Urological Research 4, 111-117 (1976)

Urological Research

Production of Specific Antibody to Purified Prostatic Acid Phosphatase

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Received: March 25, 1976

Summary. Prostatic acid phosphatase may well be a prime antigenic protein in prostatic tissue and fluid. Extraction of the enzyme in highly purified form from prostatic fluid and benign hypertrophic prostatic tissue provides a unique antigen capable of inducing a prompt and specific antibody response in the goat and rabbit as manifested by immunodiffusion, immunoelectrophoresis, and immunofluoresence techniques. In prostatic cancer patients with elevated serum acid phosphatase levels it is possible to detect humoral circulating PAP antigen by standard immunoelectrophoretic methods and to confirm the existence of the enzyme by radioautography, L-tartrate inhibition, and the Gomori or Burstone staining procedures. Preliminary indirect prostatic immunofluorescence studies consistently demonstrated characteristic fluorescent foci in the paranuclear areas of benign prostatic epithelial cells, the presumed area of synthesis of prostatic acid phosphatase. Consideration has been given to the possibility of the development of a radioimmunoassay for prostatic acid phosphatase utilizing a heterologous antiserum to the enzyme extracted from human prostatic fluid.

Key words: Prostatic cancer - PAP-prostatic acid phosphatase antigen - RIA-radioimmunoassay - Prostatic acid phosphatase antibody - Immunofluorescence, prostatic.

A general interest in prostatic antigens has developed incident to the reports of Flocks, (1) Peel, (2) and Moncure (3) on the canine prostate and in the human prostate by Ablin et al., (4) Shulman et al., (5) Mattila et al., (6) and Stonington and Hemmingsen (7). These investigators initially utilized unaltered prostatic fluid and crude prostatic tissue extracts in the production of heteroimmune antibodies to prostatic proteins. The variety of precipitin patterns secured in these reports indicated the necessity for the isolation and utilization of a single tissue specific prostatic antigen capable of eliciting a quantitative antibody response in a suitable mammalian system. In a survey of potentially specific prostatic proteins we were impressed with the potentially antigenic nature of prostatic acid phosphatase (PAP) and elected initially to extract and purify the human prostatic acid phosphatase in an L-tartrate inhibitable form of this enzyme from prostatic fluid and benign hypertrophic prostatic tissue for an immunization protocol. The presence of PAP in human prostatic tissue and fluid represents a distinguishing biochemical feature, well known to clinicians since the original reports of Bodansky, (8) and Gutman and Gutman (9) and the later refinements of Fishman and Lerner on the L-tartrate inhibition of prostatic acid phosphatase (10).

MATERIAL AND METHODS

Utilizing a modification of the techniques of Ostrowsky and Tsugita, (11) and Ostrowsky and Rybarska, (12) enzymatically active PAP was purified from prostatic fluid and hypertrophic prostatic tissue. The purified PAP antigen was demonstrated on suitable immunization protocol to be capable of inducing

measurable and reproducible antibody responses in the rabbit and goat as evidenced by immunodiffusion and immunoelectrophoresis preparations. In addition, fluorescein labelled PAP antibody was found to specifically localize in the paranuclear area of benign prostatic epithelial cells on indirect fluorescence microscopy.

Preparation of PAP Antigen

Benign hyperplastic prostatic tissue was obtained at surgery and either immediately processed or quickly frozen and stored at -20°C. All subsequent steps were carried out in a cold room at 4° C. Five grams of minced prostatic tissue in approximately 40 ml of 0.3 M acetate buffer at pH 4.0 were homogenized in a Virtis homogenizer. The resulting extract was centrifuged at 3000 g and the supernatant retained. The supernatant was brought to 50 ml with the same buffer and the solution adjusted to 45.0% ammonium sulphate concentration and allowed to stand for 24 h. The resulting supernatant was saved and adjusted to 65.0% ammonium sulphate concentration. The precipitate which formed after 24 h was centrifuged at 3000 g and the supernatant discarded. The precipitate was dissolved by the addition of 2.0 ml of 0.0175 M phosphate buffer at pH 7.0, and dialyzed against the same buffer. The initial homogenate contained 158,500 Sigma units of activity and had a protein content of 500 mg of protein. The final precipitate contained 116,500 Sigma units of activity with a protein content of 93.0 mg. The specific activity was 1,250 Sigma units per mg of protein which represented an enrichment of approximately 4 fold. The yield of enzyme obtained by this procedure was 75.0%. This semipurified preparation was used for immunization and designated as Fraction I.

The semi-purified antigen, Fraction I, was further chromatographed on a DEAE cellulose column and eluted with 0.070 M Na phosphate buffer by a stepwise gradient from pH 7.0 to pH 6.0, 88,000 Sigma units of enzyme activity containing 23.4 mg of protein were recovered. One milligram of protein equalled 4150 Sigma units. The fractions were pooled and designated as purified antigen, or Fraction II.

Prostatic fluid was obtained by prostatic massage, and the collected specimens pooled to give about 4-6 ml. After adding 5 ml, 0.05 M Tris - 0.10 M KCl buffer, pH 6.5, the sample was placed on a Sephadex G-100 column previously equilibrated with the same buffer. Elution was carried out at 4° C with 0.05 M Tris - 0.10 M KCl buffer, pH 6.5. Two peaks were recovered consistently from the column. The majority of protein (80-83%) was present in the first peak. The second peak contained 95-97% of the prostatic acid phosphatase in a purified form and was utilized for immunization purposes and enzymatic determinations. The protein content was approximately 0.4 mg/-ml, a 100 fold purification.

The spectrophotometric determination of PAP enzymatic activity was carried out with p-nitrophenylphosphate as substrate (Sigma 104). Liberated p-nitrophenol was assayed in a Beckman DU Spectrophotometer at 410 mu with a p-nitrophenol reference standard. Activity was measured in micromoles of substrate liberated in 30 minutes at 37° C in 0.2 ml of sample.

Radioiodination of Prostatic Acid Phosphatase

Radioiodination was carried out in the original packaged vial of ^{125}I. Twenty-five µl of 0.5 M phosphate buffer, pH 7.5, were added to the ^{125}I. Then 5 µg of purified enzyme and 200 µg of chloramine T were added each in 25 µl of 0.05 M phosphate buffer, pH 7.5, and gently shaken. Sodium metabisulphite in a concentration of 0.48 mg/0.1 ml in 0.05 M phosphate buffer, pH 7.5, was introduced and 0.2 ml of potassium iodide solution (10 mg/ml in 0.05 M phosphate buffer, pH 7.5) added. A Sephadex G-50 column (30 cm by 0.8 cm) was equilibrated with phosphate buffer, pH 7.5, and the isotopic iodinated mixture placed on the column and washed through. Two radioactive peaks were observed, the primary representing iodinated prostatic acid phosphatase and the secondary peak representing free iodine. The iodinated prostatic acid phosphatase was stable for 4-6 weeks at 4° C. The specific activity was calculated at 80 µc per µg of prostatic acid phosphatase.

Production of PAP Antibody

The immunization protocol was initially carried out with fractions I and II. Later, a second source of PAP antigen, was obtained by the elution of human prostatic fluid with the Sephadex G-100 technique described previously. The PAP antigen fractions were homogenized 1 : 1 with complete Freund's adjuvant. Two millilitres, representing about 1.0 mg of antigen, were injected intramuscularly weekly in multiple sites, into white, female, New Zealand rabbits and alternatively into goats. Maximum antibody production occurred usually in 1-2 months, at which time very variable titres of 1 : 100 to 1 : 3.000 were obtained as measured by the method of Ouchterlony (13). Antiserum was usually obtained by heart or ear vein puncture.

Fraction II was found to manifest much less antigenicity as also reported by Ostrowsky and Tsugita, (11) but manifested very high levels of enzymatic activity. It was not routinely utilized for antibody production due to the reduced antigenicity.

Double Radial Diffusion

The Ouchterlony double diffusion technique was utilized with a centre well containing purified PAP antigen (13). The five outer wells contained varying dilutions of rabbit or goat antiserum absorbed with human female serum ranging from 1 : 10 concentration to 1 : 1.000. A similar series of Ouchterlony plates were run with concentrated rabbit or goat PAP antiserum in the outer wells. It is to be noted that the absorbed PAP antibody must be in excess for the specific precipitin line to form. The precipitin lines with PAP antigen were verified by the Burstone procedure for acid phosphatase (15). In addition the enzymatic inhibition of PAP antigen by 0.04 M L-tartrate was also utilized (10).

Immunoelectrophoresis

Microscopic slides were prepared with a coating of 1% solution of agarose in 0.05 M barbital buffer at pH 8.4. Electrophoresis was carried out in a Kallestad electrophoresis chamber for 1.5 hours at 30 milliamperes and 90 volts. The sample of test serum containing suspected PAP antigen was placed in the upper and lower wells. After electrophoresis the center trough was filled with absorbed PAP antiserum. The resulting precipitin arcs were observed to form within 48 h and were verified in situ with the Burstone or Gomori and L-tartrate techniques for acid phosphatase as noted.

Verification of PAP Complex by Gomori Procedure and L-tartrate Inhibition

Precipitin lines were incubated in a solution of 3% β-glycerophosphate and 0.1% lead nitrate phosphate buffer at pH 5.0. Incubation was maintained usually for 15-20 min. The precipitin lines were then treated with 2% ammonium sulphide solution resulting in a black to brownish-black precipitate of lead sulphide at the site of the PAP antibody complex. Prior to the above incubation and staining of the precipitin lines the plates were washed for five complete days with frequent changes of normal saline to remove unreacted protein. Verification was also secured by addition of 0.04 M L-tartrate in phosphate buffer at pH 4.8 which inhibited the development of the antigen antibody complex and characteristic Gomori effect (14).

Indirect Immunofluorescence Microscopy

Cryostat sections of fresh hyperplastic human prostatic tissue were allowed to react with the PAP antiserum produced in the goat. After 30 min incubation at room temperature the slide was washed throughly with 0.9% saline 0.02 M phosphate buffer at pH 7.0. The slide was then incubated with fluorescein labelled rabbit anti-goat gamma globulin. After 30 minutes of incubation at room temperature the slide was rinsed repeatedly with buffer and finally with distilled water. Antibody localization for human prostatic acid phosphatase was consistently observed as focal yellow-green fluorescence in the paranuclear region of the cytoplasm of the prostatic epithelial cells.

RESULTS

Double Radial Diffusion Tests

Antiserum to purified human prostatic acid phosphatase (PAP) from both the goat and the rabbit was investigated by the immunodiffusion method of Ouchterlony. (13) Antibody titers from 1 : 100 to 1 : 3000 were consistently obtained in the goat or rabbit.

Precipitin bands were readily observed by Ouchterlony immunodiffusion with PAP antigen in the centre well and serial dilutions of absorbed PAP antibody from 1 : 10 to 1 : 1000 in outer wells (Fig. 1). In general, a single precipitin line was formed with purified PAP antigen and suitable absorbed goat or rabbit antibody in the gel diffusion plate. The crude antisera gave multiple preciptin lines on most diffusion plates. However, after absorption of the crude antisera with normal human female serum, regardless of the purity of the antigen preparations (Fractions I, II, or purified prostatic fluid), a single homogeneous precipitin line resulted on gel diffusion plates. Since the bands were observed to retain their enzymatic phosphatase activity for prolonged periods at room temperature under the conditions observed, it was possible to identify and stain them for acid phosphatase in situ by the Gomori and Burstone methods. (14, 15) In addition it was feasible to inhibit the formation of the bands by the addition of 0.04 M L-tartrate in phosphate buffer at pH 4.8 (Fig. 2).

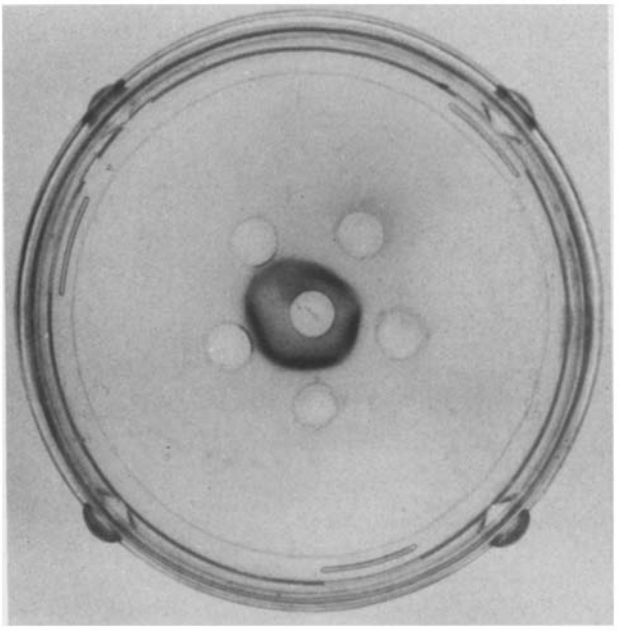

Fig. 1. Immunodiffusion discs, demonstrating the formation of precipitin lines with 2 ug of human PAP antigen in the center well and increasing dilutions of rabbit PAP antibody in the outer wells. Starting at upper right (1 : 10 dil.) proceeding clockwise antiserum dilutions of 1 : 50, 1 : 100, 1 : 500, 1 : 1000 were used

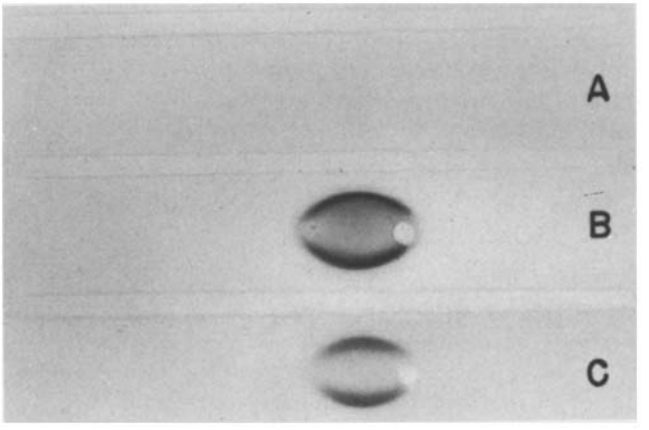

Fig. 3. Immunoelectrophoresis demonstrating the formation of antigen antibody complex. Human PAP antigen was placed in the anodic wells and after electrophoresis absorbed PAP rabbit antibody in the troughs. (A) control serum containing 0.17 Sigma units PAP antigen/ml (B) prostatic cancer serum containing 2.0 sigma units PAP antigen/ml (C) control serum containing added PAP antigen 2.5 Sigma units/ml

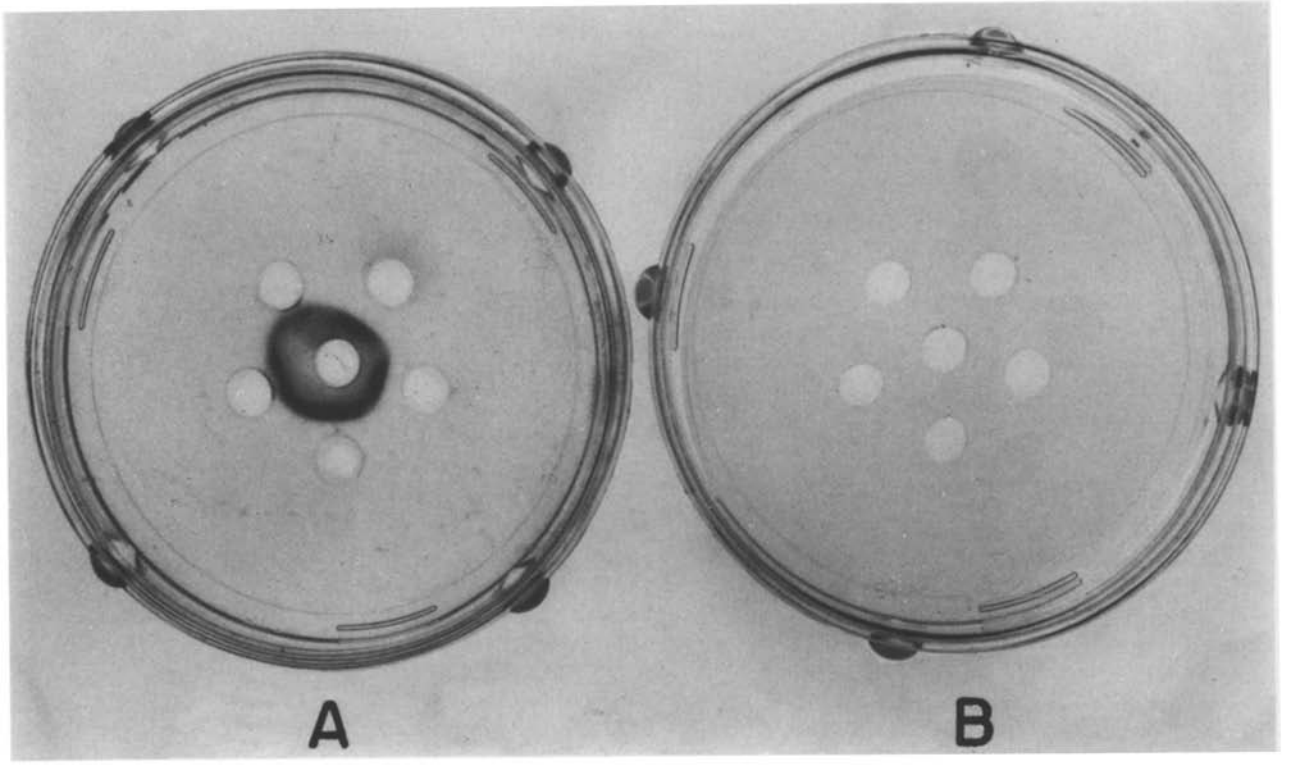

Fig. 2. Immunodiffusion discs demonstrating PAP antigen antibody complex with various dilutions of rabbit antibody (A) and inhibition by 0.04 M L-tartrate solution (B). In center well PAP (2 ug) and outer wells with diluted antibody. Starting upper right with 1 : 10 dilution and proceeding clockwise 1 : 50, 1 : 100, 1 : 500, 1 : 1000

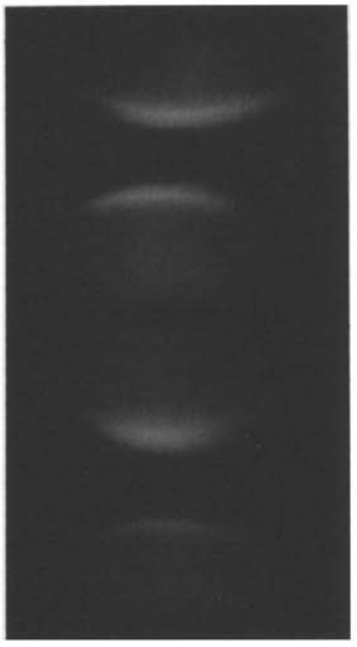

Fig. 4. Radioautograph of agarose immunoelectrophoresis. Human ^{125}I PAP antigen in the anodic wells and absorbed PAP rabbit antibody in the troughs

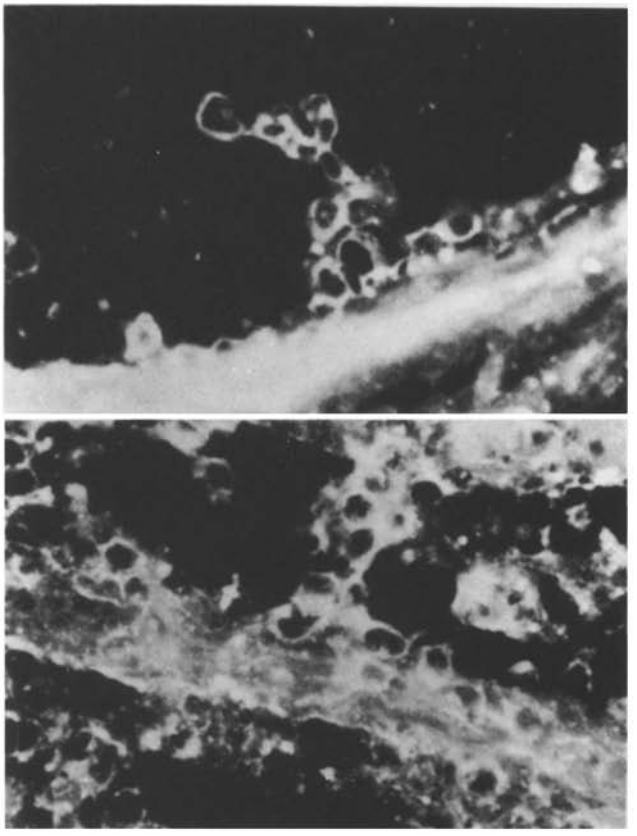

Fig. 5. Indirect immunofluorescence photomicrographs demonstrating localization of fluorescent PAP-AB in the paranuclear area of the cytoplasm of benign prostatic epithelial cells

Immunoelectrophoresis and Radioautography of PAP

Experience with the Ouchterlony double diffusion method for the detection of minute concentrations of PAP antigen in experimental and control sera stimulated the authors to examine the serum of patients with benign prostatic hyperplasia, untreated prostatic cancer, and prostatic cancer patients treated with oestrogens and bilateral corchiectomy. A series of sixty controls were secured from premarital, young males undergoing routine serologic tests for lues. These studies were carried out by slide immunoelectrophoresis in a Kallestad electrophoresis chamber with control male serum in the upper well, experimental serum in the lower well, and absorbed PAP antiserum in the center trough (Fig. 3). No precipitin bands were observed in the 60 normal young male control sera, 10 BPH sera, or in 18 histologically verified prostatic cancer patients treated or untreated presenting with normal values for total serum prostatic acid phosphatase. However, very significant precipitin bands were noted in 9 prostatic cancer patients, treated or untreated with significantly elevated serum prostatic acid phosphatase above 2.0 Sigma units/ml. The precipitin bands in the cancer patients' sera were readily stained by the Gomori and Burstone techniques and showed typical L-tartrate inhibition. When significant amounts of PAP antigen were added to control sera in a concentration above 2.0-2.5 Sigma units/ml, precipitin bands were readily observed on immunoelectrophoresis (Fig. 3 A, 3 B).

The radio-iodinated prostatic acid phosphatase antigen antibody complex was studied with a combination of agarose immunoelectrophoresis and radioautography. After immunoelectrophoresis and several days of incubation the preparation was washed, dried, and standard radioautography demonstrated single, discrete precipitin lines containing the isotopic material, indicating that the radio-iodinated prostatic acid phosphatase and antibody were monospecific (Fig. 4).

Immunofluorescence Microscopy

Cryostat sections from ten patients with benign prostatic hyperplasia were examined by means of indirect immunofluorescence microscopy (16).

In seven specimens typical yellowish-green fluorescence was noted in para-nuclear foci in the cytoplasm of the prostatic epithelial cells. No specific nuclear or cell membrane fluorescence was observed, but considerable staining occurred within prostatic ducts containing prostatic secretions (Fig. 5). Control cryostat sections of benign prostatic tissue untreated with fluorescein labelled antibody showed no typical paranuclear fluorescence.

DISCUSSION

The concept that prostatic acid phosphatase may well be the principal antigenic protein in prostatic tissue and fluid is gaining some degree of credence. The idea was originally expressed by Barnes et al., (17) and Vincent and Segonzac, (18) and supported in later years by the additional studies of Moncure, Prout, and Blaylock, (3) and Shulman, et al. (5)

In this report extensive immunodiffusion and immunoelectrophoresis studies with purified human PAP and heterologous PAP antiserum absorbed with human female serum produced single, very uniform, symmetrical precipitin lines indicating a solitary antigen antibody complex. The enzymic nature of the complex was regularly and reproducibly confirmed by Gomori or Burstone stains and L-tartrate inhibition.

The antigen antibody complex alone, as well as free PAP, was observed to hydrolyze p-nitrophenylphosphate as substrate during assay and in doing so did not undergo dissociation as previously noted by Ostrowsky, Weber, and Rybarska. (19) Thus the binding of specific PAP antibody to purified PAP antigen did not inhibit the enzymatic hydrolytic capacity of PAP in the antigen antibody complex. In fact, it is our continuing observation that PAP as an integral part of the antigen antibody complex is more enzymatically stable than the free form of serum PAP that in vitro is notoriously labile. (20)

Our experience with immuno-electrophoresis of PAP may indicate an opportunity for immunochemical detection of PAP antigen in the serum of prostatic cancer patients. In patients with elevated total serum acid phosphatase due to measurable increases in acid phosphaase of prostatic origin, significant precipitin bands were observed in fresh serum samples in the alpha 2 and gamma globulin regions of the electrophoretic slide. Such precipitin bands were verified by Gomori stain and L-tartrate inhibition. Similar findings were reported by Moncure, Prout, and Blaylock in 1965 in 2 cases of prostatic cancer. (3) The insensitivity of the method, however, and the necessity for high concentrations of PAP antigen in the serum of prostatic cancer patients for the development of significant precipitin bands is disturbing. Far more sophistication in immunologic investigative methodology is obviously necessary. Consideration might well be given to the development of a radioimmunoassay for human prostatic acid phosphatase that would provide the ultimate immunochemical sensitivity essential for the clinical determination of the enzyme. Two preliminary papers on the feasibility of a radioimmunoassay have recently been reported by the authors. (21, 22)

Finally, utilizing simple indirect immunofluorescence microscopy with suitable controls, it has been possible to consistently demonstrate that rabbit PAP antiserum harvested from the previously described immunization protocol appears to readily complex with PAP antigen within the cytoplasm of the benign human prostatic epithelial cell. Typical yellow-green fluorescence was observed in paranuclear cytoplasmic foci at the presumed sites of PAP synthesis. The fluorescent areas regularly corresponded microscopically with identical cytoplasmic areas described and stained by the Gomori and Burstone techniques. Control preparations with benign human prostate untreated with labelled antibody showed no specific fluorescence (Fig. 5).

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