Comparison of Human Prostatic Acid Phosphatase by Measurement of Enzymatic Activity and by Radioimmunoassay

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We compared results of measurement of prostatic acid phosphatase activity in serum and various tissues by enzymatic assay and radioimmunoassay. By enzymatic assay, activity in serum is lost rapidly, even at room temperature. In contrast, there was no change in antigenic activity during 48 h by radioimmunoassay. The radioimmunoassay was more specific in 12 tissues and in serum than were several enzymatic assays that make use of inhibitors of the enzyme. The enzymatic assay resulted in 26.8% (24/90) false positives from non-prostatic cancer patients. In contrast, with radioimmunoassay there were only 5.5% (5/90) false positives. We conclude that immunological detection of prostatic acid phosphatase is the more reliable technique.

Additional Keyphrases: cancer • normal values • assay specificity • diagnostic aids

Prostatic acid phosphatase (EC 3.1.3.2) is an enzyme of orthophosphoric-monoester phosphohydrolase (acid optimum). Measurement of its activity in the serum became clinically important after it was discovered that prostatic acid phosphatase activity usually increases as a consequence of prostatic carcinoma (1–5) and that the activity in the serum generally increases as the disease progresses (6). Many substrates have been used in measuring this activity in patients’ serum (7–13). Because many of these substrates are not specific for the acid phosphatase originating from the prostate, inhibitors have been introduced to decrease the effect of acid phosphatases from other tissues (14, 15).

In addition to the specificity of the substrates, the stability of the enzymatic activity of the protein is very important for a reliable diagnostic test. If enzyme activity is labile under conditions normally used for serum collection or storage, aberrant results will be obtained (4, 16). To overcome both of these insufficiencies of the enzymatic determination of prostatic acid phosphatase, we have introduced a radioimmunoassay to quantitate this enzyme in sera from patients (17, 18).

Here, we present comparative data concerning the specificity of the radioimmunoassay and of four enzymatic determinations, as well as data demonstrating the increased stability of prostatic acid phosphatase in the radioimmunoassay relative to the enzymatic activity.

Materials and Methods

Disodium p-nitrophenyl phosphate, thymolphthalein mononaphosphate (magnesium salt), phenolphthalein monophosphate (sodium salt), a-naphthyl phosphate (potassium salt), tetrazotized o-dianisidine, and Brij 35 (a surfactant, polyoxyethylene ether of lauryl alcohol; concentration, 300 g/liter) were obtained from Sigma Chemical Co., St. Louis, Mo. 63178. All other chemicals were analytical grade, from J. T. Baker Chemical Co., Phillipsburg, N. J. 08865.

The purification of human prostatic acid phosphatase and its iodination and the production of rabbit antisemum to acid phosphatase have been described (18).

Extraction of tissues: The tissue specimens were from autopsy. They were extracted twice in a homogenizer at 4 °C for 1 min, at top speed, in five volumes of saline (9 g of NaCl per liter). Platelets and erythrocytes were lysed by thrice-repeated freezing and thawing. The homogenates were centrifuged for 20 min at 3000 × g. Platelets and erythrocytes were obtained from our blood bank.

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Received Aug. 10, 1976; accepted Nov. 1, 1976.
Measurement of acid phosphatase activity: The method from Sigma Bulletin 104 (19) was adapted when p-nitrophenyl phosphate was used as substrate.

The Coleman procedure (20) was modified when we used phenolphthalein monophosphate. One milliliter of citrate buffer (0.1 mol/liter, pH 6.0) containing 5 mmol of phenolphthalein monophosphate per liter was preincubated at 37 °C, and 0.2 ml of sample or enzyme solution was added. After a further 30-min incubation, 5 ml of NaOH (50 mmol/liter) was added. The absorbance was then measured at 546 nm.

When thymolphthalein monophosphate was used as substrate, the assay procedure was that of Roy et al. (13).

The method of Amador et al. (21) was used, except that tetrazotized o-dianisidine was used (11) instead of diazotized 5-nitro-o-anisidine, when α-naphthyl phosphate was the substrate.

Radioimmunoassay procedure for human prostatic acid phosphatase: Polypropylene tubes were coated with 0.5 ml of rabbit antiserum, diluted 3000-fold, for 1 h at 4 °C. After the antiserum was aspirated, the walls of the tubes were washed twice with 1 ml of PBS, and they were allowed to stand for 45 min at room temperature with PBS containing 10 g of BSA per liter. The contents of the tubes were aspirated and 0.4 ml of saline, 0.1 ml of sample, and 10 μl of 125I-labeled antigen (2 × 10⁶ cpm) were added. The tubes were left at 4 °C for 36 h and, after the removal of the free antigen, the walls of the tubes were washed twice with PBS and counted in a gamma counter. This assay has been described in detail previously (15).

Stability of serum prostatic acid phosphatase: Blood was obtained from prostatic cancer patients by venipuncture. The serum (pH 8.4) was separated by centrifugation, divided into three portions, and incubated at either 23, 36, or 46 °C. Samples for the assay were taken at the indicated times and quickly frozen until they were measured by enzyme assay (p-nitrophenylphosphate substrate) and by radioimmunoassay. To test the enzyme's stability to freezing and thawing, serum was frozen (solid CO₂/ethanol) and thawed 14 times and the activity then determined by enzymatic assay and radioimmunoassay.

Results

Stability: The activity of serum prostatic acid phosphatase decreases rapidly at room temperature, owing to the increased pH of the serum (7). However, repeated freezing and thawing does not decrease either the enzyme activity or the immunological activity of prostatic acid phosphatase in the serum of prostatic cancer patients. The serum retained all its original prostatic acid phosphatase activity after it had been frozen and thawed 14 times. Consequently, it is valid to store a series of samples frozen and later measure their activity at one time.

Heat stability measurements were done at pH 8.4, because hemolysis does not change the pH of the serum substantially.

We saw a marked difference in the stability of prostatic acid phosphatase when serum samples (pH 8.4) of prostatic cancer patients were first incubated at 23, 36, or 46 °C and the activity of the enzyme was then measured by enzyme assay (p-nitrophenyl phosphate as substrate) and by radioimmunoassay. There was no measurable loss of activity of the prostatic acid phosphatase by radioimmunoassay when serum was incubated at 23 °C for as long as 72 h. In contrast, by enzymatic assay of the same samples there was a 30% decrease in the activity in 3 h, and by 72 h all activity of the enzyme was gone. At higher temperatures (36 or 46 °C) the enzymatic and immunological activities deteriorated much faster but the difference between the two rates was still substantial (Figure 1).

Specificity of enzymatic activity assay and radioimmunoassay: We assayed extracts from platelets, erythrocytes, nine tissues, and serum from normal males for prostatic acid phosphatase activity, with p-nitrophenyl phosphate, thymolphthalein monophosphate, phenolphthalein monophosphate, or α-naphthyl phosphate as substrates. The activity of the enzyme was determined in the absence (total acid phosphatase) and in the presence of L-tartrate inhibitor. (When substrates are not specific for prostatic acid phosphatase, L-tartrate is incorporated to determine the contribution of other phosphatases.) Thymolphthalein monophosphate is the most nearly specific substrate for prostatic acid phosphatase of those studied; with L-tartrate present, the results were generally little changed (Table 1).

Simultaneously, the samples were measured by radioimmunoassay for prostatic acid phosphatase. Table 1 shows the results of the nonspecific hydrolysis of the substrates by various tissues. For each enzyme assay, the third column shows the relative specific activities.
of prostatic and total acid phosphatase. Total acid phosphatase activity in the different extracts is in each case quite low, but the ratio of prostatic to total acid phosphatase in serum samples varies from 10 to 25%. Thus, it is essential to be sure one is measuring only prostatic acid phosphatase when assaying for this activity in serum. The use of L-tartrate to inhibit the prostatic fraction of acid phosphatase is essential when p-nitrophenyl phosphate or phenolphthalein monophosphate is used as substrate. The phosphatase activity of nonprostatic phosphatases in tissue extracts is high when p-nitrophenyl phosphate or phenolphthalein monophosphate are used, in comparison with thymolphthalein monophosphate. The range of inhibition by L-tartrate is 30 to 90% for phenolphthalein monophosphate and p-nitrophenyl phosphate. \( \alpha \)-Naphthylphosphate is more specific, with less inhibition by L-tartrate. We found thymolphthalein monophosphate to be the most nearly specific substrate for prostatic acid phosphatase.

The bladder and the pancreas contain the greatest amount of cross-reacting protein and total acid phosphatase.

The radioimmunoassay gave the least amount of cross reactivity with other proteins in the extracts of various organs. The best overall result was obtained by radioimmunoassay.

The normal values for serum prostatic acid phosphatase are 120 ± 40 Sigma units per liter and 48 ± 8 \( \mu \)g/liter by enzyme assay and radioimmunoassay, respectively. For this study we used the mean value plus two standard deviations to define “above-normal” values; i.e., enzyme values of >200 Sigma units/liter or radioimmunoassay values >64 \( \mu \)g/liter (22).

When cancer patients with other than prostatic cancer (37 men, 53 women) were evaluated for serum prostatic acid phosphatase, 26.6% (24/90) had “above-normal” prostatic acid phosphatase values by enzymatic assay and 5.5% (5/90) had “above-normal” values by radioimmunoassay. Table 2 shows the distributions of tumors with “above-normal” serum prostatic acid phosphatase by radioimmunoassay and by enzyme assay. None of the women had “above-normal” prostatic acid phosphatase activity by radioimmunoassay. Three men had “above-normal” values by both methods: one with pharyngeal carcinoma had an extremely high value; one with epidermoid carcinoma of the lung, which metastasized to the parietal area of the brain, had moderately increased values; one with adenocarcinoma of the lung had a smaller increase. For the radioimmunoassay, 13.5% (5/37) of the male nonprostatic cancer patients had falsely positive assays for prostatic acid phosphatase by radioimmunoassay, a number that concurs with a preliminary study that showed 8 to 11% false-positive tests for normal males (22).

**Discussion**

The level of serum prostatic acid phosphatase is an important clinical diagnostic test for cancer of the prostate. Until recently, enzymatic assays have been
Table 2. Prostatic Acid Phosphatase Activities in Sera of Nonprostatic Cancer Patients

<table>
<thead>
<tr>
<th>Site of malignancies</th>
<th>&quot;Above-normal&quot; by radioimmunoassay</th>
<th>&quot;Above-normal&quot; by enzyme assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Male 3/11 Female 0/8</td>
<td>Male 5/11 Female 3/8</td>
</tr>
<tr>
<td>Cervix</td>
<td>Male — Female 0/9</td>
<td>Male — Female 1/9</td>
</tr>
<tr>
<td>Breast</td>
<td>Male — Female 0/9</td>
<td>Male — Female 1/9</td>
</tr>
<tr>
<td>Endometrium</td>
<td>Male 0/6 Female 0/1</td>
<td>Male 2/6 Female 1/1</td>
</tr>
<tr>
<td>Larynx</td>
<td>Male 0/3 Female 0/2</td>
<td>Male 0/3 Female 1/2</td>
</tr>
<tr>
<td>Bladder</td>
<td>Male 0/1 Female 0/4</td>
<td>Male 1/1 Female 0/4</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td>Male 0/2 Female 0/4</td>
<td>Male 0/2 Female 0/4</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>Male 0/2 Female 0/4</td>
<td>Male 1/2 Female 0/4</td>
</tr>
<tr>
<td>Nasal</td>
<td>Male 1/2 Female 0/0</td>
<td>Male 0/2 Female 0/0</td>
</tr>
<tr>
<td>Brain</td>
<td>Male 0/1 Female 0/1</td>
<td>Male 0/1 Female 1/1</td>
</tr>
<tr>
<td>Pharynx</td>
<td>Male 1/1 Female 0/0</td>
<td>Male 1/1 Female 0/0</td>
</tr>
<tr>
<td>Leukemia</td>
<td>Male 0/1 Female 0/1</td>
<td>Male 0/1 Female 1/1</td>
</tr>
<tr>
<td>Pituitary</td>
<td>Male 0/0 Female 0/2</td>
<td>Male 0/0 Female 1/2</td>
</tr>
<tr>
<td>Others: (renal; uterine; ovary; etc.)</td>
<td>Male 0/9 Female 0/5</td>
<td>Male 0/9 Female 0/5</td>
</tr>
<tr>
<td>Total</td>
<td>Male 5/37 Female 0/53</td>
<td>Male 10/37 Female 14/53</td>
</tr>
</tbody>
</table>

used exclusively for this diagnostic test. However, increased temperature inactivates the enzyme at the pH of normal serum; therefore, results obtained by these methods might not reflect the true activity of this enzyme in serum if scrupulous precautions have not been followed in preparing the serum. Such considerations, as well as questions of substrate specificity, have led to an immunological quantitation of prostatic acid phosphatase. The immunological reactivity of prostatic acid phosphatase is apparently more consistent than enzymatic activity. Therefore, serum samples do not require special treatment (e.g., pH adjusting) and handling.

Normal serum contains several acid phosphatases of different origin. Consequently, a specific assay that detects enzymes of prostatic origin is required. The acid phosphatase derived from the prostate—as opposed to extraprostatic sources—that is present in the serum evidently comprises only about 10 to 25% of the total prostatic acid phosphatase.

Extracts from platelets, erythrocytes, and various tissues were evaluated for the total and prostatic fractions of acid phosphatase as defined by enzyme inhibitor studies and for prostatic acid phosphatase as assayed by radioimmunoassay. The highest specificity for the enzyme was demonstrated by radioimmunoassay. Among the enzyme substrates, thymolphthalein monophosphate has the highest specificity for prostatic acid phosphatase—no inhibitor has to be used with this substrate. However, there is a significant disadvantage to this method (23). The mean value for normal males is 260 ± 120 Sigma units per liter (24). Using 0.2 ml of serum, as described for routine analysis of prostatic acid phosphatase, one would obtain an absorbance reading of 0.006 to 0.017 at 590 nm. Experimental error with such a range of readings is extremely high (24). Therefore, small changes in the serum acid phosphatase (e.g., at stage I or II of the disease) cannot be accurately measured because of this limited sensitivity. An increase in the prostatic acid phosphatase activity from 400 to 500 Sigma units per liter, for example, results in a change in absorbance of only 0.005. Similarly, in the case of p-nitrophenyl phosphate, the normal value for males is 130 to 630 Sigma units per liter (19), corresponding to an absorbance of 0.050 to 0.180 at 410 nm. Because prostatic acid phosphatase is inhibited by L-tartrate, the ratio prostatic acid phosphatase/total acid phosphatase varies from 1/10 to 1/4, with absorbance readings in the presence of L-tartrate ranging from 40 to 145 Sigma units/liter. The level of prostatic acid phosphatase is estimated by subtracting these two values, which gives an even smaller number; such a procedure introduced a great deal of error into the assay.

Certain anticoagulants (e.g., oxalate, fluoride) inhibit prostatic acid phosphatase activity. Many such compounds that inhibit the enzymatic activity of the prostatic fraction of acid phosphatase, have no effect on the antibody reaction (Foti et al., in preparation). Similarly, drugs and other substances may affect the assay.

An extended study is under way to determine what types of drugs and other pathological conditions may interfere with the detection of prostatic carcinoma by radioimmunoassay.

This work was supported in part by Southern California Permanent Medical Group and by contract E (04-1) Gen-12 between the U. S. ERDA and the Regents of the University of California.

References