Serum Prostate-Specific Acid Phosphatase: Development and Validation of a Specific Radioimmunoassay

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We describe radioimmunoassay for human prostatic acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] in serum, with use of monospecific antisera raised in rabbits against highly purified acid phosphatase from human prostates. The antisera did not cross react with partly purified acid phosphatases from human spleen, erythrocytes, or synovial tissues. 125I-labeled acid phosphatase was prepared by a Chloramine T method, and the bound and free antigen was separated in the assay by use of anti-rabbit gammaglobulin raised in sheep. Uniform low nonspecific binding of the [125I]acid phosphatase was achieved by using acid-phosphatase-free serum to prepare standard curves and diluted samples of serum with high acid phosphatase activities. Concentrations of immunoreactive acid phosphatase in the serum of healthy men ranged from <1 to 10 μg/liter and for 12 patients with advanced prostatic carcinoma between 100 and 500 μg/liter. The concentrations of the enzyme in sera of patients with benign prostatic hyperplasia were very similar to those in sera of the reference group.

Additional Keyphrases: normal values · benign prostatic hyperplasia · prostatic carcinoma

Since the original findings by Gutman and his associates (1–4) and of Huggins and of Hodges (5) about 40 years ago that serum acid phosphatase (EC 3.1.3.2) activity is markedly increased in patients with prostatic carcinoma, determination of this enzyme activity has been widely used to detect prostatic cancer and in monitoring the effects of therapy for this disease (6). When the assay is based on measurement of catalytic activity, phosphatases of nonprostatic origin in the serum contribute to the results (7). To overcome this, specific substrates and inhibitors for the acid phosphatase of prostatic origin have been utilized, but with limited success. Measurement of the tartrate-labile serum acid phosphatase activity currently considered to be the most reliable index for the prostate-specific phosphatase, but even the validity of this tartrate-labile enzyme activity in prostatic disease is still controversial, because some falsely positive and negative cases have been observed (7, 8). Another factor complicating the present techniques for assay of serum acid phosphatase is the great instability of the catalytic activity of the enzyme (9, 10). In short, if the measurement of serum acid phosphatase is based on its catalytic activity, it is subject to many potential errors that may limit the clinical usefulness of this enzyme, which is considered to be one of the most promising indicators of tumor (11).

Immunological measurement of acid phosphatase protein may be clinically more useful than measurement of catalytic activity (12). Because circulating acid phosphatase is a mixture of isoenzymes originating from several tissues (7), it is essential to pay great attention to the purity of the antigen and tracer used if one is to have an organ-specific determination of serum acid phosphatase. To allow a specific and sensitive estimation of prostate-specific acid phosphatase in serum, we have developed a radioimmunoassay (RIA) for this purpose. The antigen we used was purified to homogeneity from human prostatic tissue (13), and subsequently used to raise monospecific antibodies. Here, we report the characteristics and validation of the prostate-specific RIA for acid phosphatase, and give data on concentrations of immunoreactive enzyme in normal men, as well as in patients with benign prostatic hyperplasia and advanced prostatic cancer.

Materials and Methods

Chemicals

Na125I (specific activity, 11–17 kCi/g) was purchased from the Radiochemical Centre, Amersham, U.K. Sephadex gels (G-50, G-100, and G-200) and AH-Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. Freund’s complete and incomplete adjuvants were supplied by Difco Laboratories, Detroit, Mich. 48223, bovine serum albumin by Sigma Chemical Co., St. Louis, Mo. 63178, and p-nitrophenyl phosphate from Boehringer Mannheim GmbH, Mannheim, G.F.R. Diaflo PM 10 ultra-filtration membranes were purchased from Amicon Corp., Lexington, Mass. 02173. Other chemicals were from Merck AG, Darmstadt, G.F.R., and were of the highest grade of purity available.

Serum Specimens

The group of 53 normal subjects, 30 to 60 years of age, has been described in detail earlier (14). In short, subjects who had previously experienced any form of urinary difficulty or urogenital disease were excluded. Only subjects whose prostate size was normal as judged by rectal palpation were accepted for a final screening process, which involved several hematological and clinical chemical routine analyses.

Our patient group came from patients attending the Department of Urology, Oulu University Central Hospital, for treatment or observations in connection with either benign hypertrophy (n = 11) or carcinoma (n = 12) of the prostate. In addition to clinical investigation, the diagnosis was based on histopathological examination of a prostate specimen in every subject. All patients with prostatic carcinoma were at...
Stage 4 of the disease. The metastases were revealed by bone scan or roentgenographic bone survey, or both.

About 10 ml of blood were drawn from antecubital vein of control subjects and patients before rectal examination or eventual biopsy of the prostate. Blood samples were allowed to stand at room temperature for 30 min and the serum was harvested by centrifugation (1500 × g, 15 min). The samples were stored at −20 °C until used. Serum samples from normal women (20–30 years of age) for the preparation of acid phosphatase-free serum were obtained in a similar fashion.

**Enzyme Purifications**

Fresh human prostatic tissue was obtained during operations on patients with prostatic hypertrophy. The tissue was handled and processed for purification of acid phosphatase as previously reported in detail (13). In brief, the enzyme purification involved precipitation with ammonium sulfate, affinity chromatography on an L(+)-tartrate-AH-Sepharose 4B column, gel filtration on Sephacry G-200, and isoelectric focusing on a preparative column. An enzyme fraction with a pI of 4.9 was collected from the isoelectric focusing column and filtered on a Sephadex G-50 column. The fractions with the highest acid phosphatase activity were pooled and concentrated by ultra-filtration through Diaflo PM 10 membranes under nitrogen pressure. The concentrated enzyme fraction was re-filtered on a Sephadex G-50 column and the fraction with peak activity was concentrated once again by filtration through Diaflo PM 10 membranes. The purified preparation was homogeneous when analyzed by polyacrylamide gel electrophoresis. The specific activity of the final enzyme preparation was 4018 μmol/(min × mg) under the conditions previously described. The overall purification was 1900-fold as compared to the activity in the ammonium sulfate precipitate.

Acid phosphatase from human seminal fluid of healthy men's ejaculates was partly purified by the method of Foti et al. (15). Human synovial tissue was obtained from operations on joints, and spleen samples from splenectomies. Erythrocyte preparations were obtained from the Finnish Red Cross Transfusion Service, Helsinki, Finland. The acid phosphatasases from human synovial tissue, spleen, and erythrocytes were purified by essentially the same technique as used for prostatic tissue. Isoelectric focusing yielded two peaks of acid phosphatase activity from synovial tissue and spleen, termed enzyme I and enzyme II. The purity of these acid phosphatasases was evaluated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (13), as follows. Synovial tissue I and spleen I about 45%, synovial tissue II 70%, seminal fluid 70%, and spleen tissue II 90% pure by the criterion of acid phosphatase enzyme protein.

**Preparation of Antiserum against Human Prostatic Acid Phosphatase**

Homogeneous acid phosphatase from human prostate was dialyzed against 0.15 mol/liter NaCl for 24 h, then mixed with an equal volume of complete Freund's adjuvant. Rabbits were initially immunized with 0.25 mg of the enzyme preparation, intradermal injections being made at about 10 sites. Four weeks later, the rabbits were injected with the same amount of enzyme preparation mixed with an equal volume of incomplete Freund's adjuvant. After this, booster injections were given every two weeks with 0.12 mg of acid phosphatase emulsified in incomplete Freund's adjuvant. About one week after each booster injection the rabbits were bled and the serum was stored at −20 °C until dilution and use.

Antiserum against rabbit gamma-globulins, raised in sheep, was purchased from Oy Finnsorbents Ab, Espoo, Finland.

**Radioiodination of Acid Phosphatase**

Purified human prostatic acid phosphatase was radiolabeled with Na125I by the Chloramine T technique, essentially as described by Greenwood et al. (16). Twenty micrograms of acid phosphatase (in 40 μl of 0.5 mol/liter sodium phosphate buffer, pH 7.6 at 20 °C) was mixed with 2 mCi of Na125I in 20 μl of the sodium phosphate buffer and 30 μg (12 μl) of Chloramine T. The reaction was allowed to proceed for 30 s at 20 °C, then terminated by adding 300 μg (30 μl) of sodium metabisulfite. The whole reaction mixture was then applied to a Sephadex G-100 column (2 × 50 cm), which had been equilibrated with 50 mmol/liter sodium phosphate buffer—0.15 mol/liter NaCl, pH 7.6, containing 20 g of bovine serum albumin per liter. The radioactive fractions eluted in the void volume contained 125I-labeled acid phosphatase and were pooled and stored in small aliquots at −20 °C until used. To remove aggregated material formed during storage, we purified 125I-labeled acid phosphatase preparations at one-month intervals by filtration on a Sephadex G-200 column, under conditions described above for the Sephadex G-100 column chromatography.

**Radioimmunoassay Procedure**

For the radioimmunoassay of human prostatic acid phosphatase we used 12 × 75 mm polypropylene tubes. Approximately 70 000 cpm of the 125I-labeled acid phosphatase in 0.2 ml of sodium phosphate buffer (50 mmol/liter, pH 7.5, containing 3 mmol of sodium azide per liter) and 0.2 ml of diluted anti-acid phosphatase serum were added to 0.1 ml of the unknown samples or standards. The usual working dilution of the antisera was 3000-fold in the sodium phosphate buffer. To improve the sensitivity of the assay for acid phosphatase in sera of healthy men and women, we used an antiserum dilution of 10 000-fold on some occasions. The reaction was allowed to proceed for 48 h at 20 °C, and then 50 μl of anti-rabbit γ-globulin (diluted 5-fold with 0.05 the sodium phosphate buffer) was added. The tubes were then incubated for 48 h at 20 °C. After the second incubation, the tubes were centrifuged (3000 × g, 30 min), the supernates aspirated by water suction and pellets containing the antibody-bound 125I-labeled acid phosphatase were counted for radioactivity in a gamma counter (LKB-Wallac Rack Gamma, Turku, Finland). Each unknown sample was assayed in duplicate and every series of assays also included acid phosphatase standard curve tubes with the following amounts of the purified non-labeled enzyme in quadruplicate: 0, 1, 2, 3.9, 7.8, 15.5, 31, 62, 124, 248, and 496 μg/liter. Both the standards and serum samples (when needed) were diluted with acid-phosphatase-free human female serum (see below). The concentrations of acid phosphatase in sera were calculated with the aid of standard curves.

**Other Techniques**

**Assay of catalytic activity of acid phosphatase.** The acid phosphatase activity was assayed, with 5.5 mmol/liter p-nitrophenyl phosphate as the substrate, in 50 mmol/liter citrate buffer, pH 4.8, at 37 °C, as described previously (13).

**Protein determinations.** During purification of acid phosphatase from human tissues, elution of proteins from the columns was monitored by measuring the absorbance at 280 nm. Protein was measured by either the method of Lowry et al. (17) or by the method of Bramhall et al. (18), with crystalline bovine serum albumin as the standard.

**Preparation of acid-phosphatase-free serum.** Acid phosphatase was removed from women's serum by affinity chro-
matography on a column of L(+)-tartrate-AH-Sepharose 4B, under conditions previously described for purification of acid phosphatase from human sources (13). Such a pool of serum from women had an acid phosphatase activity of less than 1 U/liter before passage through the affinity column; no acid phosphatase activity could be detected in the flow-through fractions, which were pooled and termed acid-phosphatase-free serum.

Immunodiffusion. Double immunodiffusion was performed according to standard procedures on microscope slides covered with agar, 10 g/liter, in 0.15 mol/liter NaCl (19).

Immunoelectrophoresis. Microscope slides were covered with agar, 10 g/liter of 20 mmol/liter barbital buffer, pH 8.6, and used for electrophoresis at 44 mA and 130 V for 3 h (20). After antibody was added, precipitation arcs were observed within 48 h and were made better visible by staining with Coomassie Brilliant Blue. When acid phosphatase activity was localized after immunoelectrophoresis, the slides were stained with a-naphthyl phosphate coupled to Fast Garnet GBC in sodium acetate buffer (50 mmol/liter, pH 5.0).

Results

Properties of the Antiserum

On double-immunodiffusion and immunoelectrophoresis a single precipitation line formed with the pure prostatic acid phosphatase and the partly purified seminal fluid acid phosphatase (Figure 1), also identical when the samples were treated to show acid phosphatase activity (Figure 1). When enzyme preparations from spleen, synovial tissue, and erythrocytes were examined with double-immunodiffusion or immunoelectrophoresis with the antiserum, no precipitation lines were seen, indicating that the antiserum was specific for acid phosphatase of prostatic origin.

Characteristics of the Radioimmunoassay

As indicated above, the antiserum raised against homoge-

eous prostatic acid phosphatase did not detectably cross react with acid phosphatas from other tissues investigated, when evaluated by double-immunodiffusion or by immunoelectrophoresis. To more sensitively estimate the specificity of the antiserum, we repeated the cross-reactivity experiments, this time by the radioimmunoassay technique. Acid phosphatase isoenzymes partly purified from spleen, erythrocytes, or synovial tissue exhibited minimal or no competition for the binding of $^{125}$I-labeled acid phosphatase to the antiserum (Figure 2).

Figure 2 also shows a typical standard curve for the radioimmunoassay of serum prostate-specific acid phosphatase. With the current antiserum dilution and utilizing 0.1-ml serum samples, the lowest measurable acid phosphatase was 3.5 μg/liter, but this could be decreased to 1 μg/liter by using a more dilute antiserum solution (not shown). With the present technique, acid phosphatase concentrations up to 200 μg/liter can be reliably measured and thus the assay allowed a convenient determination of the enzyme protein in 0.1-ml samples of serum from most of the normal subjects, as well as from patients with prostatic carcinoma. Whenever the concentration of acid phosphatase exceeded this upper limit, dilution of an aliquot of the sample with acid-phosphatase-free serum and re-analysis of the diluted sample allowed quantification of concentrations many-fold higher than shown in Figure 2.

The concentration of acid phosphatase as measured by the present radioimmunoassay technique was strictly proportional to the amount of serum added to the assay (Figure 3). In this experiment, 3- to 100-μl portions of serum from a patient with prostatic carcinoma were taken for the assay, and a straight line was obtained when immuno-reactive acid phosphatase was plotted vs. volume of serum.

Within- and between-assay coefficients of variation of the present radioimmunoassay, evaluated by repeated measuring of acid phosphatase in four different serum pools, were between 6 and 11% (Table 1). When higher accuracy and precision are needed at high enzyme concentrations, the sample should be diluted with acid-phosphatase-free serum to optimize the use of the standard curve (see Figure 2).

Acid-phosphatase-free serum from women was also used to dilute the standards, to minimize variations in the protein concentration of the standards and samples. In this way we avoided differences in nonspecific binding (2%) between
position of these precipitation lines was identical with zones associated with the catalytic activity of the enzyme.

The RIA technique developed was sensitive enough to measure immunoreactive acid phosphatase in sera of most of the apparently healthy men studied. Further, the method was satisfactorily reliable as judged by within- and between-assay variation coefficients. The use of acid-phosphatase-free serum samples with a high acid phosphatase minimized variation in protein concentration within the assay tubes and, therefore, a uniform low nonspecific binding of the 125I-labeled acid phosphatase was achieved.

The values for immunoreactive acid phosphatase we have measured by our RIA in sera of healthy individuals and of patients with prostatic diseases were lower than those reported by Foti et al. (12, 15) and Choe et al. (21): for healthy men <1 to 10 μg/liter (mean 6.0 μg/liter) as compared to 24.2 ± 6.5 (SD) and 16.8 ± 8 (SD) μg/liter, respectively. Although we have so far studied only a few patients with advanced prostatic carcinoma, our values have been repeatedly below 500 μg/liter for these patients, while Foti et al. (12, 15) and Choe et al. (21) measured concentrations up to 1000–2000 μg/liter. At present, it is difficult to explain our lower values. One possible explanation is that we used an antigen for immunization that appears to be, according to all the available information, 100% pure (13). Furthermore, this protein was purified directly from prostatic tissue and not from prostatic fluid, which was the enzyme source used by Foti et al. (15). We have tried to apply the simple purification technique of Foti et al. (15) to isolate acid phosphatase from seminal fluid and have not been able to obtain a homogeneous protein preparation; the acid phosphatase fraction was contaminated by at least two additional protein bands when studied by polyacrylamide gel electrophoresis under denaturing conditions (unpublished observations). The extent to which different purification techniques and variable sources of the antigens explain the disagreement in the values for acid phosphatase remains to be elucidated.

Possibly, exclusion of acid phosphatases of nonprostatic origin in the measurement of the prostatic enzyme will increase the diagnostic sensitivity of acid phosphatase measurements as markers of early stages of prostatic carcinoma.

The concentrations of immunoreactive acid phosphatase in sera of patients with histologically verified benign prostatic hyperplasia were very similar to those in sera of apparently healthy men (Figure 4). Even though the number of such patients was few in our series, the results suggest a minimal incidence of falsely positive increases in serum acid phosphatase if the assay is performed by a specific RIA, which may not hold true when catalytic activity is assayed (12, 22).

As for the large-scale use of the current RIA technique, the major disadvantage seems to be the rather long time needed

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**Table 1. Within-assay and Between-assay Means, SD's, and CV's in the Radioimmunoassay of Serum Acid Phosphatase.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Within-assay Mean ± SD (n) μg/liter</th>
<th>Between-assay Mean ± SD (n) μg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool I</td>
<td>12.70 ± 0.95 (18)</td>
<td>36.0 ± 2.07 (8)</td>
</tr>
<tr>
<td>Pool II</td>
<td>77.73 ± 6.69 (19)</td>
<td>176.12 ± 18.50 (8)</td>
</tr>
</tbody>
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**Discussion**

On several grounds, our RIA seems to be specific for acid phosphatase of prostatic origin. First, the antigen was purified to homogeneity from human prostatic tissue (13) and we believe it to be the most nearly pure acid phosphatase so far described, as judged from its specific activity (13). Second, the antiserum raised against this homogeneous acid phosphatase did not cross react with partly purified acid phosphatases from other human sources (cf. Figures 1 and 2). Third, the antiserum was monospecific, forming only a single line of precipitation in immunodiffusion or immunoelectrophoresis vs. prostate homogenates or human seminal fluid. Finally, the

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**Fig. 3.** Relation between the measured immunoreactive acid phosphatase and amount of serum used in the assay. Different portions (0.003–0.1 ml) of serum from a patient with advanced prostatic cancer were used for the assay. The serum samples were diluted to 0.1 ml with pooled acid-phosphatase-free serum before the RIA. Each point is a mean of duplicate assays.

**Fig. 4.** Immunoreactive acid phosphatase in sera of healthy men (n = 53), of patients with histologically-proven benign prostatic hyperplasia (BPH, n = 11), and of patients with metastatic prostatic carcinoma (CA, n = 12). The symbols on the abscissa show samples with an immunoreactive acid phosphatase content of <1 μg/liter.
to complete this assay (about four days). Studies aimed at simplifying the method are currently in progress in our laboratory.

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References