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Advances in Brief

Monoclonal Antibodies to the Extracellular Domain of Prostate-specific Membrane Antigen Also React with Tumor Vascular Endothelium[1]

He Liu, Peggy Moy, Sae Kim, Yan Xia, Ayyappan Rajasekaran, Vincent Navarro, Beatrice Knudsen, and Neil H. Bander[2]

From the Departments of Urology [H. L., P. M., S. K., Y. X., V. N., N. H. B.], Cell Biology [A. R.], and Pathology [B. K.], New York Hospital-Cornell Medical Center, New York, New York 10021, and the Ludwig Institute for Cancer Research, New York Branch [N. H. B.], New York, New York 10021

Abstract

Prostate-specific membrane antigen (PSMA), initially defined by monoclonal antibody (mAb) 7E11, is a now well-characterized type 2 integral membrane glycoprotein expressed in a highly restricted manner by prostate epithelial cells. 7E11 has been shown to bind an intracellular epitope of PSMA that, in viable cells, is not available for binding. Herein, we report the initial characterization of the first four reported IgG mAbs that bind the external domain of PSMA. Competitive binding studies indicate these antibodies define two distinct, noncompeting epitopes on the extracellular domain of PSMA. In contrast to 7E11, these mAbs bind to viable LNCaP cells *in vitro*. In addition, they show strong immunohistochemical reactivity to tissue sections of prostate epithelia, including prostate cancer. These mAbs were also strongly reactive with vascular endothelium within a wide variety of carcinomas (including lung, colon, breast, and others) but not with normal vascular endothelium. These antibodies should prove useful for *in vivo* targeting to prostate cancer, as well as to the vascular compartment of a wide variety of carcinomas.

Introduction

PSMA[3] is a highly restricted prostate epithelial cell membrane glycoprotein of approximately 100 kDa (1, 2). The PSMA gene has been cloned, sequenced (2), and mapped to chromosome 11q14 (3). In contrast to other highly restricted prostate-related antigens such as PSA, prostatic acid phosphatase, and PSP, which are secretory proteins, PSMA is an integral membrane protein. Among the reasons for significant interest in PSMA is that it is ideal for *in vivo* prostate-specific targeting strategies. In addition to its prostate specificity (1, 2, 4, 5), PSMA is expressed by a very high proportion of PCas (6); this expression is further increased in higher-grade cancers, in metastatic disease (6), and in hormone-refractory PCa (5–7).

The initial validation of PSMA as an *in vivo* target has been borne out by imaging trials with mAb 7E11/CYT-356 (8–11). However, epitope mapping indicates that 7E11/CYT-356 targets an intracellular epitope (12, 13). In viable cells, this binding site is not accessible to an antibody (1, 13). Successful imaging with 7E11/CYT-356 probably relates to the targeting of dead/dying cells within tumor sites (6, 12, 13). It has been noted (2, 12–14) that a mAb to the extracellular domain would provide benefits, including improved *in vivo* localization and enhanced imaging and therapy. In this study, we report the development of four IgG mAbs to the external domain of PSMA. These mAbs also have been found reactive to vascular endothelium within a wide range of carcinomas but not with normal endothelial cells.

Materials and Methods

Generation and Production of mAb. BALB/c mice were immunized three times with LNCaP cells or a primary culture of PCa epithelial cells. Spleen cells were fused with X63.Ag.653 mouse myeloma cells using standard hybridoma technique. Clones that were reactive against LNCaP but unreactive against tissue sections of normal kidney (with the exception of some proximal tubule reactivity) and colon were subcloned. Murine ascites fluid was produced, and mAbs were purified using protein G (Pharmacia LKB Biotechnology, Piscataway, NJ). Purified mAb 7E11 was a generous gift from Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA).

Immunohistochemical Staining. Normal and cancer tissues were precooled in liquid nitrogen, snap-frozen in OCT compound (Miles Inc., Elkhart, IN) on dry ice, and stored at −80°C. Cryostat tissue sections (5 μm) were fixed in cold acetone (4°C) for 10 min. mAbs (5 μg/ml or hybridoma supernatants) were incubated for 1 h at RT. Antibody binding was detected using rabbit antimouse immunoglobulin-peroxidase (DAKO Corp., Carpinteria, CA) as a secondary antibody and diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as chromogen. Isotype-matched irrelevant antibody was used as a negative control.

Cross Immunoprecipitation. LNCaP cells were lysed in lysis buffer [20 mM Tris/HCl, pH 8; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; and 1% (v/v) Triton X-100]. The resulting lysate was precleared by incubation with protein G beads overnight at 4°C, then incubated with mAb for 2 h. Protein G beads were added for 90 min prior to further washing. The beads were resuspended and boiled for 5 min in 1× Laemmli sample buffer containing 2-mercaptoethanol at 5% final concentration. The samples were centrifuged, and supernatant was recovered and placed on a 12% SDS-PAGE gel. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5% dry milk/TBST (Tris-buffered saline-Tween 20), incubated with primary mAbs × 60 min and followed by sheep antimouse immunoglobulin-peroxidase (Amersham Corp., Cleveland, OH). After washing, the membranes were developed using the enhanced chemiluminescence method (Amersham). Either 0.15 μg/ml J591 or 0.5 μg/ml 7E11 was used as a probe to detect the protein that was immunoprecipitated by 7E11, J591, J533, J415, and E99, respectively. Isotype-matched irrelevant antibody (I56; reactive to PSP) was used as a negative control.

IF Assay. LNCaP cells were grown on glass coverslips. IF assays were performed using either viable or fixed cells, the latter being either permeabilized or nonpermeabilized. For fixation, cells were treated with 2% paraformaldehyde-PBS (PBS, pH 7.4) for 30 min at RT, which does not permeabilize the cell membrane, washed with 1% BSA-PBS, quenched for 10 min in 50 mm NH_4Cl in PBS, and rinsed with 1% BSA-PBS. Where cell membrane perme-

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[2] To whom requests for reprints should be addressed, at New York Hospital-Cornell Medical Center, Box 23, 525 East 68th Sreet, New York, NY 10021. E-mail: nhbander@mail.med.cornell.edu.

[3] The abbreviations used are: PSMA, prostate-specific membrane antigen; PSA, prostate-specific antigen; IEM, immunoelectron microscopy; IF, immunofluorescence; mAb, monoclonal antibody; PCa, prostate cancer; PSP, prostate secretory protein; RT, room temperature.

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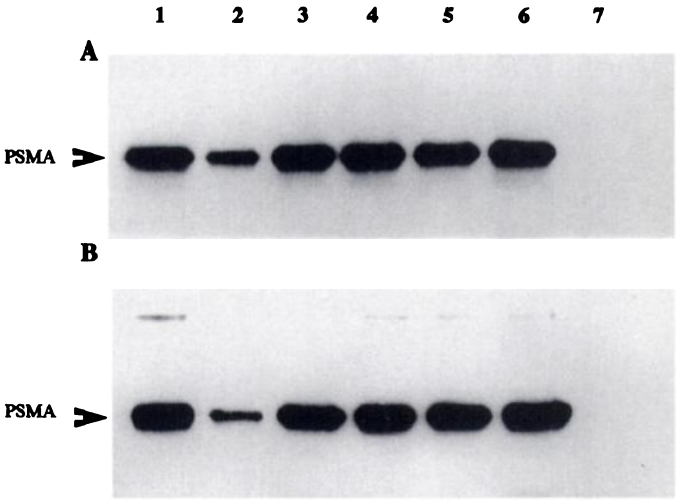

Fig. 1. Cross immunoprecipitation shows mAbs J591, J415, J533, E99, and 7E11 recognize the same antigen. *Lane 1*, crude LNCaP lysate. In *Lanes 2-7*, LNCaP lysate was immunoprecipitated with 5 μg/ml mAb 7E11 (*Lane 2*); 5 μg/ml J591 (*Lane 3*); 10 μg/ml J533 (*Lane 4*); 10 μg/ml J415 (*Lane 5*); 10 μg/ml E99 (*Lane 6*); and 10 μg/ml I56 (to PSP; *Lane 7*). Immunoprecipitates were immunoblotted with 0.15 μg/ml mAb J591, (*A*) or 0.5 μg/ml 7E11 (*B*).

abilization was desired, 0.075% saponin (Sigma) in 1% BSA-PBS was added for 15 min at RT.

Primary mAb at 4 μg/ml in BSA-PBS (plus saponin in cases of permeabilization) was incubated for 60 min at 4°C in the case of viable cells or at RT for fixed cells. After primary mAb incubation, viable cells were fixed in cold methanol for 20 min. FITC-goat antimouse secondary antibody (1:100 in BSA:PBS ± saponin; Jackson ImmunoResearch, West Grove, PA) was incubated for 30 min and washed extensively in 1% BSA-PBS. Slides were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA).

IEM Microscopy. The IEM procedure was similar to the nonpermeabilized IF assay above. LNCaP cells were grown in 35-mm culture dishes and incubated with 10 μg/ml J591 or 10 μg/ml 7E11 for 45 min at 4°C, fixed with 2% paraformaldehyde, washed, and quenched as above. After 1% BSA washes, cells were incubated with 15-nm gold-conjugated goat antimouse IgG (Amersham) for 1 h. After extensive washing, cells were fixed in 2.5% glutaraldehyde for 15 min, gently scraped, pelleted, and processed for IEM as described previously (15). Electron micrographs were taken with a Joel 100CX electron microscope.

Competitive Binding Assay. Biotinylated mAbs were prepared by incubating 1 mg/ml mAb with 0.1 ml of biotinamidocaproate *N*-hydroxysuccinamide ester (1 mg/ml; Sigma) in DMSO for 4 h at RT. Unbound biotin was removed by dialysis against PBS. 7E11 (10 μg/ml) was coated onto Terasaki plates. Plates were washed with 1%BSA-PBS-0.1% Tween 20. LNCaP membrane preparations were added to wells for 1 h at RT. After washing, serial dilutions of unlabeled (competing) antibody were added to duplicate wells for 1 h. Biotinylated antibody was added to each well and incubated for an additional 1 h followed by avidin-alkaline phosphatase (Sigma). After wash-

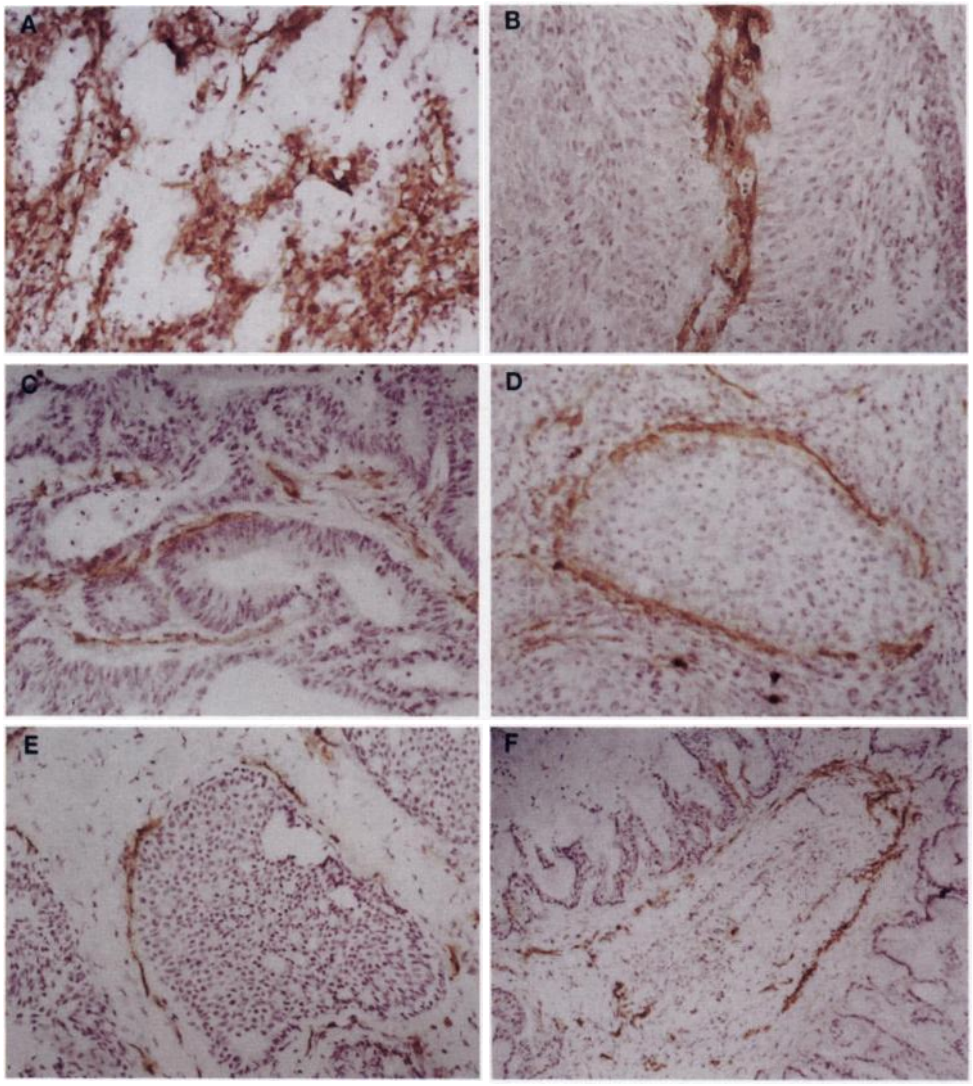

Fig. 2. Immunohistochemical reactivity of mAb J591 to neovasculature of renal (*A*), urothelial (*B*), colon (*C*), lung (squamous; *D*), breast carcinomas (*E*), and metastatic adenocarcinoma to liver (*F*). ×250.

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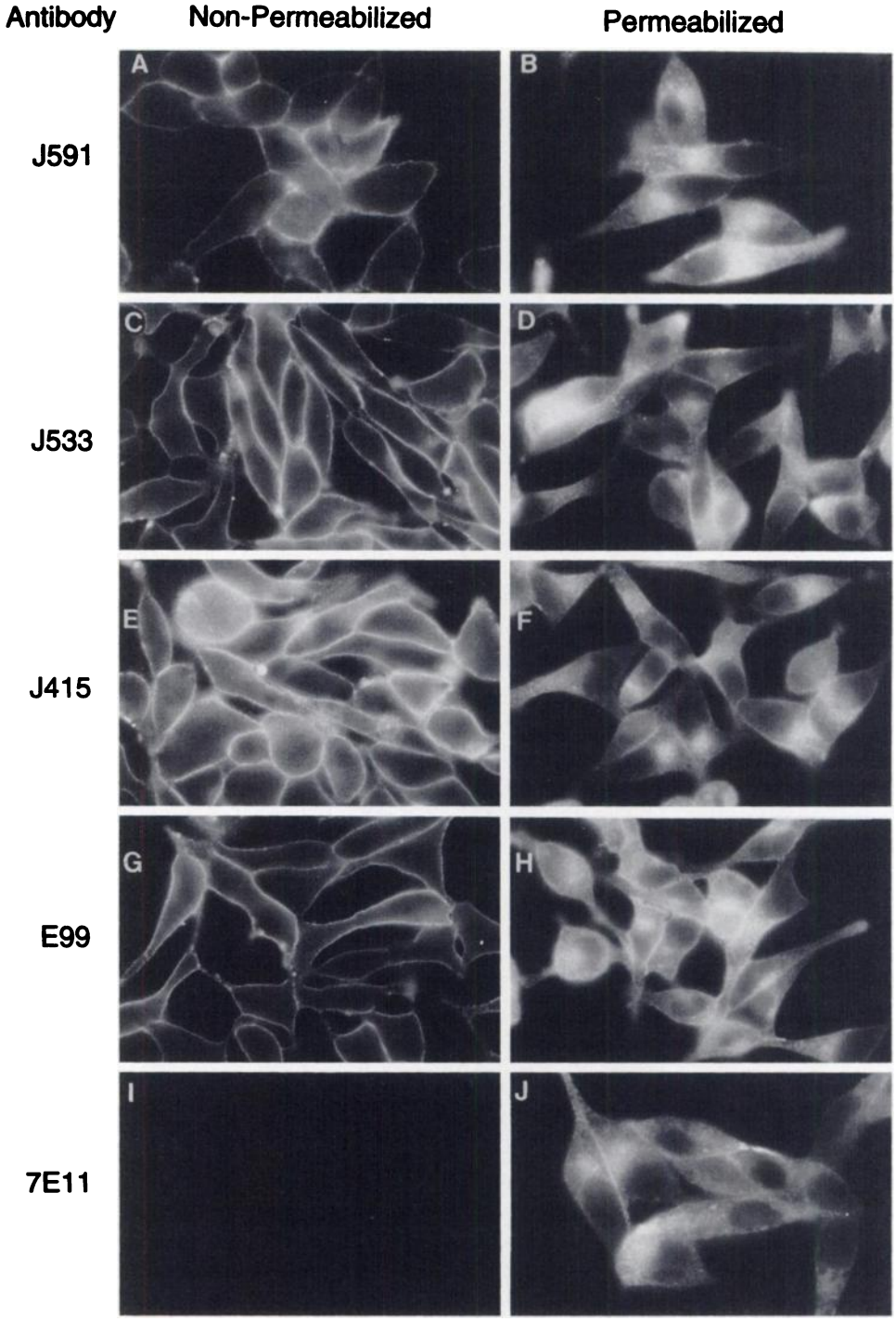

Fig. 3. Immunofluorescence assay comparing the reactivity of mAbs J591, J415, J533, and E99 to 7E11 on nonpermeabilized and permeabilized LNCaP cells. Intact, nonpermeabilized cells are reactive with mAbs J591 (*A*), J533 (*C*), J415 (*E*), and E99 (*G*) but not 7E11 (*I*). Reactivity is limited to the cell membrane without cytoplasmic staining, as mAbs do not enter the intact cells. Failure of 7E11 to bind (*I*) is consistent with the intracellular location of its epitope. When the cells are permeabilized prior to mAb incubation (*B, D, F, H, J*), reactivity to both cytoplasmic and membrane PSMA is seen. After the permeabilization and exposure of the intracellular PSMA epitope, 7E11 does bind. ×1500.

ing, substrate (para nitrophenylphosphate) was added, and reactivity was read at $A_{405\ nm}$. Irrelevant antibody (I56) was used as a control.

Results

From over 2000 clones screened, 4 clones that reacted with a 100-kDa band on Western blots and that produced strong immunohistochemical staining of prostate epithelium were selected for further characterization.

Immunoprecipitation/Immunoblot. In Western blot analysis, mAbs J591 (IgG1), J533 (IgG1), J415 (IgG1), and E99 (IgG3), as well as 7E11, identified a 100-kDa band from LNCaP lysate but not from the PSMA-negative PC3 lysate (data not shown). To confirm that mAbs J591, J533, J415, and E99 detected the same antigen as 7E11, a cross-immunoprecipitation experiment was performed. Fig. 1 illustrates that the 100-kDa band that was immunoprecipitated by mAbs J591, J533, J415, E99, or 7E11 was detectable by immunoblot using either J591 or 7E11 as a probe (Fig. 1, *A* and *B*, respectively). Sequential immunoprecipitation studies (data not shown) also demonstrated that 7E11 and the four new mAbs can preclear reactivity to one another.

Immunohistochemical Reactivity. The reactivity of mAbs J591, J533, J415, and E99 with normal human tissues and cancers, with rare exception (*vide infra*), were similar to 7E11. Normal tissues with similar immunohistochemical reactivity included prostate (normal and hyperplastic glands demonstrated heterogeneous, weak to moderate staining intensity), kidney (subset of proximal

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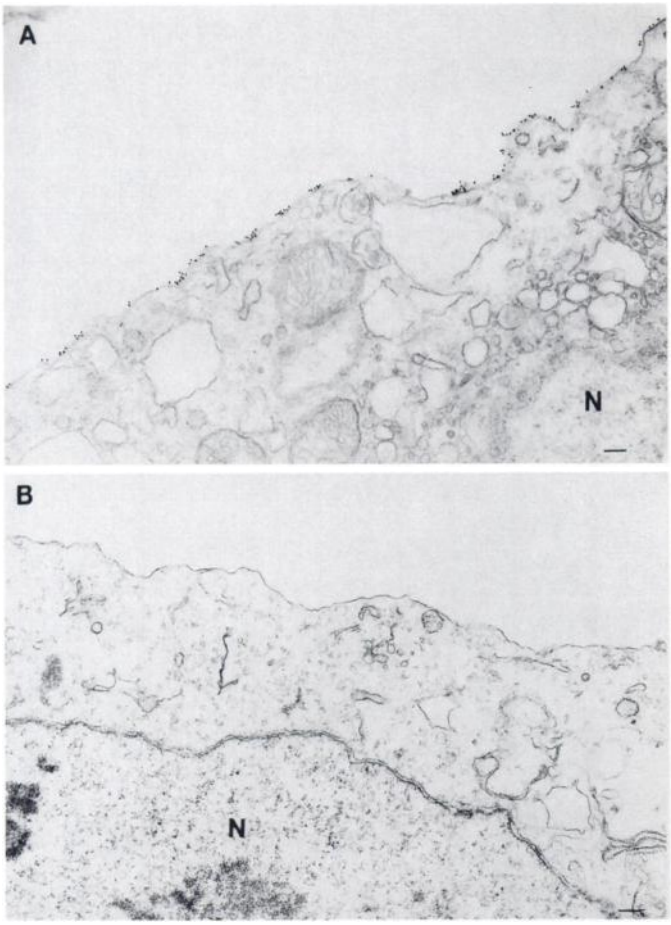

Fig. 4. IEM showing the reactivity of mAbs J591 (*A*) and 7E11 (*B*) to viable LNCaP cells. mAb J591 localizes to the extracellular surface of the plasma membrane, whereas 7E11 demonstrates no binding. *Bar*, 0.6 μ (*A*) and 1.0 μ (*B*). *N*, nucleus.

tubules), and duodenum (weakly reactive). The only normal tissue in which we found any difference in reactivity was striated muscle. Although 7E11 was strongly reactive to striated muscle, mAbs J591, J533, J415, and E99 demonstrated no reactivity. In neoplastic tissues, findings were again similar when comparing 7E11 to mAbs J591, J533, J415, and E99. All 21 PCas studied were strongly reactive with mAbs J591, J533, J415, and E99, being somewhat more intense and more homogeneous than 7E11. As reported previously (17), we found 7E11 reacted with vascular endothelium in a subset of tumors. However, mAbs J591, J533, J415, and E99 reacted more strongly with vascular endothelium in all 23 carcinomas studied (Fig. 2), including 9 of 9 renal, 5 of 5 urothelial, 6 of 6 colon, 1 of 1 lung, 1 of 1 breast and 1 of 1 metastatic adenocarcinoma to the liver.

Immunofluorescence Staining of LNCaP Cells. We compared, by indirect immunofluorescence, mAbs J591, J533, J415, and E99 to mAb 7E11 on viable or fixed, permeabilized or nonpermeabilized LNCaP cells (Fig. 3). LNCaP cells with intact plasma membrane (*i.e.*, either viable [data not shown] or fixed without permeabilization) demonstrated cell surface reactivity with mAbs J591, J533, J415, and E99 (Fig. 3, *A-C, E, G*), but not with mAb 7E11 (Fig. 3*I*). Only after LNCaP cells were permeabilized could 7E11 reactivity be demonstrated (Fig. 3*J*). Once permeabilized, the reactivity of all mAbs appeared both in the cytoplasm and on the plasma membrane.

Immunoelectron Microscopy. IEM similarly demonstrated immunoreactivity of mAb J591 (Fig. 4*A*) but not 7E11 (Fig. 4*B*) with viable LNCaP cells. Furthermore, the IEM photomicrographs of mAb J591 show the gold particles localized to the extracellular face of the plasma membrane, confirming reactivity with the extracellular domain of PSMA.

Competitive Binding Assay. A double antibody sandwich competition ELISA was used to determine whether the four mAbs recognize the same or different epitopes (Fig. 5). Each unlabeled mAb was able to block its biotinylated counterpart serving as a positive control. An unrelated IgG1 antibody (I56) did not block any of the mAbs to PSMA. J591, J533, and E99 were each able to block each other, but were not blocked by J415. Conversely, J415 was blocked only by its unlabeled counterpart but not by any of the other three mAbs. These results indicate that J591, J533, and E99 recognize the same epitope that is distinct and noncross-reactive with the epitope recognized by J415.

Discussion

This study defines four new IgG mAbs that detect two distinct extracellular epitopes of PSMA ($PSMA_{ext1}$ and $PSMA_{ext2}$). The reactivity of these mAbs with PSMA has been defined by immunoprecipitation and immunoblotting studies and reactivity against cell lines (data not shown) and tissue sections using the 7E11 mAb as a reference. Immunoprecipitation and immunoblotting studies demonstrate identical reactivity to that seen with 7E11. Reactivity *in vitro* (data not shown) and on tissue sections of normal and neoplastic specimens demonstrates nearly identical results. The exceptions in immunohistochemical reactivity were limited to striated muscle and tumor vascular endothelium. Striated muscle is reactive with 7E11 but not with mAbs J591, J415, J533, or E99. 7E11 reactivity with striated muscle had been reported previously by Lopes *et al.* (4) who, like the present study, used frozen sections but has been reported as negative by Silver *et al.* (17) who studied paraffin sections. This discrepancy is most likely explained by some loss of 7E11/PSMA immunoreactivity in the fixation/embedding process. The difference in reactivity of 7E11 and the present mAbs to striated muscle, both herein studied on frozen sections, may represent differences in the posttranslational processing of PSMA (the external domain of which is heavily glycosylated) occurring in prostate as compared to muscle.

Reactivity of 7E11 with tumor but not normal vascular endothelium also was noted previously by Silver *et al.* (17), although 7E11 reactivity was reported in only half of their renal and urothelial cancers (15 of 30) and 3 of 19 colon carcinomas. In the present study, mAbs J591, J415, J533, and E99 demonstrate reactivity with tumor vasculature in all 23 nonprostate carcinomas tested. Some of the increased reactivity seen herein may represent the benefit of studying frozen as compared to paraffin sections. Within this study, when comparing mAbs J591, J415, J533, and E99 to 7E11 using a constant tissue preparation (frozen sections), we found stronger reactivity with mAbs J591, J415, J533, and E99 than with 7E11. The most likely explanation for the generally stronger reactivity seen with these new mAbs is that they were selected for, among other features, strong immunohistochemical reactivity. We have not studied the immunohistochemical reactivity of mAbs J591, J415, J533, and E99 on paraffin sections.

The initial study with 7E11 (1) indicated reactivity to fixed, but not viable, LNCaP cells later explained by epitope mapping studies indicating the 7E11 epitope to be intracellular (12). A more recent study by Troyer *et al.* (13) studying ultrathin sections by IEM demonstrated 7E11 reactivity on the cytoplasmic aspect of LNCaP

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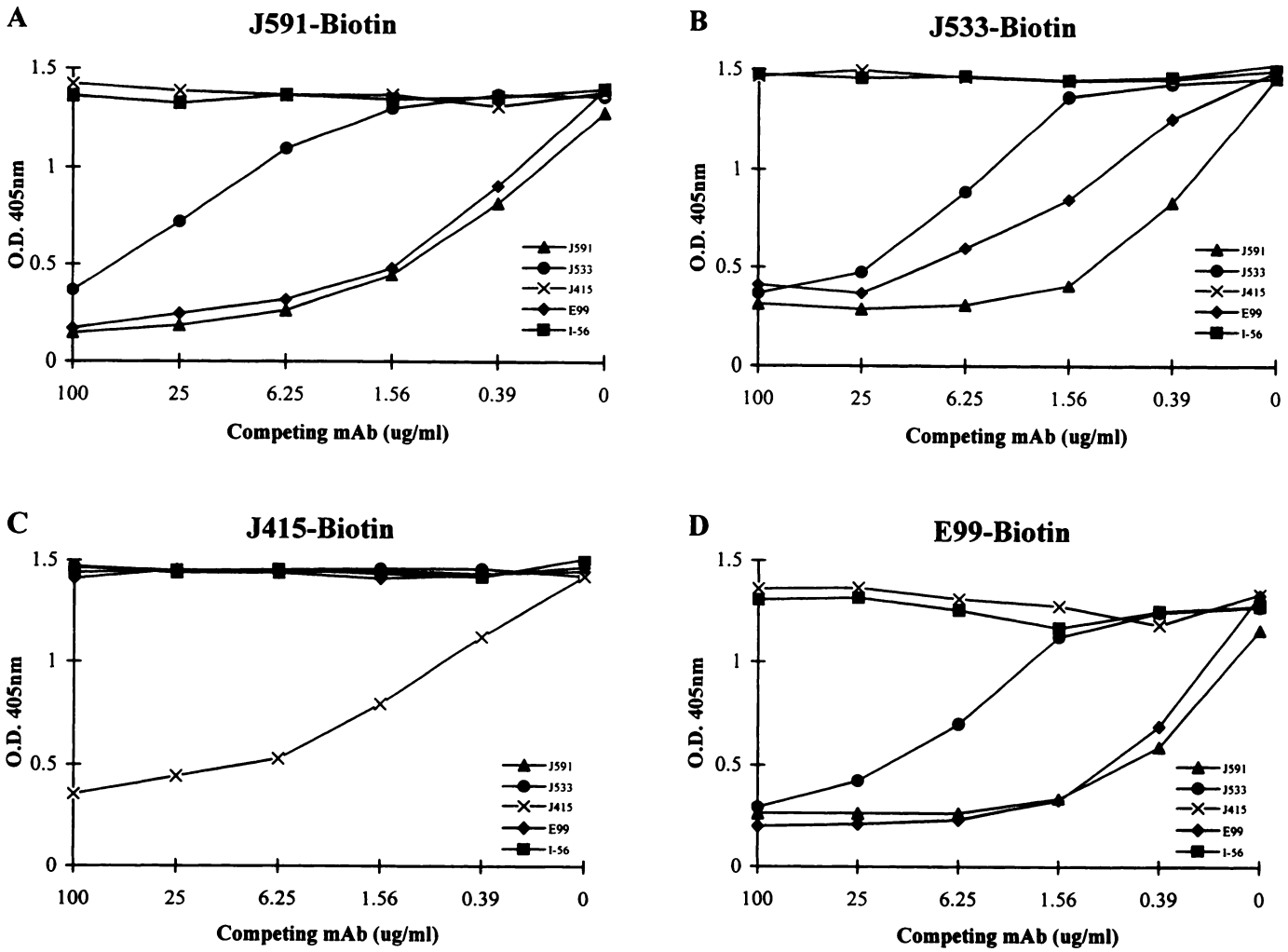

Fig. 5. Competitive binding assay of biotinylated mAbs. PSMA was first captured by 7E11, then unlabeled blocking mAb was added, followed 1 h later by the indicated biotinylated mAb: 0.3 μg/ml J591-biotin (*A*); 1.25 μg/ml J533-biotin (*B*); 0.2 μg/ml J415-biotin (*C*); and 1.25 μg/ml E99-biotin (*D*). Each unlabeled mAb blocks its biotinylated counterpart (positive control); mAb I56 (to PSP) did not block (negative control). mAbs J591, J533, and E99 compete with each other for binding, whereas J415 does not.

cells plasma membrane. Troyer *et al.* (13) also confirmed 7E11 reactivity with permeabilized but not with nonpermeabilized LNCaP cells. Our studies comparing 7E11 with the present mAbs by immunofluorescence assays on viable and fixed, permeabilized and nonpermeabilized LNCaP cells confirmed the data published previously that 7E11 detects an intracellular epitope not available for mAb binding unless the cell membrane is disrupted. A recent report by Barren *et al.* (18) represents the sole study indicating that 7E11 can react with viable LNCaP cells. The report by Barren *et al.* is inconsistent with other published work (1, 12, 13), as well as the results reported here, and may be due to a technical point. Barren *et al.*, after incubating 7E11 with viable LNCaP cells, harvested LNCaP for flow cytometry by scraping the cells in the presence of 7E11. As scraping can disrupt cell membranes, this would have provided 7E11 access to its intracellular epitope, which likely accounts for the reactivity reported. Importantly, mAbs J591, J415, J533, and E99, unlike 7E11, can bind to either viable or nonpermeabilized cells consistent with targeting accessible epitopes on the extracellular domain of PSMA. Our IEM finding of mAb J591 localization on the extracellular aspect of the plasma membrane (Fig. 4*A*), in contrast to the intracellular localization of 7E11 on IEM reported by Troyer *et al.* (13), provides further evidence of reactivity of the present mAbs to the extracellular domain of PSMA.

Epitope mapping of the four present mAbs demonstrates that J591, J533, and E99 each bind to a single epitope ($PSMA_{ext1}$), whereas J415 binds to a different, noncompeting site ($PSMA_{ext2}$). This will allow the development of a "sandwich" assay to determine the presence and measure the level of PSMA in serum, which is an area of some current controversy (14, 16).

By allowing the study of viable cells, these mAbs will be useful for studies of PSMA function and PCa cell biology. Recent work indicates that PSMA has glutaminase (19, 20) activity. Studies are under way to determine whether mAbs to $PSMA_{ext1}$ and/or $PSMA_{ext2}$ can block this enzymatic activity and, if so, the effect of such a blockade on normal and neoplastic prostate physiology.

Given prior understanding of PSMA specificity and expression and the established ability of 7E11/CYT-356 to localize *in vivo* to a substantially less available epitope, one would anticipate the likelihood that these new mAbs might demonstrate significantly improved *in vivo* targeting for imaging and therapy. The immunoreactivity of these mAbs to vascular endothelium of a wide variety of cancers may significantly broaden their *in vivo* utility.

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