INFECTION AND IMMUNITY, Oct. 1991, p. 3708–3714
0019-9567/91/103708-07$02.00/0

Vol. 59, No. 10

Efficacy of Enteric-Coated Protease in Preventing Attachment of Enterotoxigenic *Escherichia coli* and Diarrheal Disease in the RITARD Model

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Received 26 April 1991/Accepted 1 July 1991

In this study, we report on a novel approach based on modification of the intestinal surface to prevent diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC). The removable intestinal tie adult rabbit diarrhea (RITARD) model was used to test the efficacy of an enteric-coated protease preparation (Detach; Enzacor Technology Pty. Ltd.) in the prevention of bacterial attachment and diarrheal disease caused by colonization factor antigen I-positive (CFA/I$^+$) *E. coli* H10407. Protease was administered orally to rabbits 18 h prior to challenge with 10^{11} bacteria. Four groups of rabbits were inoculated with different ETEC strains which produced different combinations of adhesin and enterotoxin or with sterile phosphate-buffered saline. Occurrence of diarrhea during the subsequent 24-h incubation period was recorded. Oral administration of protease was successful in reducing diarrhea and diarrhea-induced death in six of seven (86%) rabbits infected with CFA/I$^+$, heat-stable and heat-labile toxin-positive *E. coli* (H10407). Seven of eight (87%) rabbits not protected by protease treatment died or developed severe diarrhea. Quantitative analysis of bacterial cultures obtained from the small intestine of rabbits showed a significant ($P < 0.001$) 2,000-fold reduction in CFU per centimeter of intestine following treatment with protease. The efficacy of protease treatment was 99.5%, with very wide confidence limits (>0 to 99.9%). The data indicate that the use of protease to prevent ETEC diarrheal disease has considerable potential.

The role of enterotoxigenic *Escherichia coli* (ETEC) as an important etiologic agent in human diarrheal disease is well established (31). These organisms are characterized by their ability to produce heat-labile toxin (LT) and/or heat-stable toxin (ST) (11). Some strains also produce pilus adhesins called colonization factor antigens (CFAs). These adhesins permit attachment of ETEC strains to the intestinal mucosa, thereby facilitating colonization and delivery of enterotoxin to target epithelial cells.

CFAs may be of use as vaccine candidates and for this reason have attracted considerable attention. Those identified to date include CFA/I and CFA/III and two multivariant antigen groups known as CFA/II and CFA/IV. Each of the last two possesses a combination of three pilus adhesins, designated coli surface antigens (CS). CS1, CS2, and CS3 are present on CFA/II *E. coli*, and CS4, CS5, and CS6 are present on CFA/IV *E. coli* (7, 16, 21, 28). Other putative colonization factors have also been described previously (5, 14, 34, 36). As colonization factors are antigenically distinct, potential vaccines must be multivalent. A prototype ETEC vaccine that contains known CFAs and outer membrane antigens associated with ETEC is being developed (32).

Significant protection against diarrhea caused by ETEC following vaccination with a combination of formalin and heat-inactivated whole *Vibrio cholerae* cells and the purified B subunit of cholera toxin has been reported recently (4). *V. cholerae* and ETEC produce heat-labile enterotoxins which are structurally, functionally, and immunologically similar and consist of a combination of a biologically active A subunit and five B subunits (12, 15, 22).

Other efforts to develop vaccines against ETEC infection have given variable results (8, 18, 19), and while the prospect of effective immunization against diarrheal diseases of humans is appealing, effective vaccines are not expected to be available in the near future (16a). Alternative approaches to the prevention of ETEC diarrhea are therefore being pursued.

Studies with pig ETEC that possess the K88 adhesin have indicated that the interaction between ETEC and pig brush border membranes can be modified by the use of proteases (27). Preliminary challenge experiments and field trials (2a) have indicated that an enterically protected protease preparation (Detach; Enzacor Technology Pty. Ltd., Melbourne, Australia) administered orally is able to modify the mucosal surface of the piglet small intestine such that diarrhea is less likely to occur.

A number of studies have described similarities in mechanisms of pathogenesis of ETEC infection in humans and animals (11, 31). This has led us to investigate the use of protease in the prevention of human diarrheal diseases. Recent experiments (23a) have indicated that binding of CFA/I and CFA/II to human intestinal mucosa can be prevented in vitro by the use of protease. An effect of protease in reducing the binding of LT was also demonstrated. It appears that modification of intestinal mucosa to prevent human diarrheal diseases has considerable potential. This report describes an experiment in which the removable intestinal tie adult rabbit diarrhea (RITARD) model described by Spira et al. (29) was used to test the efficacy of exogenous protease (Detach) in reducing attachment of CFA/I-positive *E. coli* to rabbit intestinal mucosa in vivo. The work represents a novel approach to preventing attachment of ETEC to intestinal cells and therefore preventing diarrheal disease.

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MATERIALS AND METHODS

Animals. Forty-four New Zealand White breed rabbits of both sexes from a single breeder were used for the experiment. Their weights ranged from 1.5 to 2.7 kg. Animals were acclimatized to their animal housing facility for at least 1 week prior to the start of experimentation.

Bacteria. ETEC strains used in this study were originally isolated in Bangladesh from patients with diarrhea. Strain H10407 (serotype O78:K80:H11) and a mutant derivative of this strain, H10407-P, were kindly provided by D. G. Evans (The University of Texas Medical School at Houston, Houston). Strain E1392/75 7A (serotype O6:K15:H16) was kindly provided by B. Rowe (Division of Enteric Pathogens, London, United Kingdom). Strain H10407 produces both ST and LT and possesses CFA/I. Strain H10407-P produces both ST and LT but does not produce CFA/I (7). Strain E1392/75 7A is a CFA-negative, nontoxigenic spontaneous laboratory derivative of CFA/II$^+$ *E. coli* 1392 (17) and has been shown not to colonize or induce diarrhea in the RITARD model (35).

Stock cultures of all strains were suspended in Trypticase soy broth (Oxoid) containing 15% (vol/vol) glycerol and stored in multiple aliquots at −80°C. A new aliquot was used for each experiment. Bacteria were inoculated onto CFA agar (6) and grown at 37°C overnight. Cultures were harvested by means of a flamed Pasteur pipette, washed in sterile phosphate-buffered saline (0.01 M, pH 7.2; PBS), and diluted to yield the desired optical density measurements. The bacterial concentration was confirmed by viable cell count on duplicate blood agar plates after serial dilution in PBS. Prior to the inoculation of rabbits, all cultures were checked for the presence of CFA/I and LT by a specific enzyme immunoassay (EIA).

Antigens. CFA/I was purified as previously described (6). Purified LT (unnicked) was kindly provided by J. D. Clements (Walter Reed Army Institute of Research, Washington, D.C.).

Antisera. Specific CFA/I antiserum was produced by giving rabbits three subcutaneous injections with 60 μg of purified CFA/I at intervals of 4 weeks. For the first immunization, the antigen was emulsified with Freund's complete adjuvant. Subsequent immunizations were emulsified with Freund's incomplete adjuvant. The animals were bled by the ear vein 2 weeks after the final immunization. LT-specific antiserum was similarly produced by injecting 60 μg of purified LT. The immunoglobulin G fraction was prepared by protein A affinity chromatography with protein A–Sepharose CL-4B (Pharmacia) as specified by the manufacturer.

Detection of CFA/I and LT by EIA. Disposable polystyrene microtiter plates (Nunc, Roskilde, Denmark) were used for all assays. CFA/I- or LT-specific immunoglobulin G was diluted in sodium bicarbonate buffer (0.1 M, pH 9.6) and adsorbed to wells (100 μl per well) by incubation overnight at 4°C. For CFA/I detection, bacteria were diluted to approximately 2.5×10^9 cells per ml ($A_{625} = 1.0$) in working dilution buffer containing PBS (0.01 M, pH 7.2), bovine serum albumin (0.25%; Fraction V; Sigma), Tween 20 (0.05%; Sigma), disodium salt EDTA (0.0075%), and sodium azide (0.02%). For LT detection, supernatant material was obtained following centrifugation (at 12,000 × *g* for 15 min) of overnight cultures of bacteria grown in CYE medium (9).

The bacterial suspension or culture supernatant was inoculated into duplicate wells (100 μl per well). Bound material was detected with urease-conjugated (CSL, Melbourne, Australia) specific immunoglobulin G diluted in working dilution buffer containing 1% hen egg albumin (Fraction II; Sigma). Urea substrate (0.008% bromocresol purple [Sigma], 0.1% urea [Bio-Rad], 0.0075% disodium salt EDTA; pH 4.8, 100 μl) was used, and a positive reaction was indicated by a color change from yellow to purple. During each of the above steps, plates were incubated at 37°C for 30 min. Between each of the steps, supernatant liquid was removed from the wells, which were then washed with washing buffer (0.01 M PBS, 0.05% Tween 20). Prior to the addition of substrate, wells were washed with distilled water to remove residual buffer. Development of purple color was monitored at A_{540}.

RITARD model procedure. The RITARD model developed by Spira et al. (29) was used with slight modifications. At 18 h prior to challenge, half of the rabbits from each group (see Table 1) were given a single oral dose of 0.42 g of enteric-coated protease granules (Detach containing 25% protease; Enzacor Technology Pty. Ltd.). The protease granules were placed in gelatin capsules and administered by placing the capsules at the back of the throat. Food was withheld after dosing, but water was made available ad libitum. Before surgery, animals were anesthetized with 16 mg of xylazine (Rompun; Bayer) and 100 mg of ketamine (Ketapex; Apex Laboratories Pty. Ltd) intramuscularly. The incision site was anesthetized with 2 ml of lignocaine with adrenalin (20 mg of lignocaine hydrochloride per ml, 0.01 mg of adrenalin [as bitartrate] [Apex Laboratories Pty. Ltd] per ml). The cecum was exteriorized through a midline incision and ligated permanently as close to the ileo-cecal junction as possible with no. 11 umbilical tape (Ethicon). At this time the ileum was temporarily obstructed approximately 10 cm proximal to the cecal tie with a slipknot tie with umbilical tape. Inoculum (10 ml) containing 10^{11} bacteria diluted in sterile PBS or sterile PBS alone was injected into the duodenum. The intestine was returned to the peritoneal cavity, which was sutured with one end of the temporary tie left accessible through the incision. The ileal tie was gently removed 2 h after bacterial challenge, and the rabbits were returned to their cages, where food and water were available ad libitum. All surviving animals were killed 24 h postchallenge by means of barbiturate overdose; some animals infected with the CFA/I$^+$ bacteria died within the 24 h. Immediately after death, each animal was autopsied and the peritoneal cavity was swabbed for bacterial culture.

The *E. coli* enterotoxin and adhesin combinations were selected to include a CFA-positive ETEC strain (H10407), a CFA-negative ETEC strain (H10407-P), and a CFA-negative, nontoxigenic strain (E1392/75 7A). A PBS control was included to enable the effect of surgery and protease treatment in the absence of bacterial challenge to be monitored. The challenge rate of 10^{11} bacteria was based on earlier experiments in which such a dose of organisms was required to induce diarrhea in 90% of infected animals (1). In the present study, surgery was performed on rabbits in groups of eight over a period of 4 weeks (two rabbits from each group each week; see Table 1). Two weeks later, 12 rabbits were challenged with H10407.

Monitoring of infection. Rabbits were observed hourly for the 24-h postchallenge period for diarrhea, weakness, or death. Diarrhea was scored as follows: 0, no diarrhea; 1, mild diarrhea with feces softer than normal; 2, moderate diarrhea with at least three watery stools; and 3, severe diarrhea with multiple watery stools. Fecal swabs were collected when feces were passed, and rectal swabs were taken from rabbits which did not pass feces. Challenge organisms were identified by means of typical *E. coli* colony

TABLE 1. Diarrheal response in rabbits treated with Detach or untreated and challenged with different ETEC strains

Strain	Adhesin	Toxin	Treatment[a]	Diarrheal response[b]	Small intestine fluid vol (ml)
H10407[c]	CFA/I$^+$	ST$^+$ LT$^+$	D	1/7[d]	35–50
			C	7/8[e]	20–105[f]
H10407-P	CFA/I$^-$	ST$^+$ LT$^+$	D	1/4[g]	10–50
			C	1/4[g]	10–50
E1392/75 7A	CFA/II$^-$	ST$^-$ LT$^-$	D	0/4	15–50
			C	0/4	15–50
Nil (PBS)			D	0/4	8–12
			C	0/4	24–40

[a] D, rabbits treated with Detach; C, untreated rabbits.
[b] Number of animals with diarrhea or death/total number tested. For H10407-challenged rabbits, $P < 0.001$: one of seven Detach-treated animals died compared with seven of eight untreated animals challenged with the same dose rate.
[c] Five rabbits were omitted from the analysis because of death not related to diarrhea.
[d] One Detach-treated rabbit died; CFU/cm at S3 was 1.2×10^7.
[e] One rabbit survived infection; CFU/cm at S3 was 5.8×10^9.
[f] The total volume of fluid accumulated in small and large intestines combined was 130 to 165 ml.
[g] Mild diarrhea (score 1).

morphology and enzyme-linked immunosorbent assay nitrocellulose replica methods as described previously (20).

Collection of tissue specimens. All animals were sacrificed 24 h postchallenge, and the intraluminal fluid of the small intestine was measured. Large fluid volumes (>60 ml) in the small intestine of euthanized or dead rabbits were taken as an indication that diarrhea had been a major contribution to death (29). Sections (2 by 3 cm) of small intestine were collected from five sites: duodenum (S1), proximal jejunum (S2), midjejunum (S3), distal jejunum (S4), and ileum (S5). Each segment was opened longitudinally and washed extensively in sterile PBS to determine the numbers of strongly adherent bacteria or left unwashed to determine the total bacterial numbers present. Quantitative cultures were prepared by homogenizing tissue for 1 min in a Sorvall homogenizer operated at full speed. Serial dilutions were made in PBS, and aliquots (25 μl) were plated onto sheep blood agar (5%, vol/vol) and CFA agar. After incubation at 37°C for 18 h, the number of CFU per centimeter of tissue was determined. Other specimens were processed promptly for histology following fixation in 10% neutral-buffered formalin. After specimens had been embedded in paraffin, they were stained with hematoxylin-eosin stain and tissue Gram stain.

Statistical analysis. Bacterial counts were converted by log transformation to stabilize variances and analyzed by means of Genstat V for analysis of variance. Data are expressed as the mean values ± the standard error of the mean. The efficacy of Detach (protease) protection was determined by Fortran-Finney, a program that determines efficacies (percents) from chemotherapeutic tests (10).

RESULTS

Diarrheal response. None of the rabbits challenged with nontoxigenic E1392/75 7A or given 10 ml of sterile PBS developed diarrhea (Table 1). At autopsy, the fluid volumes in the small intestine (pyloric sphincter to the ileo-cecal junction) ranged from 8 to 12 ml in the PBS-protease-treated group to 24 to 40 ml in the PBS-treated, non-protease-treated group. In rabbits challenged with E1392/75 7A, 15 to 50 ml of fluid accumulated. Two of eight rabbits that were challenged with the CFA-negative, toxigenic H10407-P passed feces that were softer than normal (score 1). This was only mild diarrhea and not considered to be important. One rabbit was in the protease-treated group, and one was in the non-protease-treated group. In rabbits challenged with H10407-P, the fluid volumes in the small intestine ranged from 10 to 50 ml. There was no significant difference between the amounts of fluid accumulated by the rabbits challenged with the two CFA-negative strains.

In total, 20 rabbits were challenged with the enterotoxigenic CFA/I-positive strain H10407. Five died within 4 h of challenge, but none of these had typical acute watery diarrhea. These rabbits were autopsied, and peritoneal swabs were plated on blood agar and MacConkey agar. Leakage of bacteria from the inoculation site was deemed to be the cause of death, as colonies of typical *E. coli* morphology were isolated from the peritoneum of these rabbits. No such bacteria were detected in peritoneal swabs from the rabbits which died in a diarrhea-related manner or in swabs from the remaining rabbits autopsied at 24 h. These five rabbits were omitted from the experiment, leaving seven protease-treated rabbits and eight untreated rabbits challenged with H10407.

Of the eight control (non-protease-treated) rabbits challenged with H10407, seven died or developed severe diarrhea. Six rabbits died 5 to 13 h postchallenge, and four of these died without passing feces. Of the two rabbits that survived the 24-h postchallenge period, one had profuse watery diarrhea 18 h postchallenge while the other did not pass feces and had no clinical signs of infection. At autopsy, the fluid volume in the small intestines of rabbits which did not pass feces ranged from 20 to 105 ml. The total volume in the small and large intestine combined, however, ranged from 130 to 165 ml (in comparison with 10 to 50 ml in rabbits inoculated with E1392/75 7A, H10407-P, and PBS). In comparison, the small intestine contained relatively little fluid (35 to 50 ml) in rabbits that had diarrhea at the time of death. The total volume of intestinal fluid was only 55 to 60 ml. A lack of fluid accumulation in rabbits that have passed feces at the time of death has been reported previously (29).

Of the rabbits treated with protease prior to H10407 challenge, only one died. This rabbit died 11 h postchallenge after passing one loose stool (score 1). Fluid volumes in the small and large intestines were 60 and 50 ml, respectively. None of the other six rabbits treated with protease had diarrhea, and the majority (four of six) had passed formed feces by 24 h. At autopsy, the contents of the large intestine were solid and fluid accumulation in the small intestine ranged from 12 to 60 ml.

Bacterial adhesion. Quantitative cultures (CFU) were prepared from all animals to determine the adhesion of challenge bacteria in different parts of the small intestine. Challenge bacteria were present at all sites, with CFA/I$^+$ H10407 being the most effective colonizer. The mean CFU levels of CFA/I$^+$ bacteria at sampling sites in non-protease-treated rabbits were lower at S1, S2, S4, and S5 (1.1×10^8, 6.7×10^7, 1.0×10^8, and 4.0×10^8 CFU/cm, respectively) than at S3 (5.5×10^9 CFU/cm). In the analysis which follows, S3 cultures are used for comparison. The recovery rates of different organisms from 1 cm of mucosa are shown in Fig. 1.

The number of CFA/I$^+$ bacteria adherent to the mucosa in the protease-treated rabbits ranged from 1.3×10^4 (minimum count) to 1.2×10^7 CFU/cm in the rabbit that died (arithmetic mean, 2.6×10^6 CFU/cm). Values for control rabbits challenged with the same strain were 2,000 times greater ($P < 0.001$). Figure 2 illustrates the differences in colony counts between protease-treated and untreated animals.

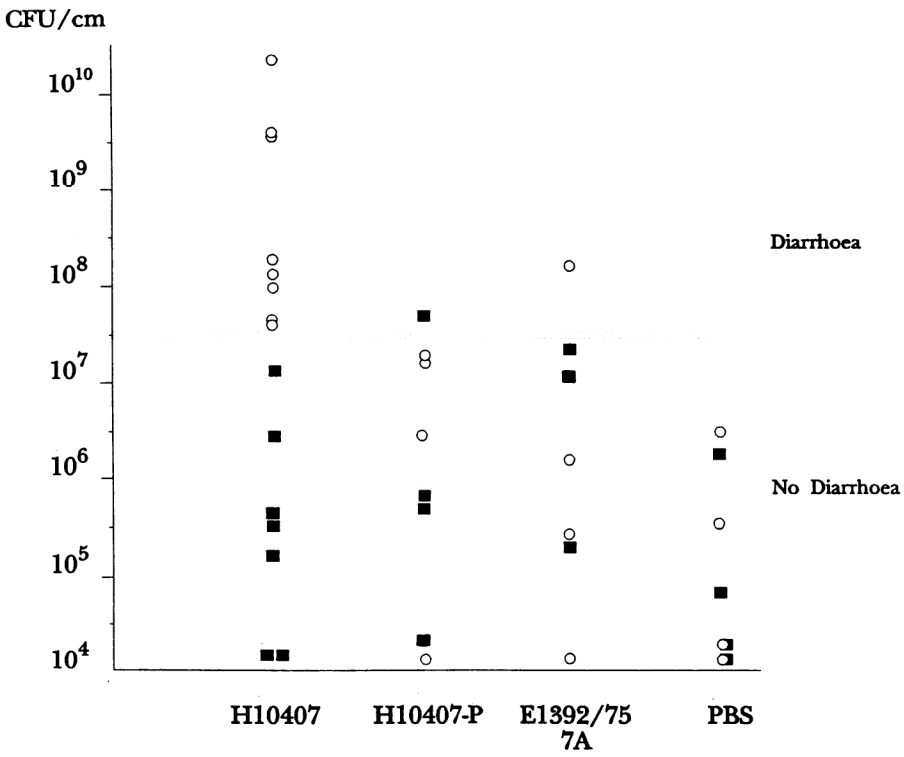

FIG. 1. Range of quantitative cultures at midjejunum (S3) of rabbits challenged with different ETEC strains or given sterile PBS. ■, rabbits treated with Detach 18 h prior to challenge; ○, untreated.

The number of bacteria bound to the small intestinal mucosa of rabbits infected with the CFA/I$^-$ strain H10407-P ranged from 1.3×10^4 CFU/cm (minimum count) to 6.6×10^7 CFU/cm in a rabbit with mild diarrhea (arithmetic mean, 1.8×10^7 CFU/cm). Counts for strain E1392/75 7A were similar, ranging from 1.3×10^4 (minimum count) to 2×10^8 CFU/cm (arithmetic mean, 3.9×10^7 CFU/cm). There were no significant differences between bacterial numbers in protease-treated and non-protease-treated animals challenged with either CFA-negative strain.

In rabbits which received sterile PBS only, there were relatively few bacteria in the small intestine (1.3×10^4 CFU/cm). There were, however, two exceptions. Two control rabbits which did not receive protease had colony counts of 4.6×10^6 and 5.2×10^5 CFU/cm at S3 (small intestinal volumes of 40 and 35 ml, respectively). One of these rabbits had 5.1×10^{10} and 4.1×10^{10} CFU/cm at S1 and S5, respectively. All colonies observed had typical *E. coli* morphology, and it seems likely that there was cross infection from one or more other bacterial challenge groups. These colonies were not checked for the presence of CFA/I. Williams-Smith and Halls (37) have reported that *E. coli* are not isolated as part of the normal rabbit flora, and it is therefore unlikely that the *E. coli* observed were the result of overgrowth of endogenous bacteria.

To assess the total number of bacteria present on the gut mucosa, sections were left unwashed. The mean values of washed versus unwashed sections from rabbits challenged with CFA/I$^+$ are shown in Table 2. These results suggest that the organisms are strongly adherent to the mucosa, as only 10^2 bacteria were washed away.

Fecal excretion of bacteria. Fecal swabs were obtained from rabbits when feces were passed. In all animals the challenge bacteria were excreted. Rectal swabs were taken at autopsy. The presence of the challenge strain in the rectum was apparent in all rabbits, including those that had not passed feces prior to termination of the experiment. In all instances, 100% of the colonies cultured were of the challenge strain.

Histology. Histological studies (light microscopy) of small intestinal tissue revealed no mucosal abnormality in any of the rabbits. Bacteria were only rarely seen on the mucosa, suggesting that bacteria bound in particular areas rather than being evenly distributed along the mucosa.

DISCUSSION

The decision to investigate the use of protease to modify the intestinal surface and thereby prevent diarrhea in humans was based on previous prevention of K88$^+$ *E. coli* infection in piglets (2a). Several studies have described similarities in mechanisms of pathogenesis of ETEC infection in humans and animals (11, 31). Most ETEC strains of human and animal origin rely on pili for adhesion and subsequent colonization of the small intestine. Also, diarrheal disease in both species is dependent ultimately on production and efficient delivery of enterotoxin.

The interaction between K88 adhesin and its intestinal receptor is one of the most studied host-pathogen associations, while relatively little is known about the interaction between CFA adhesins and human brush border membranes. The epithelial receptor for attachment of K88$^+$ ETEC to the brush border membrane of piglets is known to be a multimeric glycoprotein (30). This and other receptors

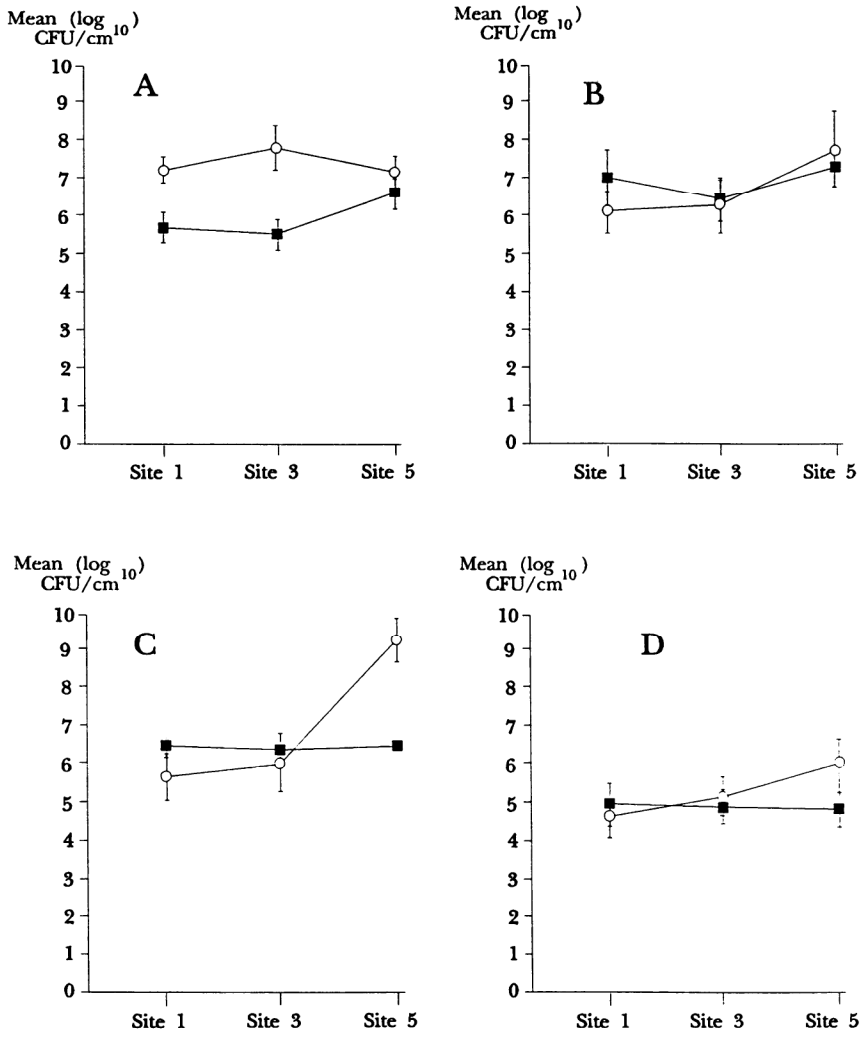

FIG. 2. Mean bacterial counts at duodenum (site 1), midjejunum (site 3), and ileum (site 5) of rabbits challenged with H10407 (A), H10407-P (B), E1392/75 7A (C), or sterile PBS (D). Each point represents the mean (log_{10}) ± standard error CFU per centimeter of tissue. ■, rabbits treated with Detach 18 h prior to bacterial challenge; ○, untreated rabbits.

involved in attachment (and colonization) have been shown to be readily inactivated by proteolytic enzymes, including those proteases that are normally active in the small intestine (23, 27, 30). This was recently investigated in experiments observing the instability of K88 receptor within pig intestines (3a). The variability of receptor activity in intestinal contents was confirmed by EIA as described previously (3), and instability of receptor activity could be controlled by addition of trypsin inhibitor to sample collection buffers. This technique was also used to demonstrate an effect of an exogenous enzyme (Detach) on a K88 intestinal glycoprotein receptor, thereby confirming the disruptive influence of protease on the binding of K88 adhesin to intestinal tissue. The efficacy of Detach under field conditions has been confirmed by field trials in commercial piggeries where large reductions in diarrhea-induced mortality and the incidence of diarrhea have been observed.

Recently we developed an in vitro (EIA) technique for monitoring the interaction among brush border preparations derived from human small intestine, ETEC, and LT. The effects of enzymatic treatment indicate that receptors for

TABLE 2. Quantitative cultures of H10407 bacteria bound at five different sites

Small intestine site[a]	Treatment[b]	Bacterial count (CFU/cm)[c]	
		Washed	Unwashed
S1	D	5.72 ± 0.40	6.96 ± 0.34
	C	7.25 ± 0.35	8.13 ± 0.44
S2	D	5.80 ± 0.28	6.90 ± 0.46
	C	7.30 ± 0.35	8.26 ± 0.31
S3	D	5.56 ± 0.40	7.04 ± 0.50
	C	7.87 ± 0.60	9.29 ± 0.38
S4	D	6.11 ± 0.38	7.20 ± 0.63
	C	7.66 ± 0.24	9.46 ± 0.29
S5	D	6.66 ± 0.42	8.80 ± 0.58
	C	7.22 ± 0.47	8.93 ± 0.36

[a] S1, duodenum; S2, proximal jejunum; S3, midjejunum; S4, distal jejunum; S5, ileum.
[b] D, Detach-treated rabbits; C, untreated rabbits.
[c] Mean log_{10} ± standard error recovered per centimeter of washed and unwashed small intestine at death or 24 h after RITARD challenge. Each mean reflects data from six or seven rabbits.

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