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Monoclonal Antibodies to a Human Prostate Antigen[1]

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ABSTRACT

Three monoclonal antibodies reactive with a purified extractable M_r 34,000 prostate antigen (PA) have been prepared by fusing splenocytes of BALB/c mice preimmunized with purified PA with the NS1 mouse myeloma cell line. The three antibodies were all of the IgG-1 subclass. The antibodies defined two non-cross-blocking unique determinants on PA; each present as one site per molecule. IF3 defined one antigenic site and 2G7 and 1C5 defined another antigenic determinant. All of the antibodies reacted with PA in a solid-phase radioimmunoassay and immunoprecipitated ^{125}I-labeled PA. Absorption and sandwich radioimmunoassays showed PA in prostate tissues but not in tonsil, liver, or kidney. Immunoperoxidase staining of formalin-fixed paraffin-embedded sections of benign prostatic hyperplasia and prostatic carcinoma revealed strong prostate epithelial reactivity. None of the antibodies showed reactivity with prostate membrane preparations. A sandwich radioimmunoassay used 2G7 as a plate coat. ^{125}I-labeled 1F3 was used to detect 5 ng PA per ml in sera of patients with prostate cancer. These results confirm previous observations regarding the specificity of PA and shed new evidence for its intracellular localization.

INTRODUCTION

Human prostatic tissues contain an extractable antigen which can be found in normal prostate tissue, BPH,[3] and prostatic carcinoma but not in other human tissues (15, 16). This antigen has been shown to be a M_r 34,000 protein with an isoelectric point of 6.8 (12, 16). The protein has been purified and designated PA (16). Using a sandwich enzyme immunoassay, it is estimated that 0.1 to 50 μg of PA are present per mg of prostate protein (4), and recently, using a rabbit antiserum to PA, the antigen was localized to epithelial cells in prostate tissues (9, 11). Cell lines of prostate carcinoma, LNCaP and PC3, have been shown to contain PA (11). Elevated levels of PA have been found in the sera of prostate carcinoma and BPH patients (4, 13).

We have recently prepared a series of monoclonal antibodies against prostate epithelial membrane antigens, none of which react with PA (1). Because of potential uses of anti-PA antibodies in serum assays for following cancer treatment (5), section staining for diagnosis of metastatic adenocarcinoma (9) and immunotherapy, as well as to facilitate further characterization of PA itself, we attempted to produce monoclonal antibodies reactive with PA. We were able to generate and characterize 3 mouse monoclonal antibodies against PA. The previously described chemical properties and tissue specificity of PA were confirmed. We demonstrated for the first time that it is not present in an antigenically detectable form on cell membranes.

With the availability of these monoclonal antibodies, we have an unlimited source of homogeneous antibodies that react specifically with PA. These antibodies should allow easier isolation and characterization of PA as well as aid in generating precisely reproducible diagnostic reagents.

MATERIALS AND METHODS

PA Purification. PA was purified as described by Wang *et al.* (16, 17). Benign hypertropic prostate tissue was homogenized in phosphate-buffered saline (0.15 M sodium chloride and 0.015 M sodium phosphate, pH 7.0) and stirred at 4° for 2 hr. The extract was precipitated with 50% saturated ammonium sulfate, and the precipitate was dissolved in 0.01 M Tris and dialyzed. The material was then centrifuged at 45,000 × *g*, and the supernatant was passed over a DEAE-Bio-Gel A column followed by a Sephadex G-100 column and another DEAE-Bio-Gel A column. The material was then concentrated with Amicon ultrafiltration membrane.

Immunizations and Fusion. Two 4-week-old female BALB/c mice were inoculated i.p. with purified PA, 50 μg, in complete Freund's adjuvant on Days 2 and 14. On Day 17, the immune splenocytes were removed and fused with NS1 mouse myeloma cells as described (10). Immune splenocytes (10^8) from the 2 mice were washed with serum-free medium and mixed with 10^8 NS1 mouse myeloma cells, also washed repeatedly in serum-free medium. The NS1 cell line is resistant to 8-azaguanine and, hence, is unable to grow in HAT medium. The cells were centrifuged, and the pellet was treated with 50% polyethylene glycol 1500 in serum-free medium for 1 min at 37°. After 5 min, 8 ml serum-free medium was added and the mixture was recentrifuged. The cells were then resuspended vigorously with serum-containing medium and aliquoted.

Screening of Hybridoma Supernatants. The fused cell suspension was aliquoted into 160 wells in 96-well Costar plates and fed HAT medium (10). The HAT medium leads to the death of remaining NS1 cells. The nonfused splenocytes have died in culture by Day 5. Only the hybrids survive and multiply in HAT medium with an average of 1 to 2 clones/well. On Day 10, postfusion supernatants from each well were screened for binding to PA in a solid-phase RIA using 30 μg PA per ml as a plate coat, and [^{125}I]iodogen-labeled rabbit anti-mouse immunoglobulin antibody as a second-step antibody (14).

Cloning of PA-reactive Wells. Fluorescence-activated cell sorter sterile cloning was performed on cell suspensions from reactive wells as described (6). The clone wells, each containing a single viable cell, were fed medium containing hypoxanthine and thymidine and screened as above (10). This medium, while supplying hypoxanthine and thymidine, allows the aminopterin to be slowly diluted out of it. Otherwise, hybridomas are exposed to some remaining aminopterin with insufficient purines for salvage. Hybridoma clones displaying PA binding were grown in flasks, and the hybridoma supernatants were analyzed.

Characterization of Hybridoma Antibodies. Isotypes of the monoclonal antibodies were determined as described (6). Chain structure of

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[3] The abbreviations used are: BPH, benign prostatic hyperplasia (also called nodular hyperplasia); PA, prostate antigen; HAT medium, medium containing hypoxanthine, aminopterin, and thymidine; RIA, radioimmunoassay.

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the hybridoma antibodies was determined by 2-dimensional gel electrophoresis of [^{35}S]methionine metabolically labeled hybridoma supernatants, as described (1). Immunoprecipitation of [^{125}I]iodogen-labeled PA was performed for each monoclonal antibody, as described by Goding *et al.* (2).

Cross-binding and Cross-blocking Studies. Each monoclonal antibody was purified from ascites by Staph A Sepharose and dialyzed against phosphate-buffered saline. The antibodies were diluted to 100 μg/ml, and 50 μl of each were aliquoted in 96-well polyvinyl chloride plates (Dynatech Laboratories, Alexandria, Va.). The wells were emptied after 12 hr at 4° and washed with RIA buffer (1% bovine serum albumin in phosphate-buffered saline). One μl of PA (50 μg/ml) was then placed in each well for 2 hr at room temperature. The wells were again washed with RIA buffer, and 50 μl of the various monoclonal antibodies, at 100 μg/ml, were incubated for 2 hr at room temperature and again washed. Finally, 50,000 cpm of [^{125}I]iodogen-labeled monoclonal antibody, prepared as described previously (1), were incubated in each well for 2 hr. The wells were washed, cut with a hot wire cutter, and counted in a γ counter.

Tissue Localization of PA. Immunoperoxidase staining of frozen sections of normal and malignant human tissues was performed as described previously (1). Formalin-fixed paraffin-embedded sections of tissue were incubated at 56° for 0.5 hr to overnight, deparaffinized, and stained in the same fashion. A light nuclear counterstain (Meyer's hematoxylin) was applied. Membrane RIA using human tissue membrane preparations was performed as described previously (1).

Absorption and Sandwich Radioimmunoassay of PA. Tissue extracts were prepared by homogenization with a Polytron, and debris was then removed at 20,000 × *g* centrifugation. Absorption studies were performed by incubating 14 μl of 1F3 hybridoma supernatant with 50 μl of extracts of various tissues in Airfuge tubes. After being left for 12 hr at 4°, the mixtures were centrifuged at 100,000 × *g*, and 40 μl were applied to polyvinyl chloride 96-well plates which had been incubated with 30 μl of PA and washed with RIA buffer. After 2 hr at room temperature, the wells were washed with RIA buffer and incubated with [^{125}I]iodogen-labeled rabbit anti-mouse Fab immunoglobulin. After 2 hr, the wells were washed with RIA buffer and cut with a hot wire device, and the wells were counted in a γ counter. Sandwich RIA used a 50-μl 2G7 (100 μg/ml) plate coat. Various tissue extracts were added. Finally, [^{125}I]iodogen-labeled 1F3 was used to detect well-bound PA. The well ^{125}I binding was tested as above.

Serum Assay of PA. The same sandwich RIA used for tissue extracts was used with serum samples.

RESULTS

Six of 160 hybridoma wells yielded antibody reactive with PA; 3 of 6 remained reactive with PA after cloning. These 3 antibody-producing clones, 1C5, 2G7, and 1F3, were further characterized.

They were all IgG-1 isotype; they each contained a heavy chain and 2 light chains on [^{35}S]methionine labeling (Fig. 1). The 4 heavy chain bands on 2-dimensional gel electrophoresis have varying amounts of sialic acid on one heavy chain (10). Each precipitated [^{125}I]PA (Fig. 2). Analysis of the 2-dimensional gel of the immunoprecipitate confirms the antigen molecular weight of 34,000 and the average isoelectric point of 6.8. Again, the sensitivity of the 2-dimensional gel electrophoresis reveals 3 charged species of PA which presumably represent variations in sialic acid or some other charged sugar moiety.

Cross-binding studies showed that binding of PA to plate coated 1F3 prevents binding of ^{125}I-1F3 but permits binding of ^{125}I-2G7 and -1C5 (Table 1). Conversely, plate coated 2G7 and 1C5, followed by PA, can bind ^{125}I-1F3 but not iodinated 2G7 or 1C5. Cross-blocking studies show that 1F3 does not block 2G7 or 1C5 binding to PA but 2G7 and 1C5 block each other and not 1F3 binding (Table 1).

Immunoperoxidase staining of frozen sections of BPH tissue with these monoclonal antibodies revealed weak, nonspecific staining. In contrast, formalin-fixed, paraffin-embedded section staining demonstrated intracellular prostatic epithelial cell staining of both benign and malignant prostate (Fig. 3). Antibody 1F3 consistently produced stronger staining than did the other 2 antibodies. All 3 antibodies were tested for reactivity on formalin-fixed, paraffin-embedded sections of normal and malignant tissues from the following organs: colon, pancreas, stomach, and kidney, as well as normal seminal vesicle. Of these, only the renal tissue stained significantly. The renal tissue examined contained normal renal parenchyma and renal cell carcinoma from each of 3 patients. Normal renal tubules (but not glomeruli) stained intensely in all 3 cases. The renal carcinomas varied in intensity of staining from faint to strong, frequently of similar intensity to that seen in prostate carcinomas. This staining was not blocked by preincubation with normal rabbit serum or hydrogen peroxide and was not produced by staining with mouse monoclonal antibody 38 (also of the IgG1 class), directed against human endothelial cell membranes, but with no reactivity with human renal tissue (1).

None of the antibodies reacted with a variety of human tissue membrane preparations, including prostate membranes (Table 2).

Absorption studies shown in Chart 1*A* show that only prostate tissue extracts and PA can absorb the reactivity of 1F3 hybridoma supernatants. The sandwich RIA shown in Chart 1*B* shows that PA is detectable only in prostate tissue. Sera analysis shown in Table 3 shows serum PA levels with the monoclonal sandwich immunoassay. Elevated levels (>5 ng/ml) are found only in prostate cancer.

DISCUSSION

We report the production and characterization of 3 mono-

Table 1

Cross-binding and cross-blocking of monoclonal antibodies to PA

The experiment was performed by incubating polyvinyl chloride 96-well plate (Cooke, Inc.) individual wells with 50 μl of 100 μg monoclonal antibody per ml in phosphate-buffered saline. After 12 hr at 4°, the wells were washed and incubated with 1 μl of 50 ng PA per ml in phosphate-buffered saline. After 2 hr at room temperature, the wells were washed and incubated with 50 μl of blocking monoclonal antibodies at 200 μg/ml in phosphate-buffered saline. After 1 hr, the wells were again washed, incubated for 2 hr with 50,000 cpm of [^{125}I]iodogen-labeled monoclonal antibody (10^7 cpm/μg). The wells were washed again with RIA buffer (1% bovine serum albumin in phosphate-buffered saline), cut with a hot wire device, and counted on a γ counter.

Plate coat antibody	Blocking antibody	Labeled antibody (cpm bound/well) 1F3	2G7	1C5
1F3	None	109	2244	1604
2G7	None	2156	119	157
1C5	None	NT[a]	119	259
1F3	1F3	91	1858	1033
2G7	1F3	112	103	177
1C5	1F3	277	116	156
1F3	2G7	87	401	196
2G7	2G7	NT	112	204
1C5	2G7	NT	84	475
1F3	1C5	118	1760	1971
2G7	1C5	NT	116	298
1C5	1C5	2404	100	260

[a] NT, not tested.

clonal antibodies which react specifically with determinants on PA. They are able to react with PA bound to a polyvinyl chloride well and immunoprecipitate ^{125}I-labeled PA. They define 2 unique non-cross-blocking determinants on PA that are each present at one site/molecule. The specificity of these antibodies was confirmed by absorption studies and sandwich RIAs with extracts of various normal tissues. In addition, antibody specificity was confirmed by the intracellular immunoperoxidase staining of formalin-fixed paraffin sections of benign and malignant prostate epithelia but not other human tissues except renal.

All 3 antibodies fail to react with membrane preparations of prostate, suggesting that there is little, if any, antigenically detectable PA in surface membranes of prostate epithelium.

A sandwich RIA using 2 of the monoclonal antibodies was used to detect levels of 5 ng PA per ml in sera. Normal female and male sera had no PA, but it was detected in the sera of patients with prostate carcinoma, confirming the earlier report of Kuriyama *et al.* (5) with a heteroantisera sandwich assay for PA with a sensitivity of 0.1 ng PA per ml.

The cross-binding studies of Table 3 demonstrated the necessity of using different monoclonal antibodies to PA in designing the sandwich RIA. With only 1 determinant/molecule, one cannot obtain binding of a monoclonal antibody to the antigen after it has been already bound to the same antibody in a well. With the sandwich RIA, the sensitivity to PA can be increased from μg PA per ml in the absorption assay to ng PA per ml.

The immunoperoxidase staining of paraffin tissue sections

Table 2

Monoclonal antibody binding to human tissue preparations

Tissue membrane preparations were made as described previously (1). Forty μl of the tissue preparation at 0.2 mg/ml were incubated at 4° for 12 hr in polyvinyl chloride 96-well plates. The plates were washed with RIA buffer, as in Table 1, and incubated with 20 μl of hybridoma supernatant. After 1 hr, the wells were washed and incubated with 20,000 cpm of [^{125}I]iodogen-labeled rabbit anti-mouse F(ab')$_2$. After 1 hr, the wells were washed, cut, and counted as in Table 1. The control PA was used at 50 μg/ml, as were the membrane preparations.

Tissue	Monoclonal antibody (cpm binding)[a]		
	1C5	2G7	1F3
Membrane preparations			
Normal prostate	250	267	300
BPH	279	269	330
Prostate cancer	230	272	260
Kidney	240	210	150
RBC	210	250	196
Spleen	211	300	278
Thymus	240	197	172
Liver	157	140	100
Pancreas	130	100	100
Brain	210	260	170
Controls			
PA	1315	1085	1084
Buffer	250	297	211

[a] Average of 3 determinations with S.D. of less than 100 cpm.

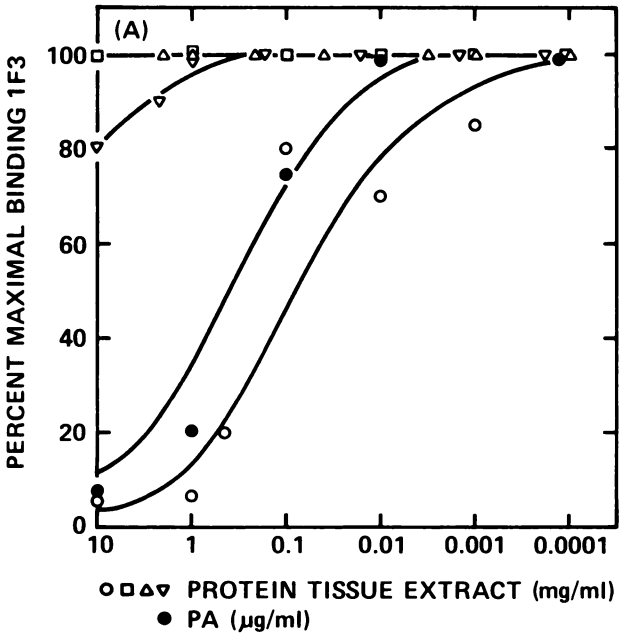
Table 3

Serum levels of PA determined by monoclonal sandwich radioimmunoassay

Assay was performed with 2G7 (100 μg/ml), 50 μl plate coat, and 50 μl serum followed by 50,000 cpm of [^{125}I]iodogen-labeled 1F3 (10^7 cpm/μg). The wells were washed, cut, and counted. Standards are run as dilutions of PA in female serum with detection of levels as low as 5 ng/ml.

Patient	PA level (ng/ml)
Normal	<5 (6/6)[a]
Prostate cancer Stage D	1500 (5/20)
	100 (2/20)
	<5 (13/20)
Other cancer patients	<5 (4/4)

[a] Numbers in parentheses, number of patients with given level/number of patients examined.



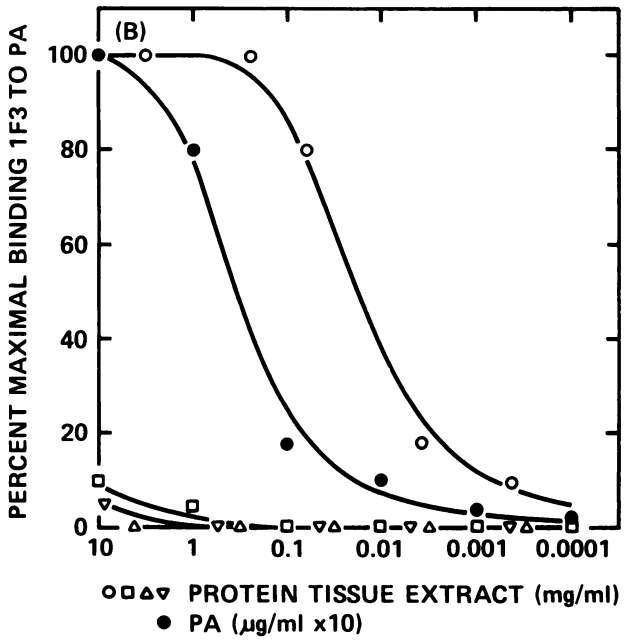


Chart 1. Absorption and sandwich RIA of PA (*A*). Absorption experiment was performed by incubating 14 μl of 1F3 hybridoma supernatant with 50 μl of tissue extract prepared by homogenization and 20,000 × *g* clarification of tissues. After 12 hr at 4°, the mixture was centrifuged at 100,000 × *g* to remove immune complexes, and 40 μl were applied to wells coated with 30 μl of PA (50 μg/ml). After being washed with RIA buffer, the wells were incubated with 20,000 cpm of rabbit anti-mouse immunoglobulin (10^7 cpm/μg) for 1 hr. The wells were then washed, cut, and counted as in Tables 1 to 3. The tissues examined were: ●, PA; ○, prostate; △, tonsil; ▽, liver; and □, kidney. Protein assays were performed by the Lowry assay. Maximal binding with PA was 2500 cpm; prostate, 2300 cpm; tonsil, 2400 cpm; liver, 2200 cpm; and kidney, 2500 cpm (*B*). Sandwich RIA was performed by coating the well with 2G7 (100 μg/ml), 50 μl, followed by 50 μl of tissue extract and, finally, 50,000 cpm of ^{125}I-labeled 1F3 antibody. The wells were cut, washed, and counted. ●, PA; ○, prostate; △, tonsil; ▽, liver; and □, kidney. Maximal binding with PA and prostate tissue was 15,000 cpm.

using monoclonal antibodies is very useful for defining the cellular localization of antigenic reactivity in a tissue. However, the sensitivity and specificity are affected by several variables. Reactivity may be lost by methods of tissue fixation. The lack of satisfactory staining on frozen sections of prostate tissue may be due to washout of antigen (compared with immobilization due to formalin fixation) or poor accessibility of the monoclonal antibody-binding sites in unfixed tissues. Reactivity with renal tissue may be due to presence of PA or its 1F3 determinant. However, no PA is detected by the absorption and sandwich RIA for renal tissue. More likely, we believe, there is a nonidiotypic binding between monoclonal immunoglobulin and a receptor on tubular epithelium. We have seen this pattern of binding for a variety of mouse monoclonal antibodies of varying isotype and specificity (1). Further experiments need to be done to explain this renal tubular binding.

The lack of detectable PA on cell membranes is of relevance to 2 of the potential applications of any tissue specific antigen: (*a*) body scanning with radioactively labeled antibodies (8) and (*b*) passively administered antibody therapy (7). Both of these applications would be expected to be more effective if the antigen being detected were available on the cell surface for reaction with circulating antibody. It may be that prostate-specific membrane antigens (1) will be more useful for these applications, although this should not affect other uses of PA for the study of prostate differentiation, serum levels in diseased patients, and tissue identification.

We were able to detect PA in the sera of patients with prostate cancer but not in the sera of other patients. However, only 25% of known prostate cancer patients give a positive test for PA. Thus, its role in the detection and monitoring of prostate cancer is yet to be defined.

Other than prostatic acid phosphatase, PA is the best characterized prostate-specific antigen which is also expressed by prostate carcinoma. We anticipate that these reagents will provide useful tools for more detailed study of the biochemistry of PA and a homogeneous source of antibody for serum and tissue section antigen detection.

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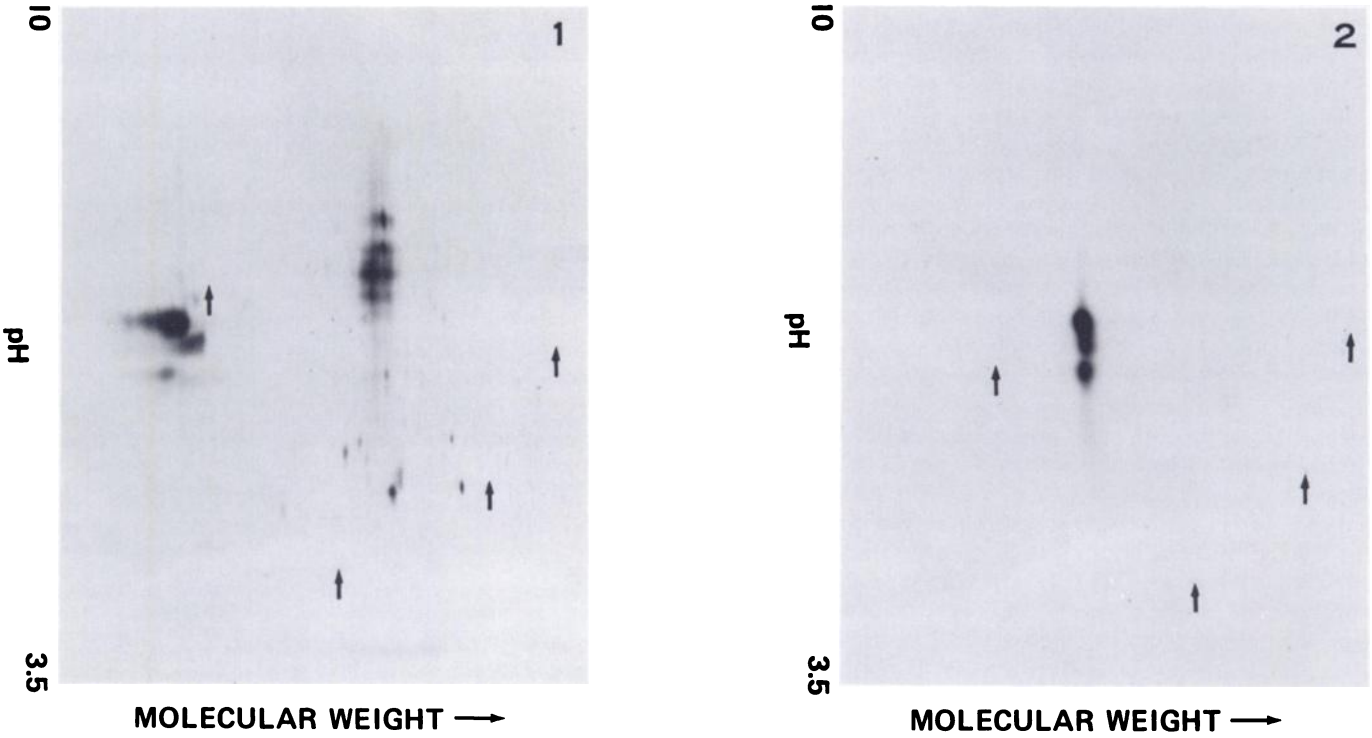

Fig. 1. Two-dimensional gel of [^{35}S]methionine-labeled hybridoma antibody, 1F3. The hybridoma cells were incubated with 0.5 mCi [^{35}S]methionine in 1 ml of methionine-free Roswell Park Memorial Institute Tissue Culture Medium 1640 with dialyzed fetal calf serum (10%). After 5 hr, the supernatant was mixed with rabbit anti-mouse immunoglobulin bound to Staph A Sepharose and immunoprecipitated. The precipitate was mixed with urea and lysis buffer and run on 2-dimensional gels as described (3). *Abscissa*, nonequilibrium isoelectric focusing; *right axis*, pH 3.5; *left axis*, pH 10. *Ordinate*, molecular weight. *Top*, *M*, 170,000; *bottom*, *M*, 10,000. *Arrows*, location of marker proteins (*M*, 30,000, 43,000, 67,000, and 94,000) run on this gel.

Fig. 2. Two-dimensional gel of ^{125}I-labeled PA immunoprecipitated with hybridoma antibody 1F3. The PA, purified as described (16), was labeled with iodogen as described (3). The unlabeled antigen was mixed with hybridoma supernatant, and the immunoprecipitate was collected, using rabbit anti-mouse immunoglobulin-coated Staph A. The Staph A was washed, and labeled immunoprecipitate was released from Staph A with lysis buffer and run on 2-dimensional gels as described (3). *Abscissa* and *ordinate*, same as for Fig. 1. *Arrows*, location of marker, as in Fig. 1.

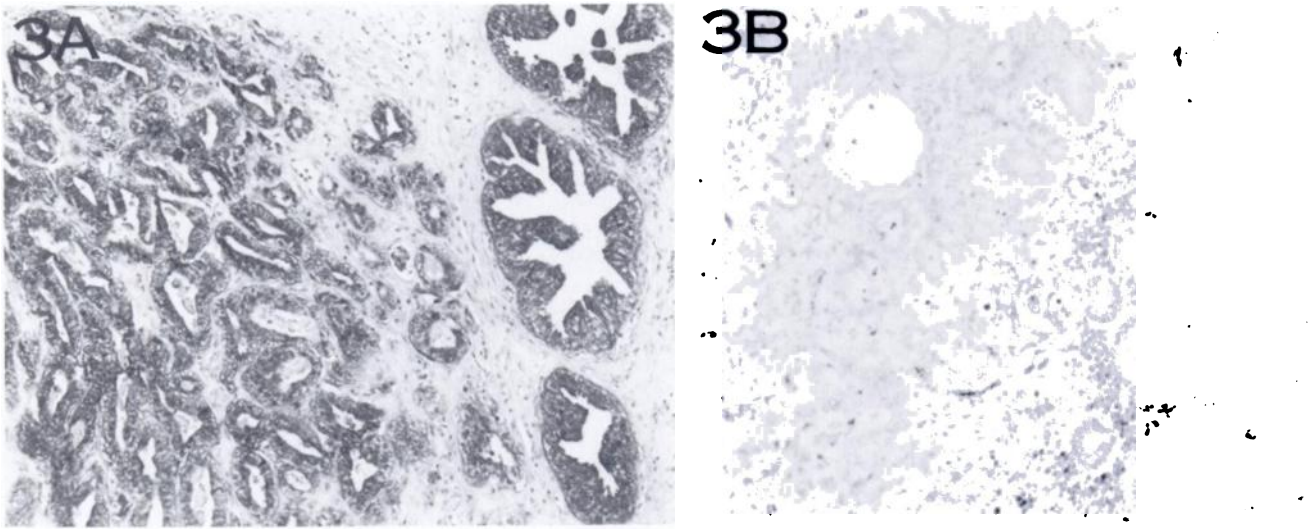

Fig. 3. Immunoperoxidase stains of formalin-fixed, paraffin-embedded sections with monoclonal antibody 1F3. *A*, both benign hyperplastic prostate (*right*) and prostate carcinoma (*left*) stain intensely; *B*, neither benign gastric epithelium (*right*) nor gastric carcinoma (*left*) stain significantly. Nuclear counterstain outlines cell patterns in each case.

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