (4) no chemical inhibitor was added for the control solution. The ocrelizumab antibody from all four mixtures was purified immediately (t=0 hr) using protein A chromatography and then again after 20 hr and 40 hr of storage in the stainless steel vials. Purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody (150 kDa). The results showed that greater than 90% intact antibody was present in all four mixtures at the initial time point (FIG. **19**). However, at the 20 hr time point, intact antibody was not detected in the control mixture (without any addition) indicating reduction of the antibody disulfide bonds. In the three other mixtures, over 90% intact antibody was still detected at both 20 hr and 40 hr time points, demonstrating the prevention of disulfide bond reduction by all three inhibitors tested.

Example 8

Inhibition of Reduction of Recombinant Antibody by Air Sparging the HCCF

One HCCF mixture generated from homogenized CCF was stored and held in two separate 10 L stainless steel fermentors. One vessel was sparged with air while the other vessel was sparged with nitrogen gas. The ocrelizumab antibody was purified immediately (t=0 hr) from the initial mixture using protein A chromatography. At selected time points, 50 mL samples were removed from each vessel and the antibody was purified using protein A chromatography. Purified protein A elution pools were then analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa. The results showed that approximately 85% intact antibody was present in the initial solution (FIG. **20**), indicating some early reduction of the antibody disulfide bonds prior to exposure to oxygen (i.e. sparged air in the fermentor). Once the mixture was sparged with air for two hours, greater than 90% intact antibody was measured for the remainder of the 36 hr study. In contrast, when the mixture was sparged with nitrogen gas, the antibody reduction event continued as measured at 2 hr (28% 150 kDa peak) and 6 hr (5% 150 kDa peak). These results demonstrated the prevention of disulfide bond reduction in the antibody when the HCCF mixture generated from homogenized CCF was exposed to oxygen.

Example 9

Design of Targeted siRNA or Antisense Nucleotide Trx Inhibitors

The design of targeted siRNAs or antisense nucleotides to the genes as found in CHO cells may be done by using publicly available sequences such as those for *E. coli* thioredoxin TrxA (SEQ ID NO:30), *E. coli* thioredoxin reductase TrxB (SEQ ID NO:31); mouse thioredoxin 1 (SEQ ID NO:32), mouse thioreodoxin 2 (SEQ ID NO:33), mouse thioredoxin reductase 1 (SEQ ID NO:34), and mouse thioredoxin reductase 2 (SEQ ID NO:35). One of ordinary skill in the art can use these sequences to select sequences to design Trx inhibitors for targeting enzymes in different organisms and/or cells, such as CHO cells.

The sequence of *E. coli* Thioredoxin TrxA is:

(SEQ ID NO: 30)

```
ATG TTA CAC CAA CAA CGA AAC CAA CAC GCC AGG CTT ATT CCT GTG GAG

TTA TAT ATG AGC GAT AAA ATT ATT CAC CTG ACT GAC GAC AGT TTT GAC ACG

GAT GTA CTC AAA GCG GAC GGG GCG ATC CTC GTC GAT TTC TGG GCA GAG

TGG TGC GGT CCG TGC AAA ATG ATC GCC CCG ATT CTG GAT GAA ATC GCT

GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAA

AAC CCT GGC ACT GCG CCG AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG CTG

CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA GTG GGT GCA CTG TCT

AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCG TAA.
```

The sequence of *E. coli* Thioredoxin TrxB is:

(SEQ ID NO: 31)

```
ATG GGC ACG ACC AAA CAC AGT AAA CTG CTT ATC CTG GGT TCA GGC CCG

GCG GGA TAC ACC GCT GCT GTC TAC GCG GCG CGC GCC AAC CTG CAA CCT

GTG CTG ATT ACC GGC ATG GAA AAA GGC GGC CAA CTG ACC ACC ACC ACG

GAA GTG GAA AAC TGG CCT GGC GAT CCA AAC GAT CTG ACC GGT CCG TTA

TTA ATG GAG CGC ATG CAC GAA CAT GCC ACC AAG TTT GAA ACT GAG ATC

ATT TTT GAT CAT ATC AAC AAG GTG GAT CTG CAA AAC CGT CCG TTC CGT CTG

AAT GGC GAT AAC GGC GAA TAC ACT TGC GAC GCG CTG ATT ATT GCC ACC

GGA GCT TCT GCA CGC TAT CTC GGC CTG CCC TCT GAA GAA GCC TTT AAA GGC
```

-continued

CGT GGG GTT TCT GCT TGT GCA ACC TGC GAC GGT TTC TTC TAT CGC AAC CAG

AAA GTT GCG GTC ATC GGC GGC GGC AAT ACC GCG GTT GAA GAG GCG TTG

TAT CTG TCT AAC ATC GCT TCG GAA GTG CAT CTG ATT CAC CGC CGT GAC GGT

TTC CGC GCG GAA AAA ATC CTC ATT AAG CGC CTG ATG GAT AAA GTG GAG

AAC GGC AAC ATC ATT CTG CAC ACC AAC CGT ACG CTG GAA GAA GTG ACC

GGC GAT CAA ATG GGT GTC ACT GGC GTT CGT CTG CGC GAT ACG CAA AAC

AGC GAT AAC ATC GAG TCA CTC GAC GTT GCC GGT CTG TTT GTT GCT ATC GGT

CAC AGC CCG AAT ACT GCG ATT TTC GAA GGG CAG CTG GAA CTG GAA AAC

GGC TAC ATC AAA GTA CAG TCG GGT ATT CAT GGT AAT GCC ACC CAG ACC

AGC ATT CCT GGC GTC TTT GCC GCA GGC GAC GTG ATG GAT CAC ATT TAT CGC

CAG GCC ATT ACT TCG GCC GGT ACA GGC TGC ATG GCA GCA CTT GAT GCG

GAA CGC TAC CTC GAT GGT TTA GCT GAC GCA AAA TAA.

The sequence of mouse thioredoxin 1 is:

(SEQ ID NO: 32)

ATGGTGAAGCTGATCGAGAGCAAGGAAGCTTTTCAGGAGGCCCTGGCCGC

CGCGGGAGACAAGCTTGTCGTGGTGGACTTCTCTGCTACGTGGTGTGGAC

CTTGCAAAATGATCAAGCCCTTCTTCCATTCCCTCTGTGACAAGTATTCC

AATGTGGTGTTCCTTGAAGTGGATGTGGATGACTGCCAGGATGTTGCTGC

AGACTGTGAAGTCAAATGCATGCCGACCTTCCAGTTTTATAAAAAGGGTC

AAAAGGTGGGGGAGTTCTCCGGTGCTAACAAGGAAAAGCTTGAAGCCTCT

ATTACTGAATATGCCTAA.

The sequence of mouse thioreodoxin 2 is:

(SEQ ID NO: 33)

ATGGCTCAGCGGCTCCTCCTGGGGAGGTTCCTGACCTCAGTCATCTCCAG

GAAGCCTCCTCAGGGTGTGTGGGCTTCCCTCACCTCTAAGACCCTGCAGA

CCCCTCAGTACAATGCTGGTGGTCTAACAGTAATGCCCAGCCCAGCCCGG

ACAGTACACACCACCAGAGTCTGTTTGACGACCTTTAACGTCCAGGATGG

ACCTGACTTTCAAGACAGAGTTGTCAACAGTGAGACACCAGTTGTTGTGG

ACTTTCATGCACAGTGGTGTGGCCCCTGCAAGATCCTAGGACCGCGGCTA

GAGAAGATGGTCGCCAAGCAGCACGGGAAGGTGGTCATGGCCAAAGTGGA

CATTGACGATCACACAGACCTTGCCATTGAATATGAGGTGTCAGCTGTGC

CTACCGTGCTAGCCATCAAGAACGGGGACGTGGTGGACAAGTTTGTGGGG

ATCAAGGACGAGGACCAGCTAGAAGCCTTCCTGAAGAAGCTGATTGGCTG

A.

The sequence of mouse thioredoxin reductase 1 is:

(SEQ ID NO: 34)

ATGAATGGCTCCAAAGATCCCCCTGGGTCCTATGACTTCGACCTGATCAT

CATTGGAGGAGGCTCAGGAGGACTGGCAGCAGCTAAGGAGGCAGCCAAAT

TTGACAAGAAAGTGCTGGTCTTGGATTTTGTCACACCGACTCCTCTTGGG

-continued

ACCAGATGGGGTCTCGGAGGAACGTGTGTGAATGTGGGTTGCATACCTAA

GAAGCTGATGCACCAGGCAGCTTTGCTCGGACAAGCTCTGAAAGACTCGC

GCAACTATGGCTGGAAAGTCGAAGACACAGTGAAGCATGACTGGGAGAAA

ATGACGGAATCTGTGCAGAGTCACATCGGCTCGCTGAACTGGGGCTACCG

CGTAGCTCTCCGGGAGAAAAAGGTCGTCTATGAGAATGCTTACGGGAGGT

TCATTGGTCCTCACAGGATTGTGGCGACAAATAACAAAGGTAAAGAAAAA

ATCTATTCAGCAGAGCGGTTCCTCATCGCCACAGGTGAGAGGCCCCGCTA

CCTGGGCATCCCTGGAGACAAAGAGTACTGCATCAGCAGTGATGATCTTT

TCTCCTTGCCTTACTGCCCGGGGAAGACCCTAGTAGTTGGTGCATCCTAT

GTCGCCTTGGAATGTGCAGGATTTCTGGCTGGTATCGGCTTAGACGTCAC

TGTAATGGTGCGGTCCATTCTCCTTAGAGGATTTGACCAAGACATGGCCA

ACAAAATCGGTGAACACATGGAAGAACATGGTATCAAGTTTATAAGGCAG

TTCGTCCCAACGAAAATTGAACAGATCGAAGCAGGAACACCAGGCCGACT

CAGGGTGACTGCTCAATCCACAAACAGCGAGGAGACCATAGAGGGCGAAT

TTAACACAGTGTTGCTGGCGGTAGGAAGAGATTCTTGTACGAGAACTATT

GGCTTAGAGACCGTGGGCGTGAAGATAAACGAAAAAACCGGAAAGATACC

CGTCACGGATGAAGAGCAGACCAATGTGCCTTACATCTACGCCATCGGTG

ACATCCTGGAGGGGAAGCTAGAGCTGACTCCCGTAGCCATCCAGGCGGGG

AGATTGCTGGCTCAGAGGCTGTATGGAGGCTCCAATGTCAAATGTGACTA

TGACAATGTCCCAACGACTGTATTTACTCCTTTGGAATATGGCTGTTGTG

GCCTCTCTGAAGAAAAAGCCGTAGAGAAATTTGGGGAAGAAAATATTGAA

GTTTACCATAGTTTCTTTTGGCCATTGGAATGGACAGTCCCATCCCGGGA

TAACAACAAATGTTATGCAAAAATAATCTGCAACCTTAAAGACGATGAAC

GTGTCGTGGGCTTCCACGTGCTGGGTCCAAACGCTGGAGAGGTGACGCAG

GGCTTTGCGGCTGCGCTCAAGTGTGGGCTGACTAAGCAGCAGCTGGACAG

CACCATCGGCATCCACCCGGTCTGTGCAGAGATATTCACAACGTTGTCAG

TGACGAAGCGCTCTGGGGGAGACATCCTCCAGTCTGGCTGCTGA

The sequence of mouse thioredoxin reductase 2 is:

(SEQ ID NO: 35)

ATGGCGGCGATGGTGGCGGCGATGGTGGCGGCGCTGCGTGGACCCAGCAG

GCGCTTCCGGCCGCGGACACGGGCTCTGACACGCGGGACAAGGGGCGCGG

CGAGTGCAGCGGGAGGGCAGCAGAGCTTTGATCTCTTGGTGATCGGTGGG

GGATCCGGTGGCCTAGCTTGTGCCAAGGAAGCTGCTCAGCTGGGAAAGAA

GGTGGCTGTGGCTGACTATGTGGAACCTTCTCCCCGAGGCACCAAGTGGG

GCCTTGGTGGCACCTGTGTCAACGTGGGTTGCATACCCAAGAAGCTGATG

CATCAGGCTGCACTGCTGGGGGGCATGATCAGAGATGCTCACCACTATGG

CTGGGAGGTGGCCCAGCCTGTCCAACACAACTGGAAGACAATGGCAGAAG

CCGTGCAAAACCATGTGAAATCCTTGAACTGGGGTCATCGCGTCCAACTG

CAGGACAGGAAAGTCAAGTACTTTAACATCAAAGCCAGCTTTGTGGATGA

GCACACAGTTCGCGGTGTGGACAAAGGCGGGAAGGCGACTCTGCTTTCAG

CTGAGCACATTGTCATTGCTACAGGAGGACGGCCAAGGTACCCCACACAA

GTCAAAGGAGCCCTGGAATATGGAATCACAAGTGACGACATCTTCTGGCT

GAAGGAGTCCCCTGGGAAAACGTTGGTGGTTGGAGCCAGCTATGTGGCCC

TAGAGTGTGCTGGCTTCCTCACTGGAATTGGACTGGATACCACTGTCATG

ATGCGCAGCATCCCTCTCCGAGGCTTTGACCAGCAAATGTCATCTTTGGT

CACAGAGCACATGGAGTCTCATGGCACCCAGTTCCTGAAAGGCTGTGTCC

CCTCCCACATCAAAAAACTCCCAACTAACCAGCTGCAGGTCACTTGGGAG

GATCATGCTTCTGGCAAGGAAGACACAGGCACCTTTGACACTGTCCTGTG

GGCCATAGGGCGAGTTCCAGAAACCAGGACTTTGAATCTGGAGAAGGCTG

GCATCAGTACCAACCCTAAGAATCAGAAGATTATTGTGGATGCCCAGGAG

GCTACCTCTGTTCCCCACATCTATGCCATTGGAGATGTTGCTGAGGGGCG

GCCTGAGCTGACGCCCACAGCTATCAAGGCAGGAAAGCTTCTGGCTCAGC

GGCTCTTTGGGAAATCCTCAACCTTAATGGATTACAGCAATGTTCCCACA

ACTGTCTTTACACCACTGGAGTATGGCTGTGTGGGGCTGTCTGAGGAGGA

GGCTGTGGCTCTCCATGGCCAGGAGCATGTAGAGGTTTACCATGCATATT

ATAAGCCCCTAGAGTTCACGGTGGCGGATAGGGATGCATCACAGTGCTAC

ATAAAGATGGTATGCATGAGGGAGCCCCCACAACTGGTGCTGGGCCTGCA

CTTCCTTGGCCCCAACGCTGGAGAAGTCACCCAAGGATTTGCTCTTGGGA

TCAAGTGTGGGGCTTCATATGCACAGGTGATGCAGACAGTAGGGATCCAT

CCCACCTGCTCTGAGGAGGTGGTCAAGCTGCACATCTCCAAGCGCTCCGG

CCTGGAGCCTACTGTGACTGGTTGCTGA.

Example 10

In Vitro Trx/Trx Reductase Studies

Materials and Methods

A commercial TrxR (rat liver) solution (4 μM) was diluted with water to yield a 2.86 μM solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μM solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μL reaction buffer (10 mM histidine, 10 mM Na2SO4, 137 mM NaCl, 2.5 mM KCl, pH 7.0), 25 μL NADPH, 16 μL formulated ocrelizumab solution (30.2 mg/mL) and 5 μL Trx were gently mixed. The reaction was initiated by the addition of 17.5 μL TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μL were taken at each sampling time-point and stored at −70° C. until analyzed by the Bioanalyzer assay.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of various inhibitors.

1. In Vitro Activity of Thioredoxin System

FIG. **24** shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, referred to as "Variant A" above) (1 mg/mL) with 0.1 μM TrxR (rat liver), 5 μM Trx (human) and 1 mM NADPH in 10 mM histidine sulfate buffer results in the reduction of ocrelizumab in less than one hour.

2. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose

Aurothioglucose (ATG) was added to the ocrelizumab mixture described above, at the following concentrations: 1 mM; 0.6 μM (6:1 ATG:TrxR); 0.4 μM (4:1 ATG:TrxR); and 0.2 μM (2:1 ATG:TrxR).

As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. **25-27**, aurothioglucose added at concentrations 1 mM, 0.6 μM, and 0.4 μM effectively inhibits the reduction of ocrelizumab by the thioredoxin system. However, as shown in FIG. **28**, under these experimental conditions aurothioglucose added at a concentration of 0.2 μM cannot inhibit ocrelizumab reduction after 24 hours.

3. In Vitro Activity of Thioredoxin System Inhibited by Aurothiomalate

Aurothiomalate (ATM) was added to the ocrelizumab mixture described above, at concentrations of 0.1 mM and 0.01 mM. As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. **29** and **30**, ATM effectively inhibits the reduction of ocrelizumab by the thioredoxin system at both concentrations tested.

4. In Vitro Activity of Thioredoxin System Inhibited by $Cuso_4$

$CuSO_4$ was added to the ocrelizumab mixture described above, at concentrations of 20 μM (4:1 Cu^{2+}:Trx); 10 μm (2:1 Cu^{2+}:Trx); and 5 μM (1:1 Cu^{2+}:Trx). As shown in FIGS. **31-33**, $CuSO_4$ effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 20 μM and 10 μM (FIGS. **31** and **32**), but the 5 μM concentration is insufficient to result in a complete inhibition of reduction (FIG. **33**).

5. In Vitro Activity of Thioredoxin System Inhibited by Cystamine

Cystamine was added to the ocrelizumab mixture describe above at the following concentrations: 532 μM (20:1 cystamine:2H7 (Variant A) disulfide); 266 μM (10:1 cystamine: 2H7 (Variant A) disulfide); 133 μM (5:1 cystamine:2H7 disulfide); and 26.6 μM (1:1 cystamine:2H7 (Variant A) disulfide). As shown in FIGS. **34-37**, cystamine effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 532 μM (20:1 cystamine:2H7 (Variant A) disulfide) and 266 μM (10:1 cystamine:2H7 (Variant A)) (FIGS. **34** and **35**) but the 133 μM (5:1 cystamine:2H7 (Variant A) disulfide) and 26.6 μM (1:1 cystamine:2H7 (Variant A) disulfide) concentrations are insufficient to inhibit the reduction of ocrelizumab after 24 hours (FIGS. **36** and **37**).

6. In Vitro Activity of Thioredoxin System Inhibited by Cystine

Cystine was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. **38**, at this concentration cystine effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted that the minimum effective concentration of cystine (just as the effective minimum concentration of other inhibitors) depends on the actual circumstances, and might be different for different proteins, such as antibodies, and might vary depending on the timing of addition. Thus, for example, if cystine is added pre-lysis, the minimum effective concentration for antibody 2H7 (Variant A) is about 1.3 mM, for Apomab about 1 mM and for antibody Variant C about 4.5 mM. When cystine is added in the cell culture medium, the minimum effective concentration typically is somewhat higher, and is about 5.2 mM for 2H7 (Variant A), 6 mM for Apomab and 9 mM for antibody Variant C. Usually, for cystine, cystamine and oxidized glutathione (see below) the minimum effective inhibitory concentration is about 40× of the antibody concentration (in μM).

7. In Vitro Activity of Thioredoxin System Inhibited by Oxidized Glutathione (GSSG)

GSSG was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. **39**, at this concentration GSSG effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted, however, that the minimum effective concentration of oxidize glutathione (just as that of the other inhibitors) depends on the actual circumstances, such as, for example, on the nature of the protein (e.g. antibody) produced and the timing of addition. For example, for antibody 2H7 (Variant A) the minimum effective concentration is about 1.3 mM for addition prior to lysis.

8. In Vitro Activity of Enzymatic Reduction System

FIG. **40** shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, Variant A) (1 mg/mL) with 10 μg/mL hexokinase, 50 μg/mL glucose-6-phosphate dehydrogenase, 5 μM thioredoxin, 0.1 μM thoredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+}, and 2 mM NADP in 50 mM histidine sulfate buffered at pH 7.38 results in the reduction of ocrelizumab in about one hour. Addition of 0.1 mM HDEA, a known glucose-6-phosphate dehydrogenase inhibitor does not inhibit the reduction.

9. In Vitro Activity of Enzymatic Reduction System Requires NADPH

As shown in the digital gel-like image from Bioanalyzer analysis of FIG. **41**, incubation of intact ocrelizumab (1 mg/mL) with 5 μM thioredoxin, 0.1 μM thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH 7.38 does not result in the reduction of the ocrelizumab antibody. Reduction of ocrelizumab could not occur without hexokinase and glucose-6-phosphate dehydrogenase and their substrates to generate NADPH.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein. The inventions have been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form the part of these inventions. This includes within the generic description of each of the inventions a proviso or negative limitation that will allow removing any subject matter from the genus, regardless or whether or not the material to be removed was specifically recited. In addition, where features or aspects of an invention are described in terms of the Markush group, those schooled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. Further, when a reference to an aspect of the invention lists a range of individual members, as for example, 'SEQ ID NO:1 to SEQ ID NO:100, inclusive,' it is intended to be equivalent to listing every member of the list individually, and additionally it should be understood that every individual member may be excluded or included in the claim individually.

The steps depicted and/or used in methods herein may be performed in a different order than as depicted and/or stated. The steps are merely exemplary of the order these steps may occur. The steps may occur in any order that is desired such that it still performs the goals of the claimed invention.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention. Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

SEQUENCE LISTING

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<212> TYPE: PRT

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain variable

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Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35 40 45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65 70 75 80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100 105

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<220> FEATURE:
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<400> SEQUENCE: 2

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
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Gly Gln Gly Thr Leu Val Thr Val Ser Ser
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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35 40 45

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```
Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 4
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 4

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 5
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 5

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

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<210> SEQ ID NO 6
<211> LENGTH: 213
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length light chain

<400> SEQUENCE: 6

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35 40 45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65 70 75 80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
210

<210> SEQ ID NO 7
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 7

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

```
Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 8
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 8

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
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```
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430
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```
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450
```

<210> SEQ ID NO 9
<211> LENGTH: 213
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length light chain

<400> SEQUENCE: 9

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
            20                  25                  30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
        35                  40                  45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
            100                 105                 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
        115                 120                 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
    130                 135                 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145                 150                 155                 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
                165                 170                 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
            180                 185                 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
        195                 200                 205

Asn Arg Gly Glu Cys
    210
```

<210> SEQ ID NO 10
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 10

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
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```
Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 11
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
```

<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 11

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Ala Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Glu Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400
```

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```
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 12
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 12

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
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```
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 13
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 13

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220
```

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```
Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Trp His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 14
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 14

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125
```

-continued

```
Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 15
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 15

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
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        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450
```

<210> SEQ ID NO 16
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 16

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser Ile Gly
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

<210> SEQ ID NO 17
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 17

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr Asp Tyr
20 25 30

Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
50 55 60

Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Asn Leu Gly Pro Ser Phe Tyr Phe Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

<210> SEQ ID NO 18
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain

<400> SEQUENCE: 18

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala
20 25 30

```
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 19
<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 19

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 20
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 20

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
1               5                   10                  15

Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
            100                 105
```

<210> SEQ ID NO 21
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 21

Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly Glu
1 5 10 15

Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met
35 40 45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
50 55 60

Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp Thr Ala Thr Tyr Phe Cys
85 90 95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
100 105 110

Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 22
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 22

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile
35 40 45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100 105

<210> SEQ ID NO 23
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 23

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr

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            20                  25                  30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
    50                  55                  60

Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 24
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 24

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 25
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 25

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr His Tyr
            20                  25                  30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
    50                  55                  60

Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val
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            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 26
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 26

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 27
<211> LENGTH: 121
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 27

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
            20                  25                  30

Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 28
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 28
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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205

Phe Asn Arg Gly Glu Cys
    210


<210> SEQ ID NO 29
<211> LENGTH: 451
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 29

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
            20                  25                  30

Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
        115                 120                 125

Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala
    130                 135                 140

Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
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145                 150                 155                 160

Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala
                165                 170                 175

Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            180                 185                 190

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His
        195                 200                 205

Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys
    210                 215                 220

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
225                 230                 235                 240

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
            260                 265                 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
        275                 280                 285

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
    290                 295                 300

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
305                 310                 315                 320

Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
                325                 330                 335

Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
            340                 345                 350

Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser
        355                 360                 365

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
    370                 375                 380

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
385                 390                 395                 400

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
                405                 410                 415

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
            420                 425                 430

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
        435                 440                 445

Pro Gly Lys
    450


<210> SEQ ID NO 30
<211> LENGTH: 384
<212> TYPE: DNA
<213> ORGANISM: E. coli Thioredoxin TrxA

<400> SEQUENCE: 30

atgttacacc aacaacgaaa ccaacacgcc aggcttattc ctgtggagtt atatatgagc     60

gataaaatta ttcacctgac tgacgacagt tttgacacgg atgtactcaa agcggacggg    120

gcgatcctcg tcgatttctg ggcagagtgg tgcggtccgt gcaaaatgat cgccccgatt    180

ctggatgaaa tcgctgacga atatcagggc aaactgaccg ttgcaaaact gaacatcgat    240

caaaaccctg gcactgcgcc gaaatatggc atccgtggta tcccgactct gctgctgttc    300

aaaaacggtg aagtggcggc aaccaaagtg ggtgcactgt ctaaaggtca gttgaaagag    360

ttcctcgacg ctaacctggc gtaa                                           384
```

<210> SEQ ID NO 31
<211> LENGTH: 966
<212> TYPE: DNA
<213> ORGANISM: E. coli thioredoxin reductase TrxB

<400> SEQUENCE: 31

atgggcacga ccaaacacag taaactgctt atcctgggtt caggcccggc gggatacacc 60

gctgctgtct acgcggcgcg cgccaacctg caacctgtgc tgattaccgg catggaaaaa 120

ggcggccaac tgaccaccac cacggaagtg gaaaactggc ctggcgatcc aaacgatctg 180

accggtccgt tattaatgga gcgcatgcac gaacatgcca ccaagtttga aactgagatc 240

atttttgatc atatcaacaa ggtggatctg caaaaccgtc cgttccgtct gaatggcgat 300

aacggcgaat acacttgcga cgcgctgatt attgccaccg gagcttctgc acgctatctc 360

ggcctgccct ctgaagaagc ctttaaaggc cgtggggttt ctgcttgtgc aacctgcgac 420

ggtttcttct atcgcaacca gaaagttgcg gtcatcggcg gcggcaatac cgcggttgaa 480

gaggcgttgt atctgtctaa catcgcttcg gaagtgcatc tgattcaccg ccgtgacggt 540

ttccgcgcgg aaaaaatcct cattaagcgc ctgatggata aagtggagaa cggcaacatc 600

attctgcaca ccaaccgtac gctggaagaa gtgaccggcg atcaaatggg tgtcactggc 660

gttcgtctgc gcgatacgca aaacagcgat aacatcgagt cactcgacgt tgccggtctg 720

tttgttgcta tcggtcacag cccgaatact gcgattttcg aagggcagct ggaactggaa 780

aacggctaca tcaaagtaca gtcgggtatt catggtaatg ccacccagac cagcattcct 840

ggcgtctttg ccgcaggcga cgtgatggat cacatttatc gccaggccat tacttcggcc 900

ggtacaggct gcatggcagc acttgatgcg gaacgctacc tcgatggttt agctgacgca 960

aaataa 966

<210> SEQ ID NO 32
<211> LENGTH: 318
<212> TYPE: DNA
<213> ORGANISM: mus musculus

<400> SEQUENCE: 32

atggtgaagc tgatcgagag caaggaagct tttcaggagg ccctggccgc cgcgggagac 60

aagcttgtcg tggtggactt ctctgctacg tggtgtggac cttgcaaaat gatcaagccc 120

ttcttccatt ccctctgtga caagtattcc aatgtggtgt tccttgaagt ggatgtggat 180

gactgccagg atgttgctgc agactgtgaa gtcaaatgca tgccgacctt ccagttttat 240

aaaaagggtc aaaaggtggg ggagttctcc ggtgctaaca aggaaaagct tgaagcctct 300

attactgaat atgcctaa 318

<210> SEQ ID NO 33
<211> LENGTH: 501
<212> TYPE: DNA
<213> ORGANISM: mus musculus

<400> SEQUENCE: 33

atggctcagc ggctcctcct ggggaggttc ctgacctcag tcatctccag gaagcctcct 60

cagggtgtgt gggcttccct cacctctaag accctgcaga cccctcagta caatgctggt 120

ggtctaacag taatgcccag cccagcccgg acagtacaca ccaccagagt ctgtttgacg 180

acctttaacg tccaggatgg acctgacttt caagacagag ttgtcaacag tgagacacca 240

gttgttgtgg actttcatgc acagtggtgt ggcccctgca agatcctagg accgcggcta 300

gagaagatgg tcgccaagca gcacgggaag gtggtcatgg ccaaagtgga cattgacgat 360

cacacagacc ttgccattga atatgaggtg tcagctgtgc ctaccgtgct agccatcaag 420

aacggggacg tggtggacaa gtttgtgggg atcaaggacg aggaccagct agaagccttc 480

ctgaagaagc tgattggctg a 501

<210> SEQ ID NO 34
<211> LENGTH: 1494
<212> TYPE: DNA
<213> ORGANISM: mus musculus

<400> SEQUENCE: 34

atgaatggct ccaaagatcc ccctgggtcc tatgacttcg acctgatcat cattggagga 60

ggctcaggag gactggcagc agctaaggag gcagccaaat ttgacaagaa agtgctggtc 120

ttggattttg tcacaccgac tcctcttggg accagatggg gtctcggagg aacgtgtgtg 180

aatgtgggtt gcatacctaa gaagctgatg caccaggcag ctttgctcgg acaagctctg 240

aaagactcgc gcaactatgg ctggaaagtc gaagacacag tgaagcatga ctgggagaaa 300

atgacggaat ctgtgcagag tcacatcggc tcgctgaact ggggctaccg cgtagctctc 360

cgggagaaaa aggtcgtcta tgagaatgct tacgggaggt tcattggtcc tcacaggatt 420

gtggcgacaa ataacaaagg taaagaaaaa atctattcag cagagcggtt cctcatcgcc 480

acaggtgaga ggccccgcta cctgggcatc cctggagaca aagagtactg catcagcagt 540

gatgatcttt tctccttgcc ttactgcccg gggaagaccc tagtagttgg tgcatcctat 600

gtcgccttgg aatgtgcagg atttctggct ggtatcggct tagacgtcac tgtaatggtg 660

cggtccattc tccttagagg atttgaccaa gacatggcca acaaaatcgg tgaacacatg 720

gaagaacatg gtatcaagtt tataaggcag ttcgtcccaa cgaaaattga acagatcgaa 780

gcaggaacac caggccgact cagggtgact gctcaatcca caaacagcga ggagaccata 840

gagggcgaat ttaacacagt gttgctggcg gtaggaagag attcttgtac gagaactatt 900

ggcttagaga ccgtgggcgt gaagataaac gaaaaaaccg gaaagatacc cgtcacggat 960

gaagagcaga ccaatgtgcc ttacatctac gccatcggtg acatcctgga ggggaagcta 1020

gagctgactc ccgtagccat ccaggcgggg agattgctgg ctcagaggct gtatggaggc 1080

tccaatgtca aatgtgacta tgacaatgtc ccaacgactg tatttactcc tttggaatat 1140

ggctgttgtg gcctctctga agaaaaagcc gtagagaaat ttggggaaga aaatattgaa 1200

gtttaccata gtttcttttg gccattggaa tggacagtcc catcccggga taacaacaaa 1260

tgttatgcaa aaataatctg caaccttaaa gacgatgaac gtgtcgtggg cttccacgtg 1320

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What is claimed is:

1. A method for the prevention of the reduction of a disulfide bond in an antibody expressed in a recombinant host cell, comprising, following fermentation, sparging the pre-harvest or harvested culture fluid of said recombinant host cell with air, wherein the amount of dissolved oxygen (dO_2) in the pre-harvest or harvested culture fluid is at least 10%.

2. The method of claim **1** wherein said air sparging is continued until the pre-harvest or harvested culture fluid is at least 30% saturated with air.

3. The method of claim **1** wherein said air sparging is continued until the pre-harvest or harvested culture fluid is between about 100% saturated to about 30% saturated with air.

4. The method of claim **1** wherein the amount of dissolved oxygen (dO_2) in the pre-harvest or harvested culture fluid is at least 30%.

5. The method of claim **1** wherein the antibody is a therapeutic antibody.

6. The method of claim **1** wherein the antibody is a biologically functional fragment of an antibody.

7. The method of claim **1** wherein the host cell is eukaryotic host cell.

8. The method of claim **7** wherein the eukaryotic host cell is a mammalian host cell.

9. The method of claim **1** wherein the host cell is prokaryotic host cell.

10. The method of claim **9** wherein the prokaryotic host cell is a bacterial cell.

* * * * *

EXHIBIT R

US009714293B2

(12) **United States Patent**
Gawlitzek et al.

(10) **Patent No.: US 9,714,293 B2**
(45) **Date of Patent: *Jul. 25, 2017**

(54) **PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA**

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(72) Inventors: **Martin Gawlitzek**, Redwood City, CA (US); **Shun Luo**, Irvine, CA (US); **Christina Teresa Bevilacqua**, San Ramon, CA (US)

(73) Assignee: **GENENTECH, INC.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/670,079**

(22) Filed: **Mar. 26, 2015**

(65) **Prior Publication Data**

US 2016/0024217 A1 Jan. 28, 2016

Related U.S. Application Data

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(60) Provisional application No. 61/232,889, filed on Aug. 11, 2009.

(51) **Int. Cl.**

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C07K 16/22	(2006.01)
C07K 14/705	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 16/2878*** (2013.01); ***C07K 14/70575*** (2013.01); ***C07K 16/18*** (2013.01); ***C07K 16/22*** (2013.01); ***C12N 5/0018*** (2013.01); ***C12N 5/0043*** (2013.01); *C07K 2317/14* (2013.01); *C07K 2319/30* (2013.01); *C12N 2500/32* (2013.01); *C12N 2500/33* (2013.01); *C12N 2500/90* (2013.01)

(58) **Field of Classification Search**

CPC C12N 5/18

See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates generally to glutamine-free cell culture media supplemented with asparagine. The invention further concerns the production of recombinant proteins, such as antibodies, in asparagine-supplemented glutamine-free mammalian cell culture.

78 Claims, 25 Drawing Sheets

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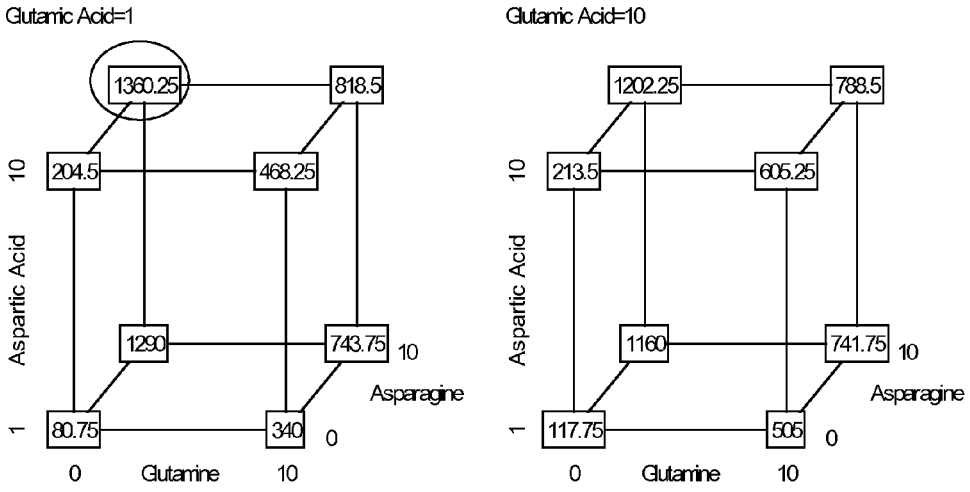
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1360.25
818.5
204.5
468.25
1290
743.75
80.75
340
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0
Glutamic Acid=10
1202.25
788.5
213.5
605.25
1160
741.75
117.75
505
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0

Figure 1.

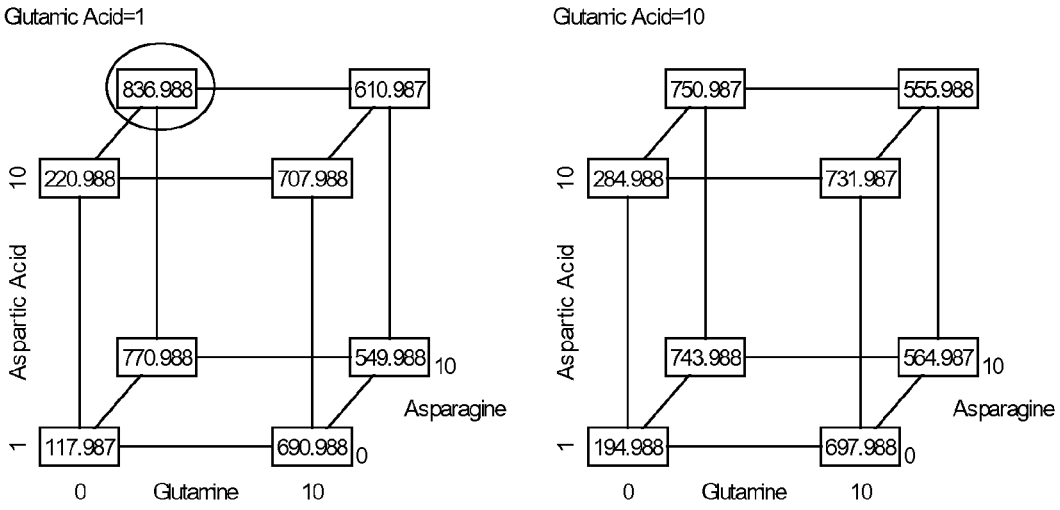

Glutamic Acid=1
836.988
610.987
220.988
707.988
770.988
549.988
117.987
690.988
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0
Glutamic Acid=10
750.987
555.988
284.988
731.987
743.988
564.987
194.988
697.988
Aspartic Acid
10
1
0
Glutamine
10
Asparagine
10
0

Figure 2.

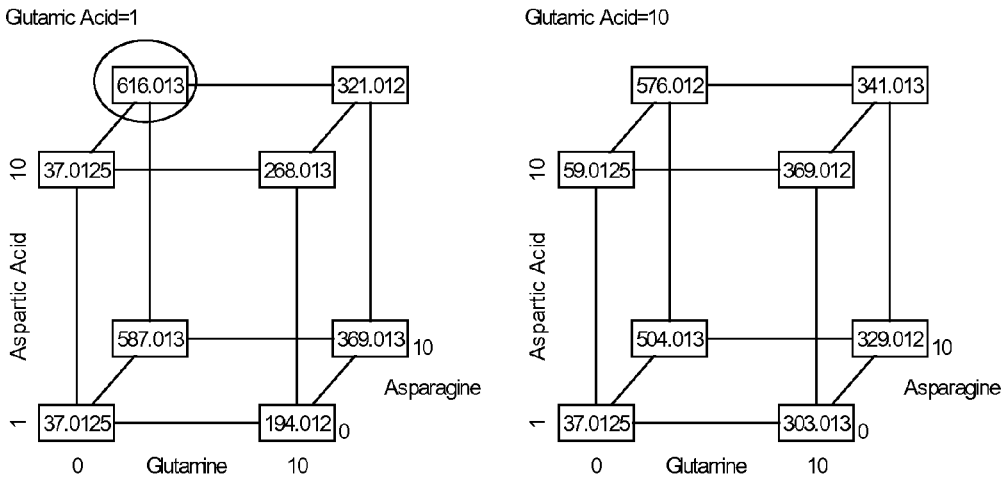

Glutamic Acid=1
616.013
321.012
37.0125
268.013
587.013
369.013
37.0125
194.012
Aspartic Acid
10
1
0
10
Glutamine
Asparagine
Glutamic Acid=10
576.012
341.013
59.0125
369.012
504.013
329.012
37.0125
303.013

Figure 3.

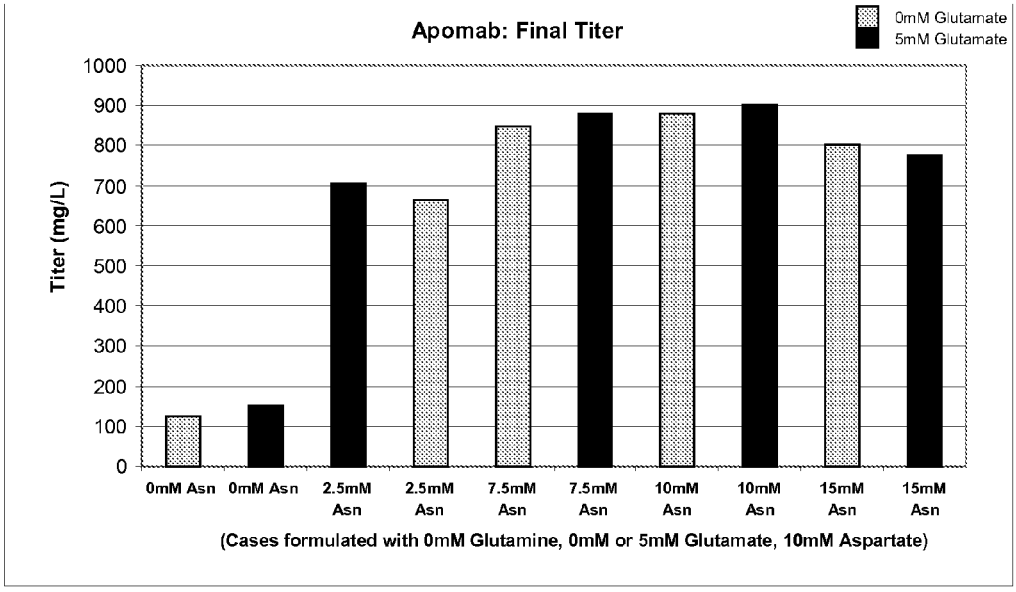
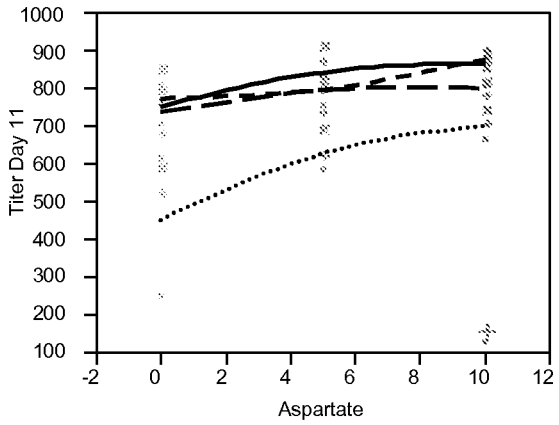

Apomab: Final Titer
0mM Glutamate
5mM Glutamate
Titer (mg/L)
1000
900
800
700
600
500
400
300
200
100
0
0mM Asn
0mM Asn
2.5mM Asn
2.5mM Asn
7.5mM Asn
7.5mM Asn
10mM Asn
10mM Asn
15mM Asn
15mM Asn
(Cases formulated with 0mM Glutamine, 0mM or 5mM Glutamate, 10mM Aspartate)

Figure 4.

Bivariate Fit of Titer Day 11 By Aspartate Glutamine=0



Smoothing Spline Fit, lambda=1 Asparagine==2.5
Smoothing Spline Fit, lambda=1 Asparagine==7.5
Smoothing Spline Fit, lambda=1 Asparagine==10
Smoothing Spline Fit, lambda=1 Asparagine==15

Smoothing Spline Fit, lambda=1 Asparagine==2.5

R-Square	0.573894
Sum of Squares Error	73461.63

Smoothing Spline Fit, lambda=1 Asparagine==7.5

R-Square	0.65596
Sum of Squares Error	11556.31

Smoothing Spline Fit, lambda=1 Asparagine==10

R-Square	0.408718
Sum of Squares Error	25684.65

Smoothing Spline Fit, lambda=1 Asparagine==15

R-Square	0.220438
Sum of Squares Error	26400.64

Figure 5.

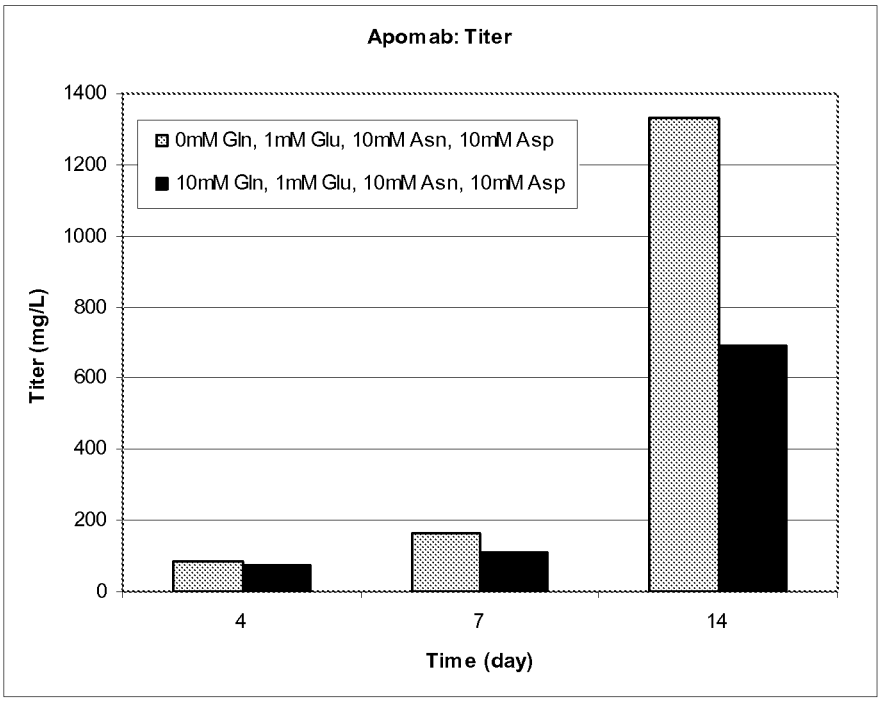

Apomab: Titer
1400
1200
1000
800
600
400
200
0
Titer (mg/L)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

Figure 6A

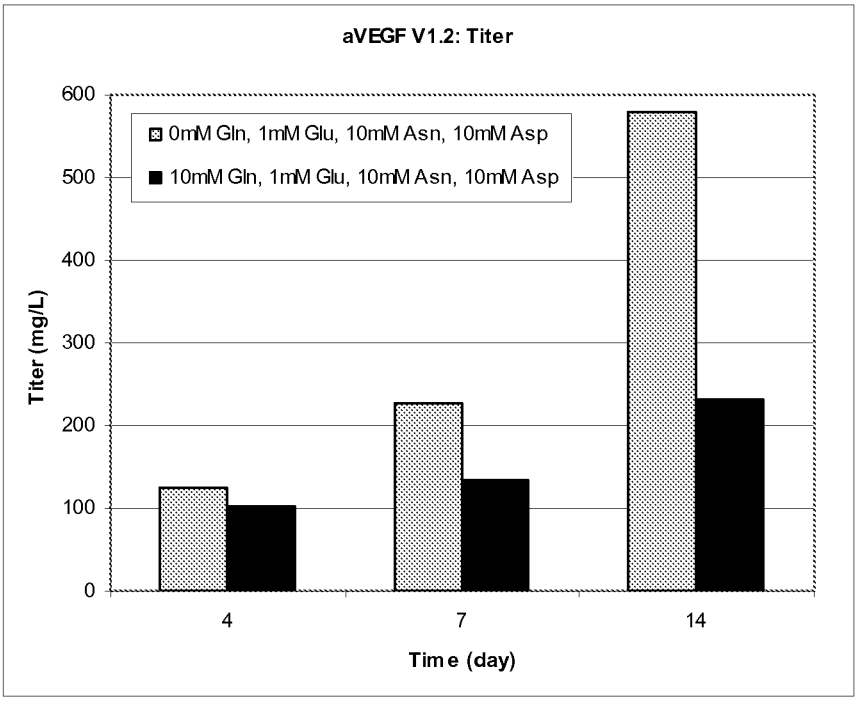

aVEGF V1.2: Titer
600
500
400
300
200
100
0
Titer (mg/L)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

Figure 6B

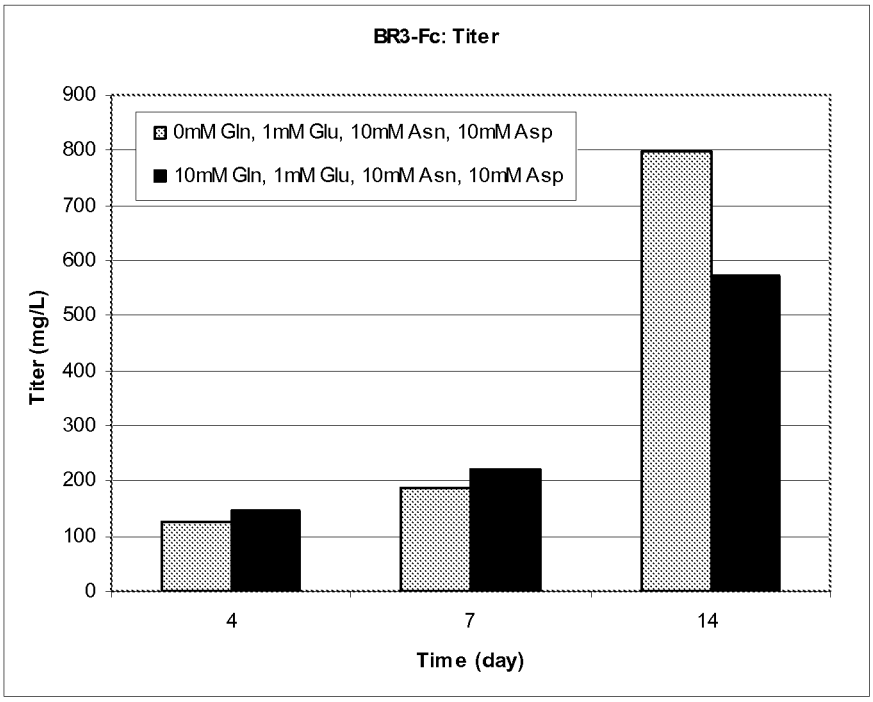

BR3-Fc: Titer
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Titer (mg/L)
900
800
700
600
500
400
300
200
100
0
4
7
14
Time (day)

Figure 6C

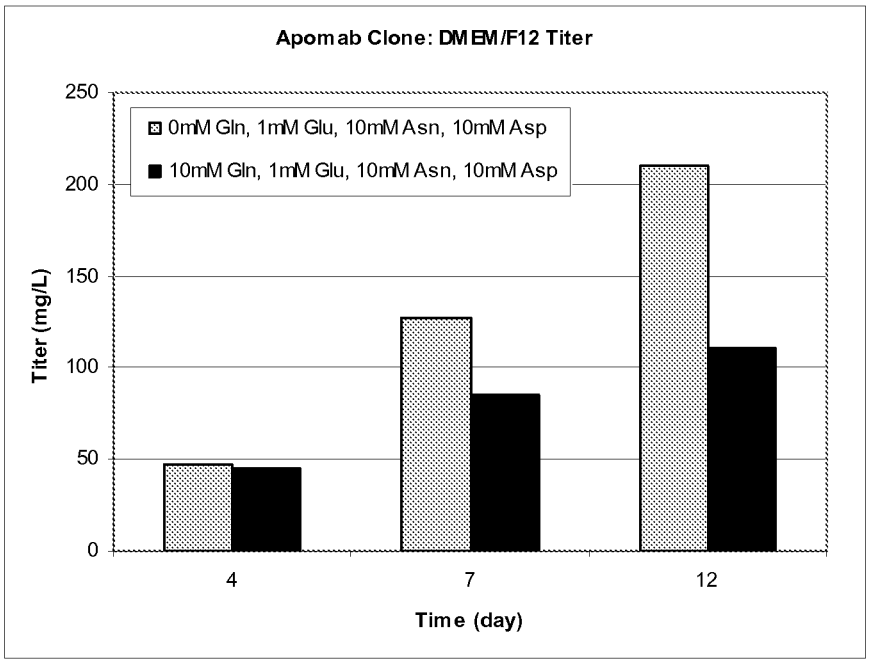

Apomab Clone: DMEM/F12 Titer
250
200
150
100
50
0
Titer (mg/L)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
12
Time (day)

Figure 7A

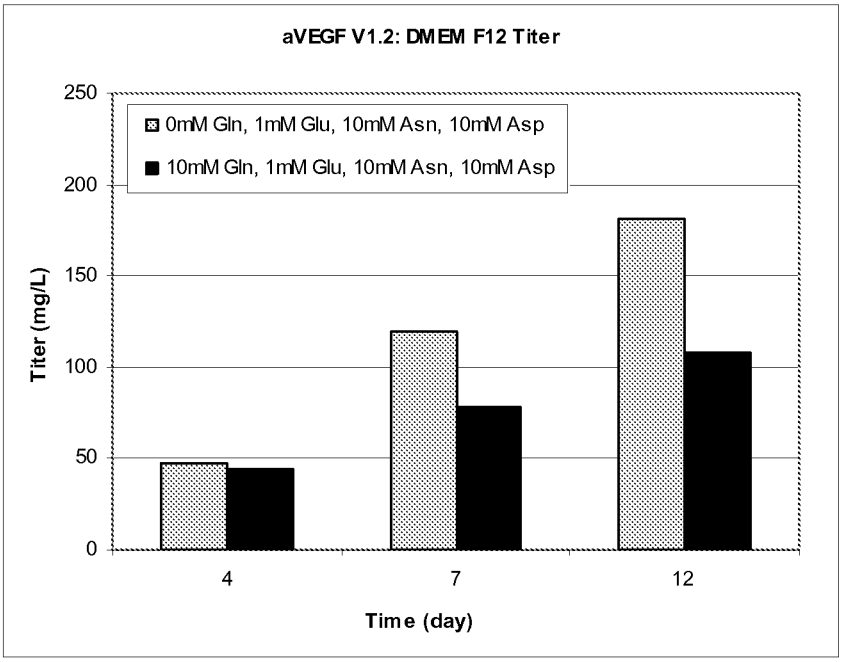

aVEGF V1.2: DMEM F12 Titer
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Titer (mg/L)
250
200
150
100
50
0
4
7
12
Time (day)

Figure 7B

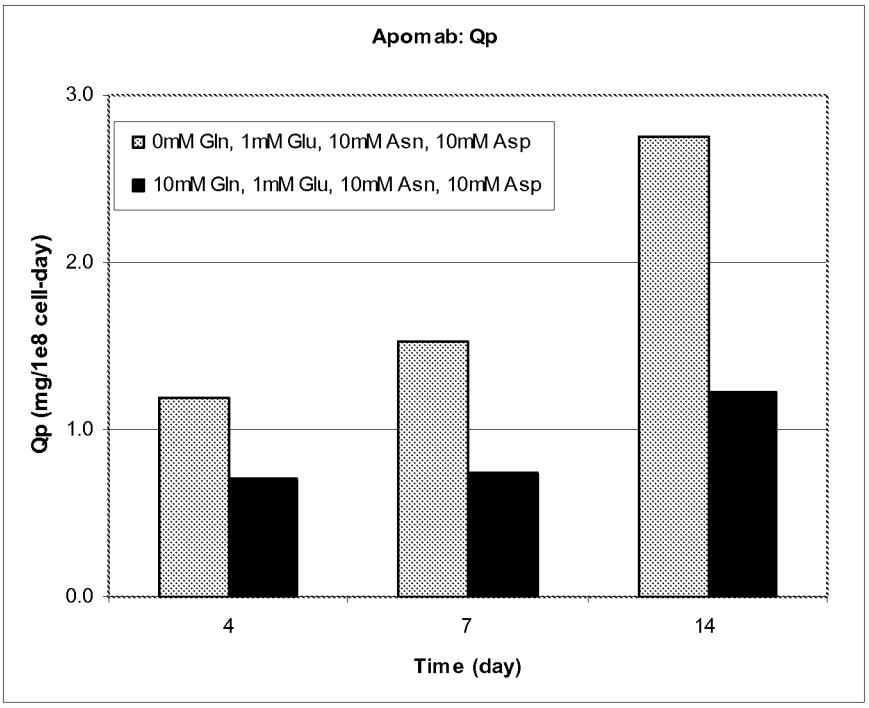

Apomab: Qp
3.0
2.0
1.0
0.0
Qp (mg/1e8 cell-day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

Figure 8A

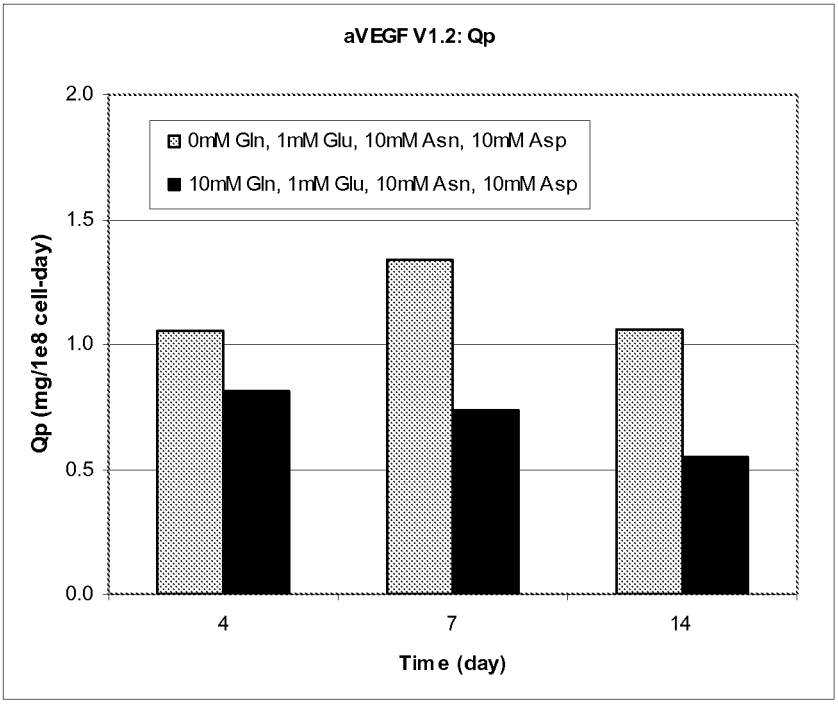

aVEGF V1.2: Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
2.0
1.5
1.0
0.5
0.0
4
7
14
Time (day)

Figure 8B

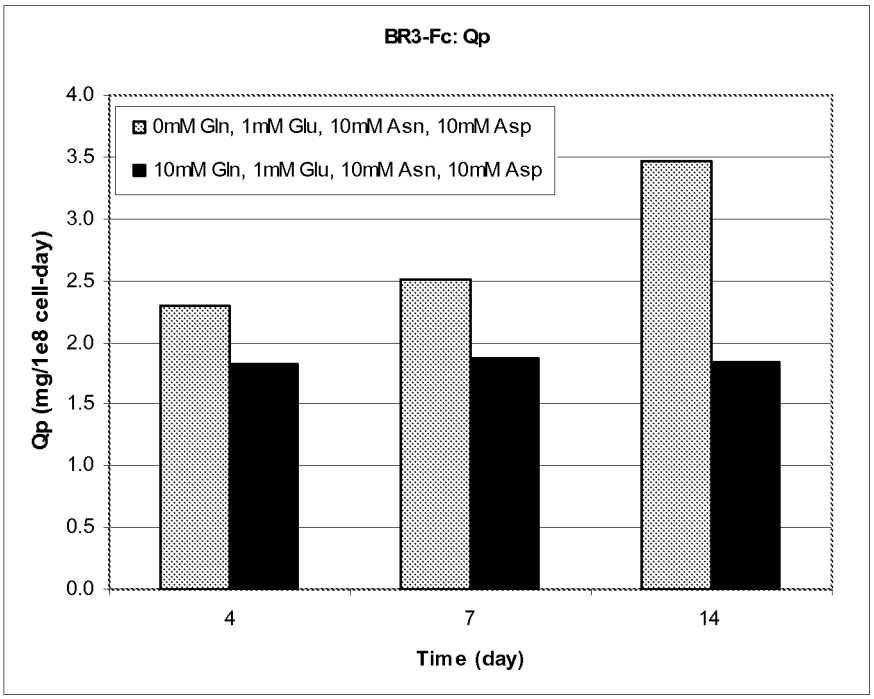

BR3-Fc: Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
7
14
Time (day)

Figure 8C

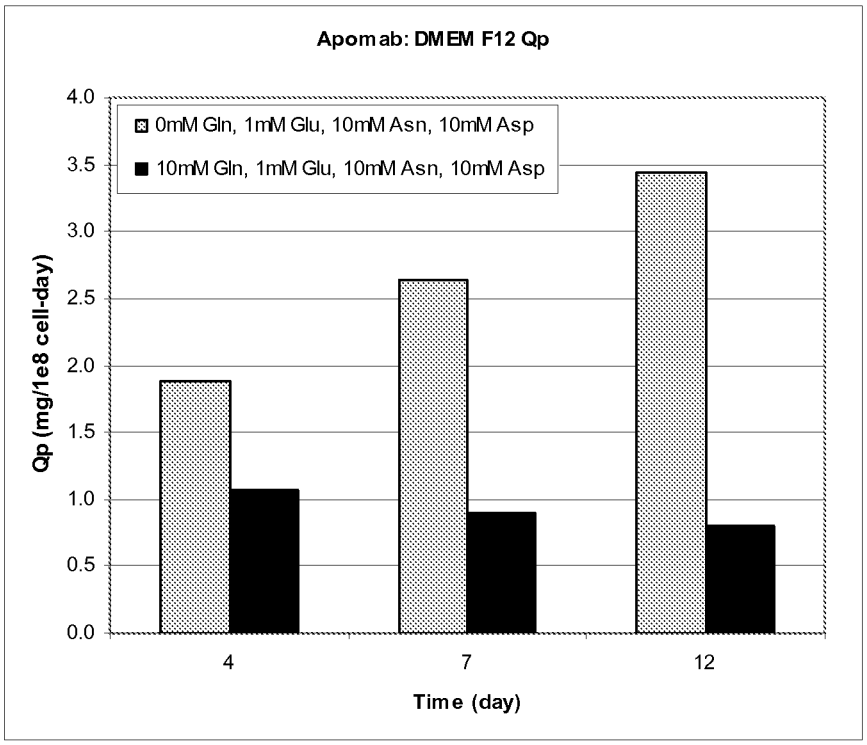

Apomab: DMEM F12 Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
7
12
Time (day)

Figure 9A

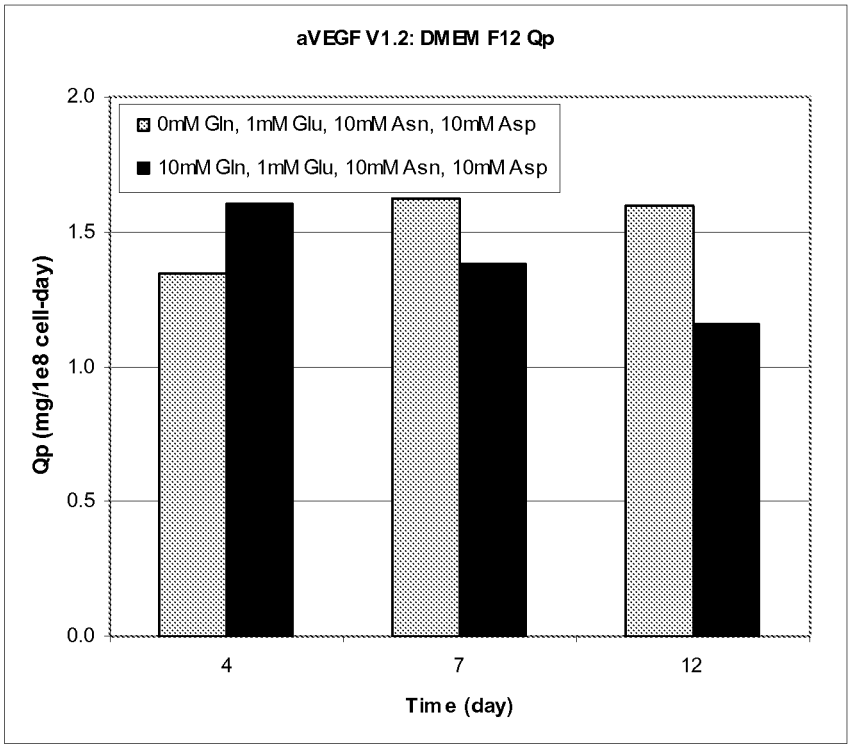

aVEGF V1.2: DMEM F12 Qp
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
Qp (mg/1e8 cell-day)
2.0
1.5
1.0
0.5
0.0
4
7
12
Time (day)

Figure 9B

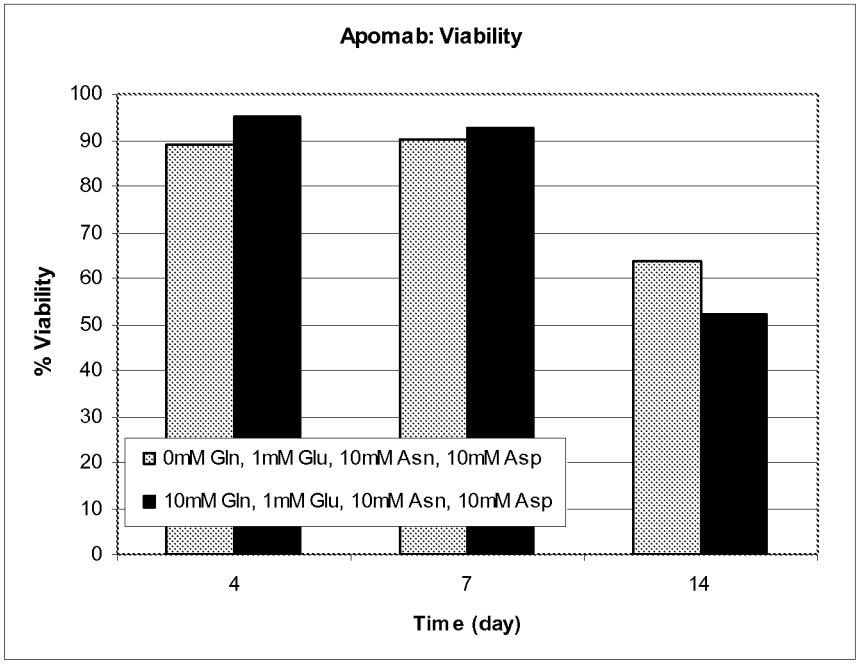

Apomab: Viability
100
90
80
70
60
50
40
30
20
10
0
% Viability
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

Figure 10A

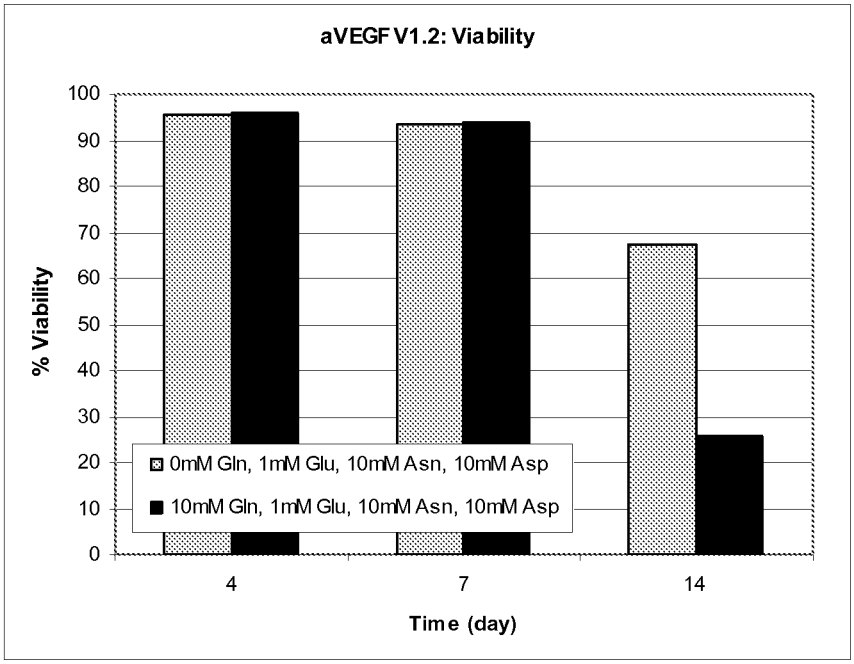

aVEGF V1.2: Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
14
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

Figure 10B

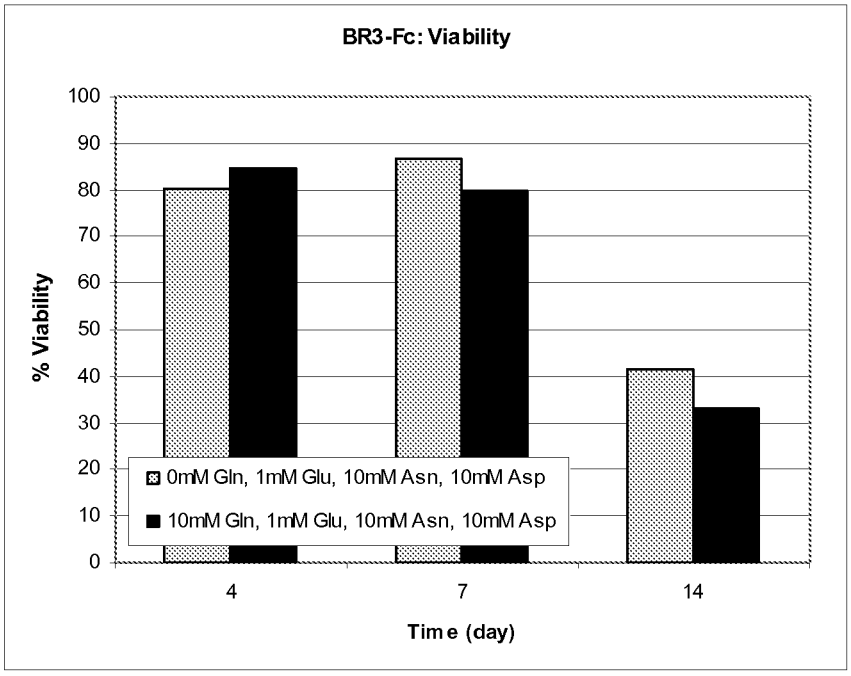

BR3-Fc: Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
14
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

Figure 10C

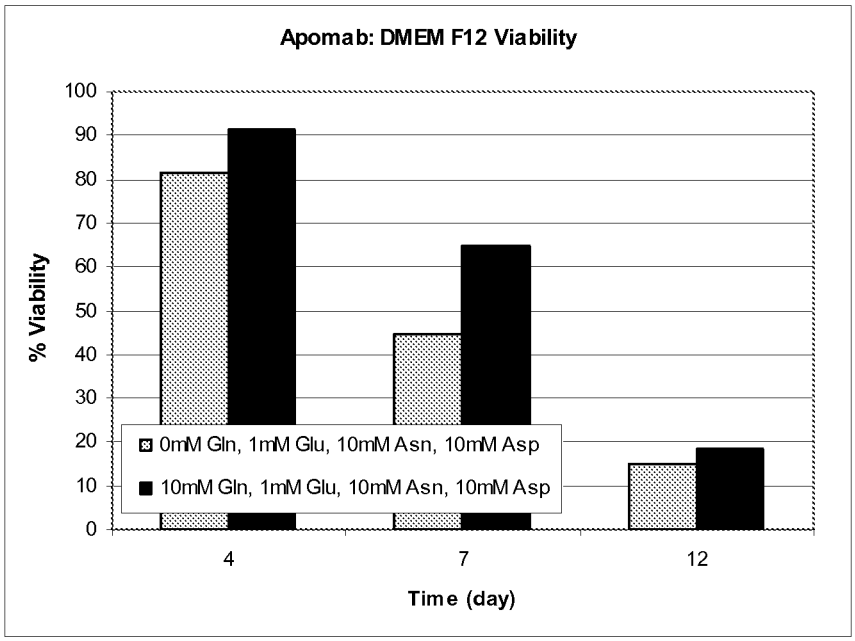

Apomab: DMEM F12 Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
12
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

Figure 11A

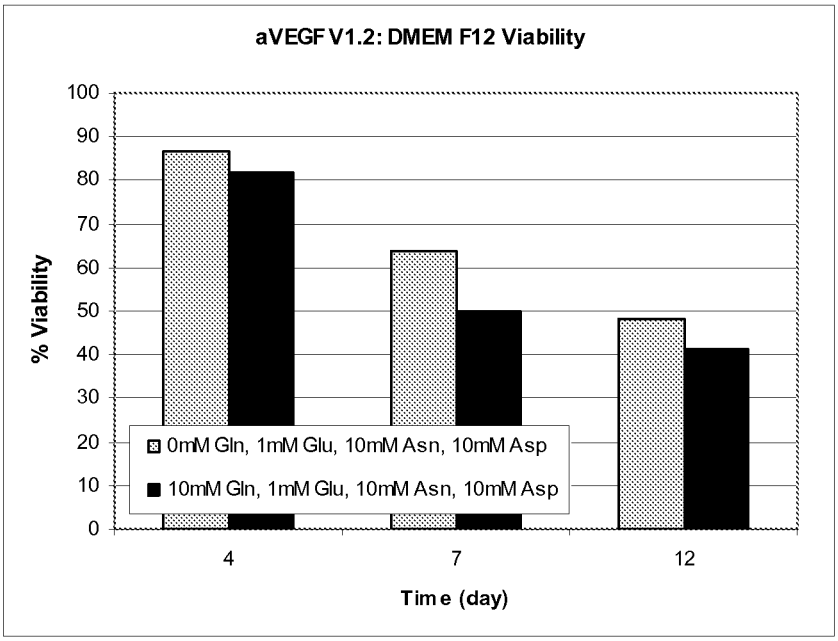

aVEGF V1.2: DMEM F12 Viability
% Viability
100
90
80
70
60
50
40
30
20
10
0
4
7
12
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp

Figure 11B

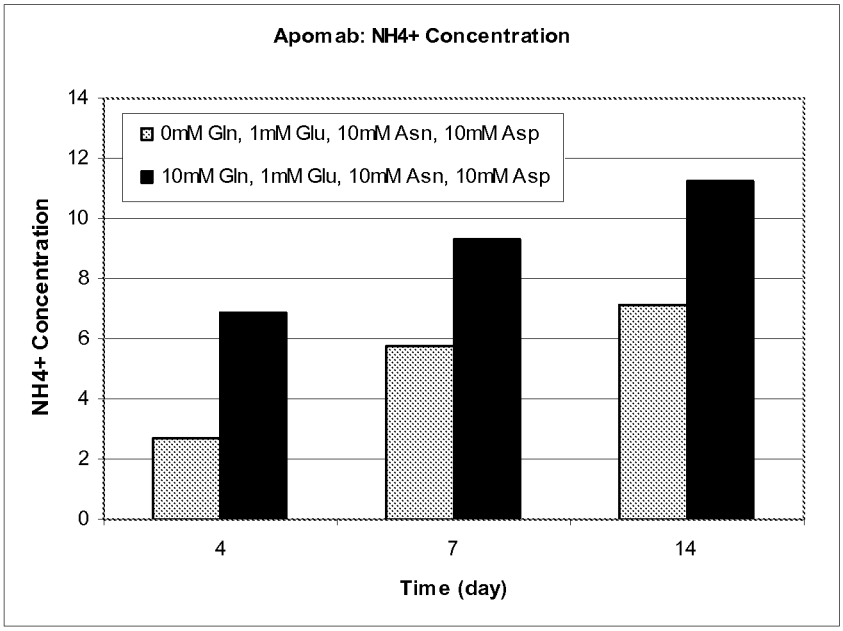

Apomab: NH4+ Concentration
NH4+ Concentration
Time (day)
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
0
2
4
6
8
10
12
14
4
7
14

Figure 12A

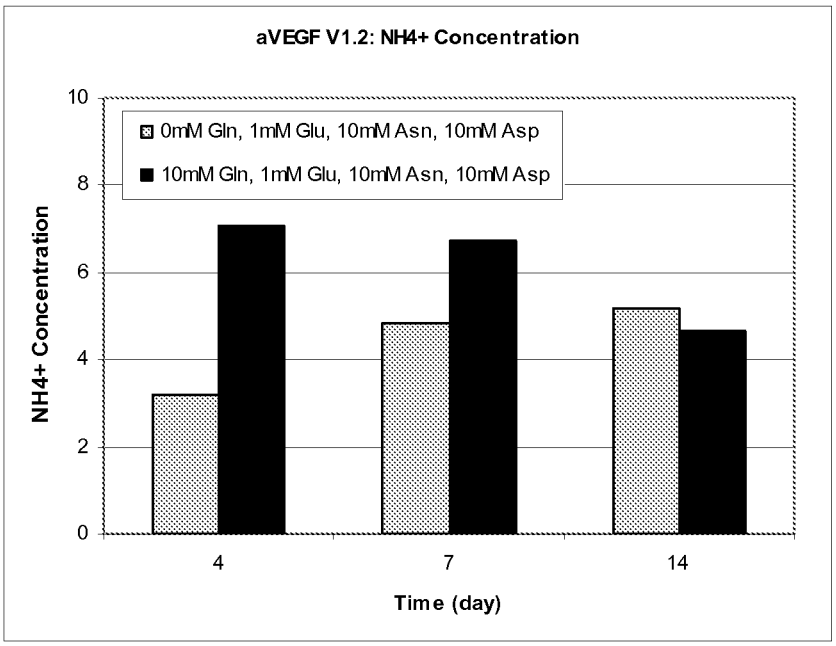

aVEGF V1.2: NH4+ Concentration
NH4+ Concentration
10
8
6
4
2
0
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
4
7
14
Time (day)

Figure 12B

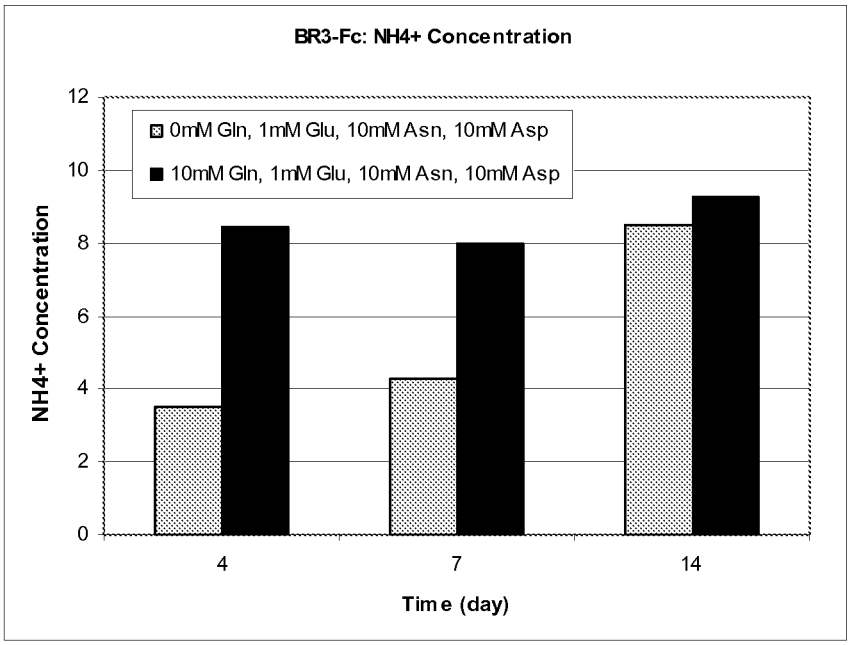

BR3-Fc: NH4+ Concentration
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
NH4+ Concentration
12
10
8
6
4
2
0
4
7
14
Time (day)

Figure 12C

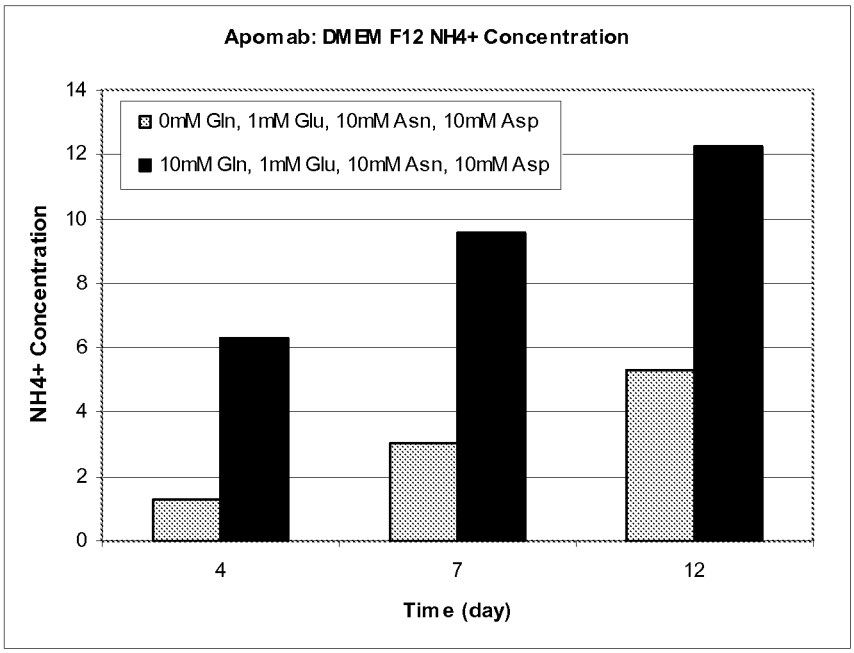

Apomab: DMEM F12 NH4+ Concentration
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
NH4+ Concentration
0
2
4
6
8
10
12
14
4
7
12
Time (day)

Figure 13A

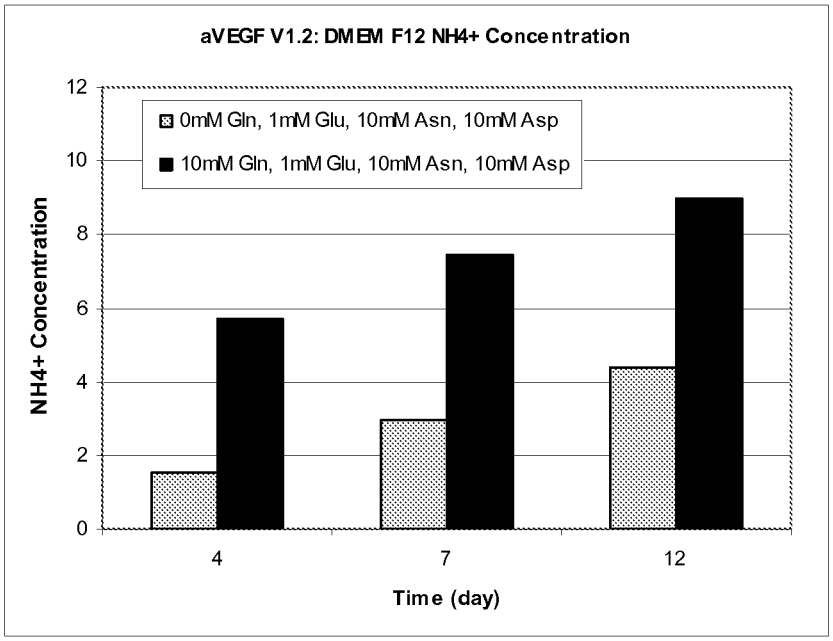

aVEGF V1.2: DMEM F12 NH4+ Concentration
0mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
10mM Gln, 1mM Glu, 10mM Asn, 10mM Asp
NH4+ Concentration
0
2
4
6
8
10
12
4
7
12
Time (day)

Figure 13B

PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 13/945,531, filed Jul. 18, 2013, now abandoned, which is a continuation of U.S. application Ser. No. 12/852,377, filed Aug. 6, 2010, now U.S. Pat. No. 8,512,983, issued Aug. 20, 2013, which claims priority under 35 USC §119(e) and the benefit of U.S. Provisional application No. 61/232,889, filed Aug. 11, 2009, the contents of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. It is conventional to have glutamine in cell culture media during recombinant production of heterologous proteins, including antibodies. L-glutamine is an essential amino acid, which is considered the primary energy and nitrogen sources for cells in culture. Most commercially available media are formulated with free L-glutamine which is either included in the basal formula or added to liquid media formulations at the time of use. Thus, all mammalian cell culture media contain glutamine except those for glutamine synthetase transfected cell lines, such as GS NS0 and GS CHO cell lines, where the cells themselves produce the glutamine needed for growth. Glutamine is widely used at various concentrations typically from 1 to 20 mM in base media and much higher concentration in feeds for fed-batch process. For example, the concentration of L-glutamine is 0.5 mM in Ames' Medium and 10 mM in MCDP Media 131. DMEM/Ham's Nutrient Mixture F-12 (50:50) is often used as a starting formulation for proprietary media used with Chinese Hamster Ovary (CHO) cells. L-glutamine in DMEM/Ham's Nutrient Mixture F-12 is 2.5 mM. L-glutamine concentration in Serum-Free/Protein Free Hybridoma Medium is 2.7 mM. L-glutamine in DMEM, GMEM, IMDM and H-Y medium is 4 mM, of which IMDM is often used as a starting formulation for proprietary hybridoma cell culture media. It is generally held that hybridoma cells grow better in concentrations of L-glutamine that are above the average levels found in media. (Dennis R. Conrad, Glutamine in Cell Culture, Sigma-Aldrich Media Expert)

It was shown that glutamine is the main source of ammonia accumulated in cell culture (see review by Markus Schneider, et. al. 1996, *Journal of Biotechnology* 46:161-185). Thus, lowering glutamine in cell culture media significantly reduced the accumulation of NH_4^+ level, resulting in lower cytotoxicity (see Markus Schneider, et. al. 1996, supra). Reduced NH_4^+ cytotoxicity resulted in higher cell viability, thus extended culture longevity. Based on an estimated glutamine consumption study using CHO cells, it was suggested that cells may consume glutamine at a rate of 0.3-0.4 mM per day (Miller, et. al. 1988, Biotechnol. Bioeng. 32: 947-965). Altamirano et al. (2001, *J. Biotechnol.* 110:171-9) studied the effect of glutamine replacement by glutamate and the balance between glutamate and glucose metabolism on the redistribution of CHO cells producing recombinant human tissue plasminogen activator (rhut-PA). When glutamine was replaced with glutamate and balanced with glucose catabolism (carbon and nitrogen ratio, C/N ratio), cell metabolism was found redistributed and forced to utilize carbon and energy source more favorably to production of rhut-PA. It was also reported that CHO cells in adherent cultures can grow in the absence of added glutamine due to endogenous glutamine synthetase activity that allowed cells to synthesize glutamine from glutamic acid in the medium (Sanfeliu and Stephanopoulos, 1999, *Biotechnol. Bioeng.* 64:46-53). However, compared to control cultures in glutamine-containing media, the cell growth rate in glutamine-free media was slower with an increased fraction of cells distributed in the G0/G1 phase. The depletion of both glutamine and glutamic acid did cause cell death.

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the unexpected finding that not only can recombinant proteins be produced in a mammalian host cell using a glutamine-free production medium without any significant adverse effect, in fact the use of a glutamine-free medium in the production phase significantly increases cell viability, culture longevity, specific productivity and/or the final recombinant protein titer.

The present invention is also based on the unexpected finding that the addition of asparagine to a glutamine-free production medium can further enhance the cell viability, culture longevity, specific productivity and/or the final recombinant protein titer in a mammalian host cell using a glutamine-free production medium without any significant adverse effect.

In one aspect, the invention concerns a process for producing a polypeptide in a mammalian host cell expressing said polypeptide, comprising culturing the mammalian host cell in a production phase of the culture in a glutamine-free production culture medium supplemented with asparagine.

In one embodiment, the mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

In another embodiment, the mammalian host cell is a $dhfr^-$ CHO cell.

In yet another embodiment, the production medium is serum-free.

In a further embodiment, the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

In a still further embodiment, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

In all embodiments, the production phase may, for example, be a batch or fed batch culture phase.

In all embodiments, the process may further comprise the step of isolating said polypeptide.

In a further embodiment, isolation may be followed by determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

In a still further embodiment, at least one of the cell viability, culture longevity, specific productivity and final recombinant protein titer is increased relative to the same

polypeptide produced in a glutamine-containing production medium of the same composition.

In a further aspect, the invention concerns a ready-to-use glutamine-free cell culture medium for the production of a polypeptide in a production phase.

In yet another embodiment, the polypeptide is a mammalian glycoprotein.

In other embodiments, the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', $F(ab)_2$, scFv, $(scFv)_2$, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

In a still further embodiment, the antibody or antibody fragment is chimeric, humanized or human.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha_4\beta_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In other embodiments, the therapeutic antibody is an anti-BR3 antibody or BR3-Fc immunoadhesin.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell. such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

These and other aspects will be apparent from the description below, including the Examples and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Apomab antibody cube plot analysis of titer results from a Full Factorial Design of Experiment (DOE) evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **2**. BR3-Fc immunoadhesin cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **3**. anti-VEGF antibody cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **4**. Effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

FIG. **5**. Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free

and low Glutamate conditions. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

FIGS. **6**. A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **7**-A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM F12 medium.

FIGS. **8** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **9** A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

FIGS. **10** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. Cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **11** A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. In DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium. Viability was higher for Apomab antibody, but lower for anti-VEGF antibody compared to Glutamine containing medium.

FIGS. **12** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation Ammonia was usually lower in Glutamine-free cultures compared to Glutamine-containing cultures.

FIGS. **13** A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation. Ammonia was significantly reduces in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The terms "cell culture medium", "culture medium", and "nutrient mixture" refer to a nutrient solution used for growing mammalian cells that typically provides at least one component from one or more of the following categories:

1) an energy source, usually in the form of a carbohydrate such as glucose;

2) some or all of the essential amino acids, and usually the basic set of twenty amino acids plus cystine;

3) vitamins and/or other organic compounds typically required at low concentrations;

4) free fatty acids; and

5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range.

The nutrient mixture may optionally be supplemented with one or more component from any of the following categories:

1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor;

2) salts and buffers as, for example, calcium, magnesium, and phosphate; and

3) nucleosides such as, for example, adenosine and thymidine.

The cell culture medium is generally "serum free" when the medium is essentially free of serum from any mammalian source (e.g. fetal bovine serum (FBS)). By "essentially free" is meant that the cell culture medium comprises between about 0-5% serum, preferably between about 0-1% serum, and most preferably between about 0-0.1% serum. Advantageously, serum-free "defined" medium can be used, wherein the identity and concentration of each of the components in the medium is known (i.e., an undefined component such as bovine pituitary extract (BPE) is not present in the culture medium).

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed (host) cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "animal host cell," "animal cell," "animal recombinant host cell," and the like, encompasses invertebrate, non-mammalian vertebrate (e.g., avian, reptile and amphibian) and mammalian cells. Examples of invertebrate cells include the following insect cells: *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori*. See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in *Genetic Engineering*, Setlow, J. K. et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature,* 315:592-594 (1985).

The term "mammalian host cell," "mammalian cell," "mammalian recombinant host cell," and the like, refer to cell lines derived from mammals that are capable of growth and survival when placed in either monolayer culture or in suspension culture in a medium containing the appropriate nutrients and growth factors. The necessary nutrients and growth factors for a particular cell line are readily determined empirically without undue experimentation, as described for example in *Mammalian Cell Culture* (Mather, J. P. ed., Plenum Press, N.Y. (1984)), and by Barnes and Sato (*Cell,* 22:649 (1980)). Typically, the cells are capable of expressing and secreting large quantities of a particular protein of interest (typically a recombinant protein) into the culture medium, and are cultured for this purpose. However, the cells may be cultured for a variety of other purposes as well, and the scope of this invention is not limited to culturing the cells only for production of recombinant pro-

teins. Examples of suitable mammalian cell lines, capable of growth in the media of this invention, include monkey kidney CVI line transformed by SV40 (COS-7, ATCC® CRL 1651); human embryonic kidney line 293S (Graham et al., *J. Gen. Virolo.,* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC® CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23:243 (1980)); monkey kidney cells (CVI-76, ATCC® CCL 70); African green monkey kidney cells (VERO-76, ATCC® CRL-1587); human cervical carcinoma cells (HELA, ATCC® CCL 2); canine kidney cells (MDCK, ATCC® CCL 34); buffalo rat liver cells (BRL 3A, ATCC® CRL 1442); human lung cells (W138, ATCC® CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC® CCL 5I); rat hepatoma cells (HTC, MI.54, Baumann et al., J. Cell Biol., 85:1 (1980)); and TR-1 cells (Mather et al., *Annals N.Y. Acad. Sci.,* 383:44 (1982)) and hybridoma cell lines. Chinese hamster ovary cells (Urlab and Chasin, *Proc. Natl. Acad. Sci. USA,* 77:4216 (1980)) are a preferred cell line for practicing this invention. CHO cells suitable for use in the methods of the present invention have also been described in the following documents: EP 117,159, published Aug. 29, 1989; U.S. Pat. Nos. 4,766,075; 4,853,330; 5,185,259; Lubiniecki et al., in *Advances in Animal Cell Biology and Technology for Bioprocesses*, Spier et al., eds. (1989), pp. 442-451. Known CHO derivatives suitable for use herein include, for example, CHO/−DHFR (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 (1980)), CHO-K1 DUX B11 (Simonsen and Levinson, *Proc. Natl. Acad. Sci. USA* 80: 2495-2499 (1983); Urlaub and Chasin, supra), and dp 12.CHO cells (EP 307,247 published Mar. 15, 1989). Preferred host cells include CHO-K1 DUX B11 and dp 12.CHO cells.

"dhfr$^-$ CHO cell" refers to a dihydrofolate reductase (DHFR) deficient CHO cell. Production of recombinant proteins in mammalian cells has allowed the manufacture of a number of large, complex glycosylated polypeptides for clinical applications. Chinese hamster ovary (CHO) DHFR− cells and the amplifiable selectable marker DHFR are routinely used to establish cell lines that produce clinically useful amounts of product. (Urlab, G. and Chasin, L. A. (1980) Proc. Natl Acad. Sci. USA, 77, 4216-4220; Kaufman, R. J. and Sharp, P. (1982) J. Mol. Biol., 159, 601-621; Gasser, C. S., Simonsen, C. S., Schilling, J. W. and Schmike, R. T. (1982) Proc. Natl Sci. USA, 79, 6522-6526)

By "phase" is meant a certain phase of culturing of the cells as is well recognized by the practitioner.

"Growth phase" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are generally rapidly dividing. During this phase, cells are cultured for a period of time, usually between 1-4 days, and under such conditions that cell growth is maximized. The growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. During the growth phase, cells are cultured in nutrient medium containing the necessary additives generally at about 30-40° C., preferably about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line. Cells are maintained in the growth phase for a period of between about one and four days, usually between about two and three days.

"Transition phase" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as temperature are shifted from growth conditions to production conditions.

"Production phase" of the cell culture refers to the period of time during which cell growth has plateaued. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product.

The phrase "fed batch cell culture" when used herein refers to a batch culture wherein the animal (e.g. mammalian) cells and culture medium are supplied to the culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. Fed batch culture includes "semi-continuous fed batch culture" wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple "batch culture" in which all components for cell culturing (including the animal cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc and the culture medium is continuously or intermittently introduced and removed from the culturing vessel). However, removal of samples for testing purposes during fed batch cell culture is contemplated.

When used herein, the term "glutamine" refers to the amino acid L-glutamine (also known as "Gln" and "Q" by three-letter and single-letter designation, respectively) which is recognized as both an amino acid building block for protein synthesis and as an energy source in cell culture. Thus, the terms "glutamine" and "L-glutamine" are used interchangeably herein.

The word "glucose" refers to either of α-D-glucose or β-D-glucose, separately or in combination. It is noted that α and β glucose forms are interconvertible in solution.

The expression "osmolality" is a measure of the osmotic pressure of dissolved solute particles in an aqueous solution. The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water (1 mOsm/kg H_2O at 38° C. is equivalent to an osmotic pressure of 19 mm Hg). "Osmolarity" refers to the number of solute particles dissolved in 1 liter of solution. Solutes which can be added to the culture medium so as to increase the osmolality thereof include proteins, peptides, amino acids, non-metabolized polymers, vitamins, ions, salts, sugars, metabolites, organic acids, lipids, etc. In the preferred embodiment, the concentration of amino acids and NaCl in the culture medium is increased in order to achieve the desired osmolality ranges set forth herein. When used herein, the abbreviation "mOsm" means "milliosmoles/kg H_2O".

The term "cell density" as used herein refers to that number of cells present in a given volume of medium.

The term "cell viability" as used herein refers to the ability of cells in culture to survive under a given set of culture conditions or experimental variations. The term as used herein also refers to that portion of cells which are alive at a particular time in relation to the total number of cells, living and dead, in the culture at that time.

The terms "amino acids" and "amino acid" refer to all naturally occurring alpha amino acids in both their D and L stereoisomeric forms, and their analogs and derivatives. An

analog is defined as a substitution of an atom in the amino acid with a different atom that usually has similar properties. A derivative is defined as an amino acid that has another molecule or atom attached to it. Derivatives would include, for example, acetylation of an amino group, amination of a carboxyl group, or oxidation of the sulfur residues of two cysteine molecules to form cystine.

The term "protein" is meant to refer to a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term "therapeutic protein" or "therapeutic polypeptide" refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. "Manufacturing scale" production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

As used herein, "polypeptide of interest" refers generally to peptides and proteins having more than about ten amino acids. The polypeptides may be homologous to the host cell, or preferably, may be exogenous, meaning that they are heterologous, i.e., foreign, to the host cell being utilized, such as a human protein produced by a non-human mammalian, e.g., Chinese Hamster Ovary (CHO) cell. Preferably, mammalian polypeptides (polypeptides that were originally derived from a mammalian organism) are used, more preferably those which are directly secreted into the medium. The term "polypeptide" or "polypeptide of interest" specifically includes antibodies, in particular, antibodies binding to mammalian polypeptides, such as any of the mammalian polypeptides listed below or fragments thereof, as well as immunoadhesins (polypeptide-Ig fusion), such as those comprising any of the mammalian polypeptides listed below, or fragments thereof.

Examples of mammalian polypeptides include, without limitation, transmembrane molecules (e.g. receptors) and ligands such, as growth factors. Exemplary polypeptides include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; interferon such as interferon-α, -β, and -γ; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA), including t-PA variants; bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as β-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER1 (EGFR), HER2, HER3 or HER4 receptor; Apo2L/TRAIL, hedgehog, mitogen activated protein kinase (MAPK), and fragments of any of the above-listed polypeptides. Apo2L (TRAIL) and is variants are disclosed, for example, in U.S. Application Publication No. 20040186051. Anti-VEGF antibodies are disclosed, for example, in U.S. Pat. Nos. 8,994,879; 7,060,269; 7,169,901; and 7,297,334. Anti-CD20 antibodies are disclosed, for example, in U.S. Application Publication No. 20060246004. The BR3 polypeptide, anti-BR3 antibodies and BR3-Fc immunoadhesins are described, for example, in U.S. Application Publication No. 20050070689.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin") with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

As noted above, in certain embodiments, the protein is an antibody. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen speci-

ficity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region or intact monoclonal antibodies), antibody compositions with polyepitopic specificity, polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies) formed from at least two intact antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, F(ab')2, and Fv).

Unless indicated otherwise, the expression "multivalent antibody" is used throughout this specification to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is typically engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward et al., *Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) F(ab')2 fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (e.g. single chain Fv; scFv) (Bird et al., *Science* 242:423-426 (1988); and Huston et al., *PNAS* (*USA*) 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, e.g., EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al. *Protein Eng.* 8(10):1057 1062 (1995); and U.S. Pat. No. 5,641,870).

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO93/1161; Hudson et al., (2003) Nat. Med. 9:129-134; and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) Nat. Med. 9:129-134.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. Monoclonal antibodies are highly specific, being directed against a single antigen. In certain

embodiments, a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler and Milstein, *Nature,* 256:495-97 (1975); Hongo et al., *Hybridoma,* 14 (3): 253-260 (1995), Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage-display technologies (see, e.g., Clackson et al., *Nature,* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1991); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284 (1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, e.g., Vaswani and Hamilton, *Ann. Allergy, Asthma* & Immunol. 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409. See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.,* 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, e.g., immunized xenomice (see, e.g., U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li et al., *Proc. Natl. Acad. Sci. USA,* 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology. The humanized antibody may also include a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. *Nature Biotechnology* 14:309-314 (1996): Sheets et al. *PNAS* (*USA*) 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody rep-

ertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro). See, e.g., Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.,* 147 (1):86-95 (1991); and U.S. Pat. No. 5,750,373.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., Proc Nat. Acad. Sci. USA 91:3809-3813 (1994); Schier et al., Gene 169: 147-155 (1995); Yelton et al., J. Immunol. 155:1994-2004 (1995); Jackson et al., J. Immunol. 154(7):3310-9 (1995); and Hawkins et al., J. Mol. Biol. 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a beta-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region," "HVR," or "HV," when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. For example, the term hypervariable region refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, e.g., Xu et al., *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, N.J., 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, e.g., Hamers-Casterman et al., *Nature* 363:446-448 (1993); Sheriff et al., *Nature Struct. Biol.* 3:733-736 (1996).

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ϵ, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and Mol. Immunology, 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The term "Fc region" is used to define the C-terminal region of an immunoglobulin heavy chain which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain.

By "Fc region chain" herein is meant one of the two polypeptide chains of an Fc region.

The "CH2 domain" of a human IgG Fc region (also referred to as "Cg2" domain) is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, *Molec. Immunol.* 22:161-206 (1985). The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain.

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region. The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced "protroberance" in one chain thereof and a corresponding introduced "cavity" in the other chain thereof; see U.S. Pat. No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to make multispecific (e.g. bispecific) antibodies as herein described.

"Hinge region" herein may be a native sequence hinge region or a variant hinge region. The two polypeptide chains of a variant hinge region generally retain at least one cysteine residue per polypeptide chain, so that the two polypeptide chains of the variant hinge region can form a

disulfide bond between the two chains. The preferred hinge region herein is a native sequence human hinge region, e.g. a native sequence human IgG1 hinge region.

A "functional Fc region" possesses at least one "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

An "intact" antibody is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2 and C_H3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

A "parent antibody" or "wild-type" antibody is an antibody comprising an amino acid sequence which lacks one or more amino acid sequence alterations compared to an antibody variant as herein disclosed. Thus, the parent antibody generally has at least one hypervariable region which differs in amino acid sequence from the amino acid sequence of the corresponding hypervariable region of an antibody variant as herein disclosed. The parent polypeptide may comprise a native sequence (i.e. a naturally occurring) antibody (including a naturally occurring allelic variant), or an antibody with pre-existing amino acid sequence modifications (such as insertions, deletions and/or other alterations) of a naturally occurring sequence. Throughout the disclosure, "wild type," "WT," "wt," and "parent" or "parental" antibody are used interchangeably.

As used herein, "antibody variant" or "variant antibody" refers to an antibody which has an amino acid sequence which differs from the amino acid sequence of a parent antibody. Preferably, the antibody variant comprises a heavy chain variable domain or a light chain variable domain having an amino acid sequence which is not found in nature. Such variants necessarily have less than 100% sequence identity or similarity with the parent antibody. In a preferred embodiment, the antibody variant will have an amino acid sequence from about 75% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the parent antibody, more preferably from about 80% to less than 100%, more preferably from about 85% to less than 100%, more preferably from about 90% to less than 100%, and most preferably from about 95% to less than 100%. The antibody variant is generally one which comprises one or more amino acid alterations in or adjacent to one or more hypervariable regions thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification. In certain embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will typically possess, e.g., at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, or at least about 90% sequence identity therewith, or at least about 95% sequence or more identity therewith.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS* (*USA*) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. In certain embodiments, the cells express at least FcγRIII and perform ADCC effector function(s). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being generally preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cyto-

plasmic domain. (see, e.g., Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein.

The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward., *Immunol. Today* 18(12):592-598 (1997); Ghetie et al., *Nature Biotechnology,* 15(7):637-640 (1997); Hinton et al., *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).

Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, e.g., Shields et al. *J. Biol. Chem.* 9(2):6591-6604 (2001).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass), which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed. Polypeptide variants with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased C1q binding capability are described, e.g., in U.S. Pat. No. 6,194,551 B1 and WO 1999/51642. See also, e.g., Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al, *J. Mol. Biol.* 226:889-896 (1992).

The term "therapeutic antibody" refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell's death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A "biologically functional fragment" of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

Therapeutic antibodies of particular interest include those in clinical oncological practice or development such as commercially available AVASTIN® (bevacizumab), HERCEPTIN® (trastuzumab), LUCENTIS® (ranibizumab), RAPTIVA® (efalizumab), RITUXAN® (rituximab), and XOLAIR® (omalizumab), as well as, anti-amyloid beta

(Abeta), anti-CD4 (MTRX1011A), anti-EGFL7 (EGF-like-domain 7), anti-IL13, Apomab (anti-DR5-targeted pro-apoptotic receptor agonist (PARA), anti-BR3 (CD268, BLyS receptor 3, BAFF-R, BAFF Receptor), anti-beta 7 integrin subunit, dacetuzumab (Anti-CD40), GA101 (anti-CD20 monoclonal antibody), MetMAb (anti-MET receptor tyrosine kinase), anti-neuropilin-1 (NRP1), ocrelizumab (anti-CD20 antibody), anti-OX40 ligand, anti-oxidized LDL (ox-LDL), pertuzumab (HER dimerization inhibitors (HDIs), and. rhuMAb IFN alpha.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The term "diagnostic protein" refers to a protein that is used in the diagnosis of a disease.

The term "diagnostic antibody" refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A "biologically functional fragment" of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

"Purified" means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained. The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An "essentially pure" protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An "essentially homogeneous" protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

As used herein, "soluble" refers to polypeptides that, when in aqueous solutions, are completely dissolved, resulting in a clear to slightly opalescent solution with no visible particulates, as assessed by visual inspection. A further assay of the turbidity of the solution (or solubility of the protein) may be made by measuring UV absorbances at 340 nm to 360 nm with a 1 cm path-length cell where turbidity at 20 mg/ml is less than 0.05 absorbance units.

An "isolated" antibody or polypeptide is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "Protein A" and "ProA" are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, Pharmacia and Fermatech. Protein A is generally immobilized on a solid phase support material. The term "ProA" also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term "chromatography" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term "affinity chromatography" and "protein affinity chromatography" are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms "non-affinity chromatography" and "non-affinity purification" refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely

on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia). A "mixed mode ion exchange resin" refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J. T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

The "intermediate buffer" is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled

in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

B. Exemplary Methods and Materials for Carrying Out the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); PCR: *A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells,* 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunochemistry in Practice,* 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering,* 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology.*

1. Recombinant Production of Proteins in Mammalian Host Cells Using a Glutamine Free Cell Culture Medium

The present invention concerns the large-scale recombinant production of proteins in mammalian host cells, using a glutamine-free cell culture medium supplemented with asparagine. Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells have been approved by regulatory agencies for the production of

biopharmaceutical products, including therapeutic antibodies. Of these, Chinese Hamster Ovary Cells (CHO) are among the most commonly used industrial hosts, which are widely employed for the production of heterologous proteins. Thus, methods for the large-scale production of antibodies in CHO, including dihydrofolate reductase negative (DHFR−) CHO cells, are well known in the art (see, e.g. Trill et al., *Curr. Opin. Biotechnol.* 6(5):553-60 (1995) and U.S. Pat. No. 6,610,516).

As a first step, the nucleic acid (e.g., cDNA or genomic DNA) encoding the desired recombinant protein may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below. Optional signal sequences, origins of replication, marker genes, enhancer elements and transcription terminator sequences that may be employed are known in the art and described in further detail in PCT Publication WO 97/25428.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the protein-encoding nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the desired protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in PCT Publication No. WO97/25428.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* cells, such as *E. coli* K12 strain 294 (ATCC® 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced using standard techniques known in the art. (See, e.g., Messing et al., *Nucleic Acids Res.* 1981, 9:309; Maxam et al., Methods in Enzymology 1980, 65:499).

Expression vectors that provide for the transient expression in mammalian cells may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector (Sambrook et al., supra). Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of a desired heterologous protein in recombinant vertebrate cell culture are described in Gething et al., *Nature* 1981, 293:620-625; Mantei et al., *Nature* 1979, 281:40-46; EP 117,060; and EP 117,058.

For large-scale production, according to the present invention mammalian host cells are transfected and preferably transformed with the above-described expression vectors and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described (Shaw et al., Gene 1983, 23:315 and PCT Publication No. WO 89/05859). In addition, plants may be transfected using ultrasound treatment, PCT Publication No. WO 91/00358 published 10 Jan. 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method (Graham and van der Eb, *Virology* 1978, 52:456-457) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. For various techniques for transforming mammalian cells, see also Keown et al. *Methods in Enzymology* 1990, 185:527-537 and Mansour et al. *Nature* 1988, 336:348-352.

During large-scale production, to begin the production cycle usually a small number of transformed recombinant host cells is allowed to grow in culture for several days. Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks to begin the production phase, and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Harvesting usually includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the

purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, there is room fur further improvements in the large-scale commercial production of recombinant proteins, such as antibodies. Thus, increases in cell viability, longevity and specific productivity of mammalian host cell cultures, and improvements in the titer of the recombinant proteins produced have a genuine impact on the price of the recombinant protein produced, and, in the case of therapeutic proteins, the price and availability of drug products.

The present invention concerns an improved method for the production of heterologous proteins in mammalian cell culture, using a glutamine-free culture medium with added asparagine in the production phase of the cell culture process. The culture media used in the process of the present invention can be based on any commercially available medium for recombinant production of proteins in mammalian host cells, in particular CHO cells.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. In addition, the culture media of the present invention can be based any of the media described in Ham and McKeehan, Meth. Enz., 58: 44 (1979); Barnes and Sato, Anal. Biochem., 102: 255 (1980); U.S. Pat. No. 4,767,704; U.S. Pat. No. 4,657,866; U.S. Pat. No. 4,927,762; U.S. Pat. No. 5,122,469 or U.S. Pat. No. 4,560,655; WO 90/03430; and WO 87/00195, provided that glutamine is omitted as an ingredient.

Under Glutamine-free conditions Asparagine is required since mammalian cells can synthesize Asparagine only in presence of Glutamine. Asparagine is synthesized by amide transfer from Glutamine in the presence of Asparagine synthetase. The Asparagine is preferably added to the culture medium at a concentration in the range of 2.5 mM to 15 mM. In various embodiments of the present invention, the preferred concentration of Asparagine should be at least 2.5 mM. In preferred embodiments, the asparagine is added at a concentration of 10 mM.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in, and can be adapted for the production of recombinant proteins using the cell culture media herein.

The necessary nutrients and growth factors for the medium, including their concentrations, for a particular cell line, are determined empirically without undue experimentation as described, for example, in *Mammalian Cell Culture*, Mather, ed. (Plenum Press: NY, 1984); Barnes and Sato, *Cell,* 22: 649 (1980) or *Mammalian Cell Biotechnology: A Practical Approach*, M. Butler, ed. (IRL Press, 1991). A suitable medium contains a basal medium component such as a DMEM/HAM F-12-based formulation (for composition of DMEM and HAM F12 media and especially serum-free media, see culture media formulations in American Type Culture Collection Catalogue of Cell Lines and Hybridomas, Sixth Edition, 1988, pages 346-349), with modified concentrations of some components such as amino acids, salts, sugar, and vitamins, and optionally containing glycine, hypoxanthine, and thymidine; recombinant human insulin, hydrolyzed peptone, such as PRIMATONE HS™ or PRIMATONE RL™ (Sheffield, England), or the equivalent; a cell protective agent, such as PLURONIC F68™ or the equivalent pluronic polyol; GENTAMYCIN™; and trace elements. The formulations of medium as described in U.S. Pat. No. 5,122,469, characterized by the presence of high levels of certain amino acids, as well as PS-20 as described below, are particularly appropriate.

The glycoproteins of the present invention may be produced by growing cells which express the desired glycoprotein under a variety of cell culture conditions. For instance, cell culture procedures for the large- or small-scale production of glycoproteins are potentially useful within the context of the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, in the later two systems, with or without microcarriers, and operated alternatively in a batch, fed-batch, or continuous mode.

In a particular embodiment the cell culture of the present invention is performed in a stirred tank bioreactor system and a fed-batch culture procedure is employed. In the preferred fed-batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed-batch culture can include, for example, a semi-continuous fed-batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium Fed-batch culture is distinguished from simple-batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed-batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers, etc., and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single-step or multiple-step culture procedure. In a single-step culture the host cells are inoculated into a culture environment and the processes of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a specific aspect of the invention, fed-batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (DO_2), and the like, are those used with the particular host and will be apparent to the ordinarily-skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 40° C. and preferably about 37° C. and a suitable DO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

Production of a target protein in mammalian, e.g., CHO, cells typically employs a semi-continuous process whereby cells are culture in a "seed-train" for various periods of time and are periodically transferred to inoculum fermentors to generate enough cell mass to inoculate a production fermentor at larger scale. Thus, cells used for the production of the desired protein are in culture for various periods of time up to a maximum predefined cell age. The parameters of the cell culture process, such as seed density, pH, DO_2 and temperature during culture, duration of the production culture, operating conditions of harvest, etc. are a function of the particular cell line and culture medium used, and can be determined empirically, without undue experimentation.

According to the present invention, the cell-culture environment during the production phase of the cell culture is controlled. In a preferred aspect, the production phase of the cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged.

The desired polypeptide, such as antibody, preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the polypeptide is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g., Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When the polypeptide is produced in a recombinant cell other than one of human origin, it is free of proteins or polypeptides of human origin. However, it is usually necessary to recover or purify recombinant proteins from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the desired polypeptide. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. The heterologous polypeptide thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column such as SP-Sepharose™ or CM-Sepharose™; hydroxyapatite; hydrophobic interaction chromatography; ethanol precipitation; chromatofocusing; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G75™; and/or diafiltration.

Recombinant polypeptides can be isolated, e.g. by affinity chromatography.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for the purification and isolation of recombinant proteins, including antibodies, can be used herein, and modified if needed, using standard techniques.

Expression of the desired heterologous protein may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA* 1980, 77:5201-5205), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal.

2. Antibodies

In a preferred embodiment, the methods of the present invention are used for the recombinant production of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25,

E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_4\beta_7$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al., *Cancer Res* 55(23 Suppl):5899s-5907s (1995)); anti-Ep-CAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2):435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab. sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-1 6, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2):435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); W02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171,586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677,180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224,866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albitar et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegall); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821

(Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332,865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1) (part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" New Scientist (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): S370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.* 30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): 5197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002; Tuscano, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-CD20 antibodies. In certain embodiments, the humanized antibody composition of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized antibody compositions of the present invention include compositions of any of the preceding humanized antibodies having an Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to Fc(RIIIA (F158), which is not as effective as Fc(RIIIA (V158) in interacting with human IgG. Fc(RIIIA (F158) is more common than Fc(RIIIA (V158) in normal, healthy African Americans and Caucasians. See Lehrnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J. Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*" in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the antigens are selected from the group consisting of CD-20, CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-HER2 antibodies. HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS (USA)* 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998) are FDA approved for the treatment of cancer. In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-VEGF antibodies.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva® (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for pharmaceutical compositions comprising anti-human CD11a antibodies.

Apomab Antibodies

Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference. Apomab is a fully human monoclonal antibody which is a DR5-targeted pro-apoptotic receptor agonist (PARA) specifically designed to induce apoptosis. Apoptosis is a natural process by which damaged or unwanted cells, including those that are cancerous, die and are cleared from the body. Pro-apoptotic receptor DR5 is expressed in a broad range of malignancies.

Anti-BR3 Antibodies and Immunoadhesins

Antibodies to the BR3 (anti-BR3) antibodies and BR3-Fc immunoadhesins can also be produced in accordance with the present invention. Such anti-BR3 antibodies and immunoadhesins specifically include all variants disclosed in U.S. Application Publication No. 20050070689. The entire content of U.S. Application Publication No. 20050070689 is hereby expressly incorporated by reference.

3. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically identified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and αvγ/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the

selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of

producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be

"linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG,

IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L-AC_L;

AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

$(A\text{-}Y)_n$-$(V_LC_L\text{-}V_HC_H)_2$;

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., Cell 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

Further details of the invention are provided in the following non-limiting Examples.

All patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties.

EXAMPLES

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples,

and throughout the specification, by ATCC® accession numbers is the American Type Culture Collection, Manassas, Va.

Example 1

Production of Polypeptides in Glutamine-Free Production Medium

Materials and Methods:

Cell Lines.

In these studies, CHO host cells expressing an Apomab antibody, anti-VEGF antibody, and the fusion protein BR3-Fc, respectively were used. The host cells were adapted in suspension and serum free cultures. Frozen stocks were prepared as master or working cell banks in the media described below.

Cell line maintenance was carried out using a 250-mL or 1-Liter Corning® vented shake flasks maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37° C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform. Cell cultures were passed every 3 or 4 days with fresh media and seeded at 0.11% or 0.20% Packed Cell Volume (PCV). PCV was obtained using a glass10-mL KIMAX® USA PCV tube.

Culture Media and Conditions.

Media studies were initiated using 250-mL Corning vented shake flask inoculated in singlet, duplicate, or triplicate at 100 mL working volume at 0.20% PCV for all cases using cell culture from a source1-Liter Corning® vented shake flask with a 500-mL working volume. PCV was obtained using a glass10-mL KIMAX® USA PCV tube.

Prior to initiation of the study cell culture was centrifuged at 1000 rpm for 5-minutes in a Sorvall® RT 6000B centrifuge to complete a 100% media exchange of inoculum media containing glutamine with the respective test media. Different concentrations of Glutamine, Glutamate, Asparagine and Aspartate were evaluated in the different test media. The following concentrations were tested: Glutamine 0-10 mM, Glutamate 1-10 mM, Asparagine 0-15 mM, Aspartate 1-10 mM. Media conditions were evaluated in full factorial DOE studies.

The effect of Glutamine-free medium on was also tested in commercially available DMEM/F12 medium. The medium was used at 5× concentration (7.05 g/L) with extra Asparagine (10 mM total), Aspartate (10 mM total), Glutamine (10 mM total for the Glutamine-containing medium), Glutamate (1 mM total), and glucose (8 g/L total). Glutamine-free and Glutamine-containing medium were compared using Apomab and anti-VEGF antibody expressing cells.

Shake flasks were maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37® C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform.

The medium used contained the following components:

Organic salts and Trace Elements
Ammonium Paramolybdate, Tetrahydrate
Ammonium Vanadium Oxide
Calcium Chloride, Anhydrous
Cupric Sulfate, Pentahydrate
Ferrous Sulfate, Heptahydrate
Potassium Chloride
Magnesium Chloride, Anhydrous
Manganese Sulfate, Monohydrate
Nickel Chloride, Hexahydrate
Selenious Acid
Sodium Metasilicate, Nonahydrate
Sodium Phosphate, Monobasic, Monohydrate
Stannous Chloride, Dihydrate
Zinc Sulfate, Heptahydrate
Lipids
Linoleic Acid
Lipoic Acid (aka Thioctic Acid)
Putrescine, Dihydrochloride
Amino Acids
L-Alanine
L-Arginine, Monohydrochloride
L-Asparagine
L-Aspartic Acid
L-Cysteine, Monohydrochloride, Monohydrate
L-Glutamic Acid
L-Glutamine
L-Histidine, Monohydrochloride, Monohydrate
L-Isoleucine
L-Leucine
L-Lysine, Monohydrochloride
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine, Disodium Salt, Dihydrate
L-Valine
Vitamins
Biotin
D-Calcium Pantothenate
Choline Chloride
Folic Acid
I-Inositol
Niacinamide
Pyridoxine, Monohydrochloride
Riboflavin
Thiamine, Monohydrochloride
Vitamin B-12
Carbon Source, Growth Factors, and Miscelaneous
Fluronic F-68
D-Glucose
Sodium Bicarbonate
Sodium Pyruvate
Sodium Chloride
Sodium Hydroxide
Insulin
Galactose

The commercially-available DMEM/F-12 culture medium was also tested, having the following components;

	(mg/L)
VITAMINS	
Biotin	0.00365
D-calcium pantothenate	2.24
Choline chloride	8.98
Cyanocobalamin	0.68
Folic acid	2.65
i-inositol	12.6
Niacinamide	2.0185
Pyridoxal HCl	2
Pyridoxine HCI	0.031
Riboflavin	0.219
Thiamine HCl	2.17

-continued

	(mg/L)
AMINO ACIDS	
L-alanine	4.455
L-arginine HCl	147.5
L-asparagine monohydrate	7.5
L-aspartic acid	6.65
L-cysteine HCl monohydrate	17.56
L-cystine 2HCl	31.29
L-glutamic acid	7.35
L-glutamine	365
Glycine	18.75
L-histidine HCl monohydrate	31.48
L-isoleucine	54.47
L-leucine	59.05
L-lysine HCl	91.25
L-methionine	17.24
L-phenylalanine	35.48
L-proline	17.25
L-serine	26.25
L-threonine	53.45
L-tryptophan	9.02
L-tyrosine 2Na dihydrate	55.79
L-valine	52.85
OTHER	
Dextrose anhydrous	3151
HEPES	3575
Hypoxanthine sodium salt	2.39
Linoleic acid	0.042
DL-α-Lipoic acid	0.105
Phenol red sodium salt	8.602
Putrescine 2HCl	0.081
Sodium pyruvate	55
Thymidine	0.365
ADD: Sodium bicarbonate	1200
INORGANIC SALTS	
Calcium chloride anhydrous	116.61
Cupric sulfate pentahydrate	0.00125
Ferric nitrate nonahydrate	0.05
Ferrous sulfate heptahydrate	0.417
Magnesium chloride anhydrous	28.61
Magnesium sulfate anhydrous	48.84
Potassium chloride	311.8
Sodium chloride	6999.5
Sodium phosphate dibasic anhydrous	71.02
Sodium phosphate monobasic monohydrate	62.5
Zinc sulfate heptahydrate	0.4315

The medium for inoculum culture (as opposed for the production phase) was usually supplemented with 5 mM glutamine, 8 g/L glucose, and 75-2000 nM Methotroxate.

For studies pH adjustment was performed as needed to maintain pH value at 7.00±0.10 using 1M Sodium Carbonate. Adjustment in pH value was made in by adding 1 mL/L of 1M Sodium Carbonate to raise pH units up 0.10.

Cell culture was analyzed up to 14-days by taking a 3.5-mL sample and analyzed for viable cell count, viability, and cell size using a Beckman Coulter ViCell™-1.0 cell counter. Nutrient analysis was performed using the Nova 400 Biomedical Bioprofile®. Osmolality was measured using an Advanced® Instrument multi-sample Osmometer (Model 3900). Recombinant product titer concentration was obtained using the Agilent 1100 Series HPLC.

Recombinant Proteins.

The recombinant proteins produced were Apomab (TRAIL), anti-VEGF, and the immunoadhesin BR3-Fc.

Data Analysis.

Statistical analyses of the data were carried out using a full factorial design of experiment, which is an experiment whose design consists of two or more factors, each with discrete possible values or "levels", and whose experimental units take on all possible combinations of these levels across all such factors. A full factorial design may also be called a fully-crossed design. Such an experiment allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable.

Results

As shown in FIGS. **1-5**, use of a glutamine-free production medium increased the final recombinant protein titer of Apomab antibody, BR3-Fc immunoadhesin and anti-VEGF antibody. In each case, cube plot analysis of titer results using Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate predict that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. (FIGS. **1-3**)

The effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer is shown in FIG. **4**. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free and low Glutamate conditions is illustrated in FIG. **5**. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

The effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer is demonstrated in FIGS. **6** A-C, wherein the final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

Similar results were obtained using the commercial DMEM/F-12 culture medium. As shown in FIGS. **7** A and B, the final titer for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

As shown in FIGS. **8** and **9**, use of a glutamine-free production medium also increased specific production measured as Qp (mg/mL-cell/day). FIGS. **8** A-C illustrate that cell specific productivity (Qp) for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was significantly higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing medium. FIGS. **9** A and B illustrate that cell specific productivity for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

As shown in FIGS. **10** and **11**, use of a glutamine-free production medium was shown to improve cell viability and extend culture longevity significantly. FIGS. **10** A-C. illustrate that cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic

Acid compared to Glutamine-containing medium. FIGS. **11** A and B indicate that, in DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. Of note, viability was higher for Apomab antibody (FIG. **11** A), but lower for anti-VEGF antibody (FIG. **11** B) compared to Glutamine containing medium.

As shown in FIGS. **12** and **13**, use of a glutamine-free production medium reduced NH_4^+ accumulation significantly compared to glutamine-containing medium. FIGS. **12** A-D illustrate that ammonia levels were usually lower in Glutamine-free cultures supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing cultures. FIGS. **13** A and B illustrate that ammonia levels were significantly reduced in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention. Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

What is claimed is:

1. A process for producing a polypeptide in a host cell expressing said polypeptide, comprising culturing the host cell in a production phase of the culture in a glutamine-free production culture medium containing asparagine and aspartic acid, wherein the asparagine is added at a concentration in the range of 7.5 mM to 15 mM and wherein the aspartic acid is added at a concentration in the range of 1 mM to 10 mM.

2. The process of claim **1** further comprising the step of isolating said polypeptide.

3. The process of claim **2** further comprising determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

4. The process of claim **3** wherein at least one of the cell viability, culture longevity, specific productivity and final recombinant protein titer is increased relative to the cell viability, culture longevity, specific productivity and final recombinant protein titer in a glutamine-containing production medium of the same composition.

5. The process of claim **1** wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

6. The process of claim **1** wherein the asparagine is added at a concentration of 10 mM.

7. The process of claim **1** wherein the production medium is serum-free.

8. The process of claim **1** wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

9. The process of claim **8** wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

10. The process of claim **1** wherein the production phase is a batch or fed batch culture phase.

11. The process of claim **10**, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

12. The process of claim **11**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

13. The process of **11**, wherein the asparagine is added at a concentration of 10 mM.

14. The process of claim **11**, wherein the aspartic acid is added at a concentration of 10 mM.

15. The process of claim **11**, wherein the production medium is serum-free.

16. The process of claim **10**, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

17. The process of claim **16**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

18. The process of claim **16**, wherein the asparagine is added at a concentration of 10 mM.

19. The process of claim **16**, wherein the aspartic acid is added at a concentration of 10 mM.

20. The process of claim **16**, wherein the production medium is serum-free.

21. The process of claim 1 wherein said host cell is an eukaryotic host cell.

22. The process of claim 21 wherein said eukaryotic host cell is a mammalian host cell.

23. The process of claim 22, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

24. The process of claim 22, wherein the asparagine is added at a concentration of 10 mM.

25. The process of claim 22, wherein the aspartic acid is added at a concentration of 10 mM.

26. The process of claim 22, wherein the production medium is serum-free.

27. The process of claim 22, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

28. The process of claim 27, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

29. The process of claim 27, wherein the asparagine is added at a concentration of 10 mM.

30. The process of claim 27, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

31. The process of claim 22, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

32. The process of claim 31, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

33. The process of claim 31, wherein the asparagine is added at a concentration of 10 mM.

34. The process of claim 31, wherein the aspartic acid is added at a concentration of 10 mM.

35. The process of claim 31, wherein the production medium is serum-free.

36. The process of claim 22, wherein the production phase is a batch or fed batch culture phase.

37. The process of claim 36, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

38. The process of claim 36, wherein the asparagine is added at a concentration of 10 mM.

39. The process of claim 36, wherein the aspartic acid is added at a concentration of 10 mM.

40. The process of claim 36, wherein the production medium is serum-free.

41. The process of claim 22 wherein said mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

42. The process of claim 41 wherein the mammalian host cell is a dhfr− CHO cell.

43. The process of claim 41, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

44. The process of claim 41, wherein the asparagine is added at a concentration of 10 mM.

45. The process of claim 41, wherein the aspartic acid is added at a concentration of 10 mM.

46. The process of claim 41, wherein the production medium is serum-free.

47. The process of claim 41, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

48. The process of claim 47, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

49. The process of claim 47, wherein the asparagine is added at a concentration of 10 mM.

50. The process of claim 47, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

51. The process of claim 41, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

52. The process of claim 51, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

53. The process of claim 51, wherein the asparagine is added at a concentration of 10 mM.

54. The process of claim 51, wherein the aspartic acid is added at a concentration of 10 mM.

55. The process of claim 51, wherein the production medium is serum-free.

56. The process of claim 41, wherein the production phase is a batch or fed batch culture phase.

57. The process of claim 56, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

58. The process of claim 56, wherein the asparagine is added at a concentration of 10 mM.

59. The process of claim 56, wherein the aspartic acid is added at a concentration of 10 mM.

60. The process of claim 56, wherein the production medium is serum-free.

61. The process of claim 56, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

62. The process of claim 61, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

63. The process of claim 61, wherein the asparagine is added at a concentration of 10 mM.

64. The process of claim 61, wherein the aspartic acid is added at a concentration of 10 mM.

65. The process of claim 1 wherein the polypeptide is a mammalian glycoprotein.

66. The process of claim 1 wherein the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

67. The process of claim 66 wherein said antibody fragment is selected from the group consisting of Fab, Fab', F(ab')2, scFv, (scFv)2, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

68. The process of claim **66** wherein the antibody or antibody fragment is chimeric, humanized or human.

69. The process of claim **66** wherein said antibody or antibody fragment is a therapeutic antibody or a biologically functional fragment thereof.

70. The process of claim **69** wherein said therapeutic antibody is selected from the group consisting of anti-HER2 antibodies; anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies; anti-CD 11 a antibodies; anti-CD 18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human α4β7 integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-αvβ3 antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

71. The process of claim **69** wherein said therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, BR3 or DR5.

72. The process of claim **71**, wherein the therapeutic antibody is selected from the group consisting of bevacizumab, rituximab, and trastuzumab.

73. The process of claim **72**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

74. The process of claim **72**, wherein the asparagine is added at a concentration of 10 mM.

75. The process of claim **72**, wherein the aspartic acid is added at a concentration of 10 mM.

76. The process of claim **72**, wherein the production medium is serum-free.

77. The process of claim **1** wherein said polypeptide is a therapeutic polypeptide.

78. The process of claim **77** wherein said therapeutic polypeptide is selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

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CERTIFICATE OF SERVICE

The undersigned counsel hereby certifies that true and correct copies of the foregoing document were caused to be served on July 19, 2018 on the following counsel via email:

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