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A Prostate Antigen in Sera of Prostatic Cancer Patients[1]

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ABSTRACT

A prostate antigen has been detected by a rocket immunoelectrophoresis technique in 17 of 219 sera obtained from patients with advanced prostatic cancer. Sera from 175 patients with nonprostatic cancers, including those with late-stage disease of the breast, lung, colon, rectum, stomach, and pancreas, were antigen negative as were 20 samples each from normal adults and age-matched males. Antigen in sera showed immunological identity with antigen in prostate tissue as determined by immunoprecipitation peak enhancement experiments. Using antibody affinity chromatography and radioimmunoprecipitation techniques, the antigen in sera was purified and subjected to sodium dodecyl sulfate electrophoresis; it exhibited a molecular weight of approximately 36,000, similar to that of antigen isolated from prostatic tissue.

INTRODUCTION

In recent years, considerable effort has been made to identify enzyme or antigen markers for various types of cancers with the view towards developing specific diagnostic reagents. The ideal tumor marker would exhibit, among other characteristics, organ site or cell-type specificity and would be released into the circulation or other biological milieu which is easily obtained from individuals. Previous investigators have demonstrated the occurrence of human prostate tissue-specific antigens (1-3, 8, 11). One prostate antigen has been identified as PAP[3] (6), and other workers have indicated the presence of a species-specific prostate antigen (8). Although immunological characterization and diagnostic evaluation of PAP have been reported (6, 9), similar information is not yet available regarding prostate antigens other than acid phosphatase. We have recently developed antiserum with which we have detected and isolated a prostate antigen from prostatic tissues distinct from acid phosphatase (13). The present studies were undertaken to determine whether this antigen may be found in patients' sera.

MATERIALS AND METHODS

Production of Human Prostate-specific Antiserum. Purified preparation of prostate tissue-specific antigen was prepared as reported previously and used to immunize rabbits (13). Sera were collected, heat inactivated, and stored at −20° until use. Antibodies to normal plasma constituents were removed by treatment of the antiserum with glutaraldehyde-insolubilized normal plasma obtained from male and female adults (4). This antiserum reagent is termed "prostate antiserum."

Human Sera. Serum samples were drawn from patients with prostatic and nonprostatic cancers at the Roswell Park Memorial Institute. Normal sera were obtained from apparently healthy laboratory personnel. Age-matched sera were obtained from male volunteers, 55 years old or older, with the cooperation of the Salvation Army, Buffalo, N. Y. Chapter.

Rocket IEP. Rocket IEP was performed on cellulose acetate membranes (Bioware Inc., Wichita, Kans.) using 0.83% agarose (low electroendoosmosis; Sigma Chemical Co., St. Louis, Mo.) in 0.08 M Tris-0.024 M Tricine-0.024 M sodium barbital containing 0.3 mM calcium lactate and 0.02% sodium azide. Prostate antiserum at various final concentrations (0.5 to 2.0%) was incorporated into the agarose at 55° prior to plating (14). Samples were applied to circular wells (5.0 mm) and electrophoresed at 5 V/cm overnight at 4° using the mixture above as running buffer.

Protein Determination. Total protein determination was performed using the Bio-Rad protein assay (Catalog No. 500-0001; Bio-Rad, Richmond, Calif.).

Conjugation of Purified Prostate Antiserum (IgG) to Sepharose 4B. The method of ammonium sulfate precipitation and anion-exchange chromatography was utilized to isolate the IgG fraction of prostate antiserum (9). The purified IgG (5 to 10 mg protein per ml gel) was dissolved in 0.1 M sodium carbonate buffer, pH 8.0, containing 0.5 M NaCl and mixed with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) according to the manufacturer's procedure. The procedure resulted in coupling of approximately 85% of the IgG, as determined by spectrophotometry at 280 nm.

Purification of Prostate Antigen from Serum. Sera obtained from patients with advanced prostatic carcinoma or normal human sera were pooled (20 ml each) and applied to an immunoadsorbent consisting of prostate antiserum (IgG) coupled to Sepharose 4B. Subsequent to sample application, the adsorbent was washed with 0.05 M sodium phosphate, pH 7.2, containing 0.5 M NaCl until the column effluent showed an absorbance at 280 nm which was under 0.01. The binding fraction was eluted by the use of PB-NaCl buffer containing 3.5 M KSCN, dialyzed against H_2O at 4°, and then lyophilized.

Immunoadsorbent-purified material (25 μg) was reconstituted in 0.5 M phosphate buffer, pH 7.0, and radioiodinated by the procedure of McConahey and Dixon (10). Unreacted iodine was separated by passage over Sephadex G-25.

The affinity-purified and ^{125}I-labeled preparation was used in the following immunoprecipitation experiments. To 100 μl of radiolabeled preparation was added an excess (100 μl) of anti-human whole serum (Bio-Rad), and the mixture was incubated overnight at 22°. To the reaction mixture were added 200 μl of Pansorbin (*Staphylococcus aureus* containing Protein A; Calbiochem-Behring Corp., LaJolla, Calif.) and further incubated

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[3] The abbreviations used are: PAP, prostatic acid phosphatase; IEP, immunoelectrophoresis; PB-NaCl, 0.05 M phosphate buffer containing 0.15 M NaCl, pH 7.2.

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for 2 hr. The adsorbent along with the immune complexes were sedimented by centrifugation at 1,000 × *g* for 15 min, and the supernatant was collected. This procedure was used to remove any radioactively labeled proteins which specifically reacted with antibodies to normal serum constituents and those which nonspecifically combined with rabbit serum components or the Pansorbin reagent. The supernatant, as obtained above, was subjected to an identical immunoprecipitation except that prostate antiserum was utilized instead of anti-human whole serum. Immune complexes which bound to the Pansorbin were collected by centrifugation and washed with PB-NaCl. Elution was performed by resuspension in 100 μl of 2% sodium dodecyl sulfate with or without the addition of 2% dithiothreitol and centrifugation to remove the inert adsorbent. Samples were then analyzed by sodium dodecyl sulfate electrophoresis as previously described (13). After electrophoresis, gel slices were counted for radioactivity in an Auto-Gamma spectrometer.

Immunoprecipitation Comparison of Prostate Antigen and PAP. Double immunodiffusion analysis of the 2 prostatic antigens was performed as described (13). In some experiments, purified PAP was allowed to react with serially diluted antiserum to PAP (rabbit AP_{11}) which was preabsorbed with either purified prostate antigen (in PB-NaCl buffer) or PB-NaCl buffer alone. PAP was prepared as described previously (9) and was homogeneous as evaluated by electrophoresis. Additionally, PAP and purified preparations of prostate antigen were cross-matched against analogous antisera using immunodiffusion. After a 48-hr incubation, plates were washed and stained for the presence of acid phosphatase (6). In some cases, the plates were further stained for protein using Coomassie Blue R-250 (13).

Gel Filtration Chromatography. Serum samples (0.5 ml) previously shown to be prostate antigen positive by rocket IEP were applied to a column (0.9- x 60-cm) packed with Sephadex G-200 gel in PB-NaCl buffer. Eluted fractions were concentrated and assayed for prostate antigen using rocket IEP. Molecular weight calibration was performed using human γ-globulin, bovine serum albumin, ovalbumin, and chymotrypsinogen A (Pharmacia).

RESULTS

Immunological Reactivity of Prostate Antigen in Sera. The optimal concentration of prostate antiserum required in the rocket IEP procedure was determined by examining the migration of prostate antigen in gels containing varying concentrations of antiserum. Using this procedure, 13 ng (0.5 μg/ml) of antigen could be detected after the plates were stained for the presence of immunoprecipitated protein. Using this assay, serum samples obtained from 20 normal adults and 20 male volunteers over the age of 55 years showed no reactivity against the antiserum (Table 1). Also, serum was drawn from a total of 175 patients with various advanced cancers, including patients with cancers of the lung, colon, rectum, stomach, pancreas, thyroid, and breast and with myeloma. All sera obtained from patients with nonprostatic cancers were prostate antigen negative when assayed by the rocket IEP procedure. However, of 219 sera examined from advanced prostatic cancer patients, 17 or approximately 8% showed the presence of prostate antigen in circulation, as judged by a distinctly staining immunoprecipitation reaction. All sera exhibiting a positive reaction for prostate antigen were subsequently subjected to the same assay, and reproducibility of the test was 100%.

In order to determine the immunological relationship between the antigen in tissue and serum, immunological "peak enhancement" experiments were performed. Samples of sera and prostate tissue extracts were subjected to rocket IEP both individually and immediately after mixing, as shown in Fig. 1. When assayed individually, each sample produced a single immunoprecipitation reaction. Mixing of samples in peak enhancement experiments was also noted to produce a single reaction, and the peak height was greater than that of individual samples. In addition to peak height enhancement, immunoprecipitation fusion occurred, showing the immunological identity of antigens from sera and tissue sources (5).

Purification of Prostate Antigen from Sera. When sera from prostate cancer patients were examined by Sephadex G-200 gel filtration, the reactive antigen eluted as a single peak between M.W. 90,000 and M.W. 100,000 (Chart 1). Since antigen isolated from prostatic tissues shows a molecular weight of approximately 34,000 (13), the reactive antigen in serum was purified by antibody affinity chromatography, using prostate antiserum (IgG) coupled to Sepharose 4B for further characterization.

Table 1

Reactivity of prostate antiserum with human sera by rocket IEP

All sera were aliquoted and stored at −20° or −70° until required. Sample volumes of 25 μl were used for all studies.

Serum donors	% of positive reaction
Normal adults (male and female)	0 (0/20)[a]
Age- and sex-matched controls	0 (0/20)
Patients with advanced cancers[b]	
Lung carcinoma	0 (0/83)
Thyroid carcinoma	0 (0/1)
Colon-rectal carcinomas	0 (0/22)
Stomach-pancreas carcinomas	0 (0/34)
Breast carcinoma	0 (0/33)
Myeloma	0 (0/2)
Prostate carcinoma	8 (17/219)

[a] Numbers in parentheses, number of patients positive per total.
[b] Each case was pathologically confirmed.

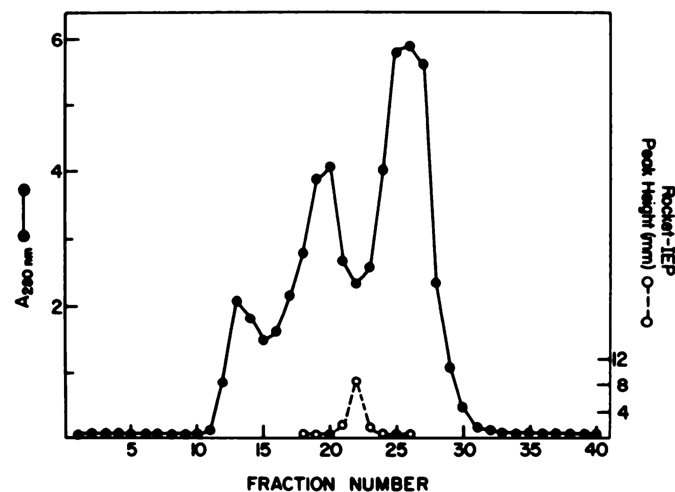

Chart 1. Sephadex G-200 elution profile of prostate antigen-positive sera from prostatic cancer patients. Antigen activity eluted between the immunoglobulin and albumin peaks (M.W. approximately 100,000). Eluted samples were analyzed for protein by absorbance at 280 nm (●) and prostate antigen (O) by rocket IEP.

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The yield of protein which bound to the immunoadsorbent after application of prostatic cancer patient sera was 75 μg and was shown by IEP to contain normal serum protein contaminants (data not shown) in addition to prostate antigen (Fig. 2). Using the same purification procedure and normal human sera, no detectable prostate antigen was isolated (Fig. 2). Immunoreactive affinity-purified material from patient sera was radiolabeled and further purified by sequential immunoprecipitations, and this preparation was examined by sodium dodecyl sulfate electrophoresis (Chart 2). The purified and radiolabeled material exhibited a molecular weight of 36,000 in the presence of reducing conditions and a similar value in the absence of dithiothreitol.

Comparison of Prostate Antigen with PAP. Since PAP has been shown to be prostate organ specific and found in the sera of patients, this antigen was immunologically compared to the presently described prostate antigen (Figs. 3 and 4). As shown in Fig. 3, the reactivity of antiserum to PAP was not diminished by pretreatment of the serum with purified prostate antigen. In a cross-match type of immunodiffusion experiment (Fig. 4), antiserum to PAP showed no reaction against 2 preparations of prostate antigen; conversely, prostate antiserum failed to react with purified PAP.

DISCUSSION

In a previous report, the detection and isolation of a prostate-specific antigen was presented (13). Other investigators have also indicated the existence of prostate-specific antigens (1, 2, 8), and the present data extend these observations to show that a prostate antigen other than acid phosphatase can be detected in sera obtained from prostatic cancer patients. Organ-specific "differentiation" antigens represent potentially valuable candidates regarding tumor detection. Variations in the release of antigens into the circulation may be related to the pathological stage of the organ, a phenomenon shown to exist for other differentiated or "eutopic" cell products, *e.g.*, PAP (6) and thyrocalcitonin (7).

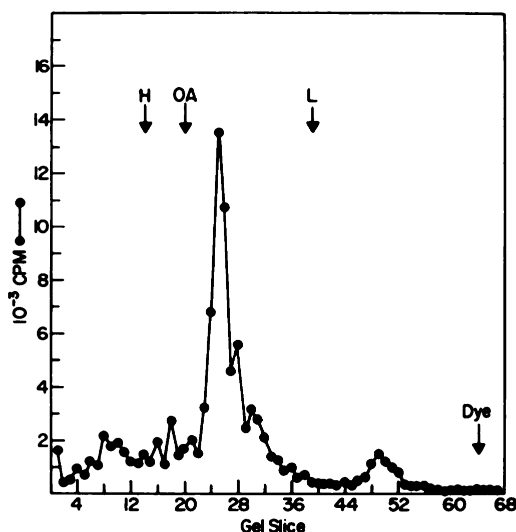

Chart 2. Sodium dodecyl sulfate electrophoresis of radiolabeled prostate antigen from serum. Pooled sera from cancer patients were subjected to immunoadsorbent chromatography and further purified by immunoprecipitation with prostate antiserum, as described in "Materials and Methods." Immune complexes after adsorption onto Pansorbin were analyzed by electrophoresis in the presence of sodium dodecyl sulfate. Molecular weight markers, run in separate gels, included H-chain of human immunoglobulin (*H*; M.W. 50,000), L-chain (*L*; M.W. 25,000), and ovalbumin (*OA*; M.W. 43,000).

To determine if the serum-borne antigen is immunologically equivalent to the antigen in tissue, antigen mixing experiments were performed in order to assess immunological peak enhancement. Results indicate immunological identity between the antigen in serum and tissue (5). Gel filtration analysis of the antigen found in serum reveals that, in the absence of denaturants or reducing agents, it exhibits a higher molecular weight than does antigen isolated from tissue (100,000 *versus* 34,000, respectively) (13). This may indicate that polymerization of the protein may have occurred. To examine this possibility, antigen in serum was purified using affinity chromatography and immunoprecipitation, subsequent to radioiodination. Electrophoresis of this preparation in the presence of sodium dodecyl sulfate revealed a molecular weight not dissimilar to that for the tissue-isolated antigen. Both lines of evidence, immunological and physicochemical, support the idea that the antigen found in patient serum is derived from prostatic cells and may represent a valuable indicator for prostatic neoplasia.

The presently described prostate antigen has been shown to be different from PAP, another prostate-specific antigen with a molecular weight of approximately 100,000 (6, 9). By using a cross-match type of gel diffusion experiment, prostate antiserum does not cross-react with purified PAP, and antiserum to PAP shows no reaction with 2 different preparations of prostate antigen. These data, therefore, rule out the possible identity between these 2 prostate organ-specific proteins.

At the present stage of development, the rocket immunoelectrophoretic procedure described here can be used to detect prostate antigen in a low percentage of sera from patients with late-stage disease. Although only 8% of these sera were immunopositive, control sera, including those from patients with late-stage nonprostatic cancers, were consistently negative. These results have prompted the development of a more sensitive assay for prostate antigen, which may be of value in detecting disease at earlier stages. Nevertheless, the present data provide additional insight into the biology of prostatic tumors and indicate that prostate-specific proteins, including prostate antigen, may be released from prostate cells during the course of tumor development.

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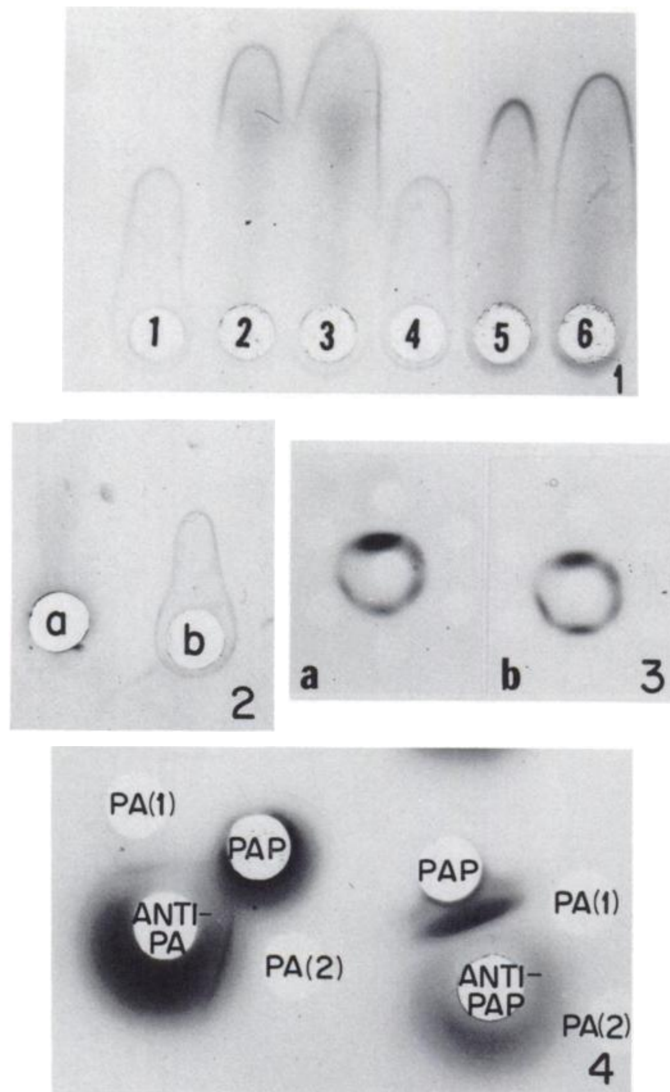

Fig. 1. Immunological relationship between prostate antigen as detected in patient serum and prostate tissue. *Sample Wells 1* and *4* contained diluted Patient Sera 4131 and 4066, respectively. *Sample Wells 2* and *5* contained diluted prostate tissue extract, prepared as described previously (13). *Wells 3* and *6* contained a mixture of patient's serum with tissue extract to assess immunoprecipitation fusion and peak enhancement. Sera and tissue extracts were at the same final protein concentration in each sample well by the use of appropriate volumes of diluting buffer (PB-NaCl). All samples were 25 µl. One % prostate antiserum was incorporated into 0.83% agarose and electrophoresed at 5 V/cm for 20 hr at 4°.

Fig. 2. Rocket IEP assay for prostate antigen. Serum samples were applied to prostate antiserum which was immobilized on Sepharose 4B, and the binding fraction was recovered as described in "Materials and Methods." Material purified from normal human serum (*Well a*) and from prostatic cancer patient serum (*Well b*) were each adjusted to 0.1 mg protein per ml buffer prior to analysis. Samples were assayed on 2 separate occasions and, in each case, antigen was detected in material purified from patient sera only. Additionally, material obtained from normal sera was tested at 0.5 mg protein per ml buffer, and no antigen was detected (not shown). Rocket IEP conditions were as indicated in Fig. 1 legend.

Fig. 3. Immunodiffusion analysis of treated antiserum to PAP. Antiserum to PAP (rabbit AP_{11}) was mixed with purified prostate antigen at a final concentration of 1.2 mg prostate antigen per ml serum. The mixture was incubated for 24 hr at 22°, centrifuged, and used in immunodiffusion experiments. Treated antiserum was applied to immunodiffusion outer wells after 2-fold serial dilutions were made with PB-NaCl buffer (starting from *top well* and proceeding clockwise). The *center well* contained purified PAP (0.1 mg/ml). Subsequent to a 48-hr incubation, the plate was extensively washed and then treated with dye-substrate to reveal the presence of acid phosphatase enzyme activity. In *a*, outer wells contained a dilution series of antiserum pretreated with prostate antigen. In *b*, outer wells contained a dilution series of antiserum diluted with PB-NaCl buffer alone. No inhibition of the reaction between PAP and analogous antiserum was observed due to pretreatment with prostate antigen.

Fig. 4. Immunological comparison of PAP and prostate antigen. Purified preparations of prostate antigen and PAP were cross-matched for their reactivity to both antisera. Reagents were plated into the immunodiffusion wells and allowed to react for 48 hr. The plates were washed and then treated first with dye-substrate to reveal enzyme activity and then with protein stain. Both antisera were noted to react with their respective antigens only. *PA(1)*, purified prostate antigen Preparation 1 (0.7 mg/ml); *PA(2)*, purified prostate antigen Preparation 2 (2.4 mg/ml); *Anti-PA*, prostate antiserum reagent; *Anti-PAP*, PAP antiserum.

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