"Sandwich" Enzyme Immunoassay for Placental Alkaline Phosphatase

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Alkaline phosphatase of the placental type in serum has been suggested as a "marker" for malignancy and pregnancy. We describe a highly sensitive enzyme-linked immunosorbent assay (ELISA) for the measurement of this enzyme in serum and ascitic fluid. The assay will detect as little as 0.4 μg/L, significantly less than with a radioimmunoassay performed with the same reagents. It is highly specific; it does not measure even above-normal concentrations of the intestinal and liver isoenzymes of alkaline phosphatase. The assay is technically simple and allows the processing of many samples in less than 10 h. We measured this isoenzyme in serum of an adult control population. The upper limit of normality is 1.85 μg/L. Interference by rheumatoid factor was eliminated. Concentrations of the analyte were increased in all pregnancy sera tested. Concentration and activity as measured by two different catalytic assays correlated well. Samples from cancer patients also showed a good correlation, with some exceptions. Possible reasons for these exceptions are discussed. The high sensitivity, specificity, and simplicity of this assay should make it a useful adjunct in monitoring cancer and pregnancy.

Additional Keyphrases: cancer • pregnancy • monitoring therapy • ascitic fluid • isoenzymes • cutoff value

Placental-type alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1.; PLAP) are produced by tumor cells in vitro (1), and the presence of these isoenzymes in the circulation of patients with different malignancies has long been recognized (1–5).

In many respects these oncofetal isoenzymes of PLAP resemble the common phenotypes of term placental alkaline phosphatase (6), but they differ in their biochemical and physical properties (7–9). Stability to heat denaturation has long been exploited in selecting for and measuring placental alkaline phosphatase (10). Recently a heat-labile PLAP has been described in patients with Hodgkin's disease (11); therefore, methods relying solely on heat stability can give falsely negative results.

Although immunochromic methods would seem to offer a useful alternative for the measurement of PLAP, radioimmunoassays so far reported are too insensitive and allow detection of PLAP in a much smaller proportion of cancer sera than do the catalytic assays, i.e., those in which activity is measured rather than concentration (12, 13).

Here we describe a highly sensitive enzyme-linked immunosorbent assay (ELISA) with which the concentrations of PLAP in even normal serum can be measured. Moreover, our preliminary data suggest the usefulness of this assay in cancer monitoring.

Materials and Methods

Serum and Ascitic Fluid Samples

Human serum specimens were stored at –20 °C until the assays were performed. Ascitic fluid specimens, from patients with different malignancies, were stored at –20 °C and were thawed only once before assay.

Immunochromic Procedures

Purification of PLAP. We purified PLAP from fresh human placentas, following the procedure of Holmgren et al. (14), with minor modifications.

Production of antisera. Rabbits were each injected subcutaneously with a total of 100 μg of purified PLAP in complete Freund's adjuvant. Blood was sampled and 50-μg booster injections were given monthly for about two years.

Preparation of Sepharose-coupled intestinal alkaline phosphatase. Portions of human small intestine obtained at autopsy were washed extensively, with care not to damage the mucosa, and stored at –20 °C until use. The intestinal mucosa was then scraped off with a scalpel, and the scrapings were extracted with n-butanol and precipitated by adding an equal volume of acetone. The precipitate was coupled to cyano-agen-bromide activated Sepharose CL-4B according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden).

Testing of antisera by radioimmunoassay. PLAP (10 μg) was labeled with 125I by use of solid-phase lactoperoxidase (15) to a specific activity of 50 Ci/g, and 0.5 ng of this labeled PLAP was used per assay tube in both binding and inhibition studies. Labeled antigen and different dilutions of antibody were incubated overnight at room temperature and the resulting antigen–antibody complex was precipitated with goat antiserum to rabbit gamma globulin (Calbiochem, La Jolla, CA 92037) in the presence of 30 g of polyethylene glycol M, 4000 (Sigma Chemical Co., St. Louis, MO 63178) per liter. Antiserum with high titer were selected and their IgG fractions were separated out on a protein A-Sepharose column (Pharmacia), then absorbed with a column of Sepharose-coupled normal human serum proteins and with a similar column of acetone-precipitated intestinal-extract proteins. The absorbed antibodies were subsequently used in a competitive radioimmunoassay for PLAP, performed as described above, and in a "sandwich" ELISA.

Coupling of anti-PLAP IgG to horseradish peroxidase (HRP). A high-titer, high-affinity antibody was selected for coupling with HRP and subsequent use in the third incubation step in the sandwich ELISA. The HRP used for coupling was a gift from the Toyobo Co. Ltd., Osaka, Japan. Five milligrams of this HRP was used per milligram of protein A-purified anti-PLAP IgG (16). The conjugate was stored at 4 °C in glycerol/water (equal volumes).

Sandwich ELISA for PLAP. The assay was performed by techniques described in ref. 16. Microtitration 96-well plates (LINBRO; Flow Laboratories Inc., Inglewood, CA 90302) were coated with 200 μL of a 10 mg/L solution of anti-PLAP IgG in 0.1 mol/L NaHCO₃. Purified PLAP, 1 g/L in distilled water, served as the standard; it was diluted with phosphate-buffered

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saline containing 0.5 mL of Tween 20 surfactant per liter, and 200 μL of each dilution was dispensed in duplicate. Two-hundred-microliter clinical samples were dispensed directly into the wells or were first diluted if the PLAP content was expected to exceed 4 μg/L (see recovery studies below). Samples and standards were incubated in the antibody-coated wells for 3 h at room temperature. After washing, a 2000-fold dilution of the anti-PLAP-HRP conjugate was prepared in phosphate-buffered saline containing 0.5 mL of Tween 20 and 0.3 g of protein A-purified rabbit IgG per liter, and 200 μL of this dilution was dispensed into each well. The plate was left at room temperature for 3 h. Enzymic activity was determined with use of citrate-phosphate buffer (0.1 mol/L, pH 5.0) containing 0.1 mL of H2O2 and 0.4 g of o-phenylenediamine per liter.

After 45 min at room temperature, the reaction was stopped by adding 50 μL of 5 mol/L H2SO4 to each well. The color was recorded with an automatic ELISA reader (Titertek Multiskan, Flow Laboratories) with the 492-nm filter.

**Catalytic Assays**

Two different catalytic assays were used for comparison with the sandwich ELISA. In both, heat inactivation supposedly eliminates interference by non-placental alkaline phosphatases. The first (method 1) is a modification (17) of the method of Hausamen that allows for kinetic determinations of PLAP activity, with p-nitrophenyl phosphate as substrate. The second (method 2) is a highly sensitive catalytic method modified according to Doellgast and Fishman (18), in which phenyl phosphate is used as substrate in a single-point type assay.

**Electrophoresis**

Electrophoresis on polyacrylamide gel was performed according to the method of Laemmli (19) in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. The protein bands in the electrophoretogram were stained with Coomassie Brilliant Blue.

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Fig. 1. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol

(A) alkaline phosphatase peak after each purification step. (1) after butanol extraction, (2) after acetone precipitation, (3) after heat denaturation, (4) after DEAE cellulose column, (5) after isoelectric focusing and G-100-200. Coomassie Blue staining

(B) Radioautography of 125I-labeled purified PLAP

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**Results**

**Analytical Variables**

**Purity of PLAP.** The purified PLAP used in this study had a high specific activity (560 kU/g) as measured under the conditions detailed in 17. On electrophoresis as described above it migrated as a homogeneous single band, even when a large amount of 125I-labeled enzyme was electrophoresed (Figure 1). Furthermore, 85% of the 125I-labeled enzyme was precipitated when excess first antibody was used in radioimmunoassay.

**Sensitivity of ELISA and radioimmunoassay.** The same reagents were used to generate inhibition curves in competitive radioimmunoassay and standard curves by sandwich ELISA (Figure 2). The minimum detectable concentration in the sandwich ELISA was 0.4 μg of PLAP per liter of serum (the concentration significantly different from 0 at the 95% confidence level). Radioimmunoassay inhibition curves gen-
erated with the same reagents showed that radioimmunoassay was less sensitive than ELISA, requiring 5 μg of PLAP per liter for detectable inhibition.

Specificity of the sandwich ELISA. Dilution of the PLAP standards with phosphate-buffered saline/Tween or pooled normal human serum give similar standard curves in ELISA, indicating that normal constituents of serum do not interfere with the assay (data not shown).

Interference by rheumatoid factor, a well-known cause of interference in immunoassays based on the sandwich principle, was initially noted. Sera with high concentrations of rheumatoid factor gave artifactualy high readings of PLAP. This interference was successfully eliminated by incorporating 0.3 g of protein A-purified normal rabbit IgG per liter at the anti-PLAP-HRP conjugate step (Figure 3).

Rabbit antibodies to PLAP do not distinguish between different allelic forms of the enzyme, but do cross react to some extent with intestinal alkaline phosphatase (20). Initial trials indicated that this cross reactivity was also present in our antisera, but it was removed by absorption of the antiserum with Sepharose-coupled intestinal extract. Liver-derived ALP did not react in our ELISA (Figure 4).

Precision and analytical recovery studies. Analysis of three different serum samples (C1, C2, and C3) containing different concentrations of PLAP gave the following within-assay variation (n = 16 determinations each): C1 = 0.83 (SD 0.06) μg/L (CV = 7.2%); C2 = 1.81 (SD 0.12) μg/L (CV = 6.6%); C3 = 8.35 (SD 0.66) μg/L (CV = 7.9%). When these samples were measured in duplicate on 10 different days the between-assay variation was: C1 = 0.89 (SD 0.09) μg/L (CV = 10.1%); C2 = 1.78 (SD 0.17) μg/L (CV = 9.6%); C3 = 8.88 (SD 1.3) μg/L (CV = 14.6%).

Analytical recovery was assessed by assaying increasing amounts of purified PLAP added to a pooled specimen of normal human serum. The different dilutions were assayed in a single plate. Recovery exceeded 95% in the range 0.4 to 4 μg/L. Samples containing more than this were diluted accordingly and re-assayed.

Clinical Studies

Basal concentrations of PLAP. Samples were obtained from 61 men and 65 women who were undergoing routine checkups at Scripps Clinic and Research Foundation. Duplicate assays of these samples for PLAP showed 70% of the population had PLAP concentrations below the limit of detection of the assay, and that all had concentrations of PLAP of <1.85 μg/L (Table 1). We used this value as the upper limit of normal.

Pregnancy sera. Serum samples from women at different stages of pregnancy were assayed for PLAP by our assay and the results were compared with those obtained by the two catalytic assays. Agreement was excellent in both cases. The correlation coefficient was \( r = 0.99 (p < 0.001) \) for the relation between ELISA and catalytic assay no. 1 (Figure 5a) and \( r = \)

![Fig. 4. Lack of cross reactivity of liver and intestinal alkaline phosphatase in the present method increasing amounts of liver, intestinal, and placental alkaline phosphatases were added to the ELISA wells. Only PLAP was detected](image)

![Fig. 3. Results of ELISA assay for PLAP in sera of patients with rheumatoid arthritis](image)

**Table 1. Distribution of PLAP Values in an Adult Reference Population**

<table>
<thead>
<tr>
<th>Intervals μg PLAP/L</th>
<th>No. Individuals</th>
<th>Frequency</th>
<th>Cumulative frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.40</td>
<td>89</td>
<td>70.6</td>
<td>70.6</td>
</tr>
<tr>
<td>0.40 to &lt;0.65</td>
<td>10</td>
<td>7.9</td>
<td>78.5</td>
</tr>
<tr>
<td>0.65 to &lt;0.85</td>
<td>4</td>
<td>3.2</td>
<td>81.7</td>
</tr>
<tr>
<td>0.85 to &lt;1.05</td>
<td>4</td>
<td>3.2</td>
<td>84.9</td>
</tr>
<tr>
<td>1.05 to &lt;1.25</td>
<td>3</td>
<td>2.4</td>
<td>87.3</td>
</tr>
<tr>
<td>1.25 to &lt;1.45</td>
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<td>7.1</td>
<td>94.4</td>
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<td>3</td>
<td>2.4</td>
<td>96.8</td>
</tr>
<tr>
<td>1.65 to &lt;1.85</td>
<td>4</td>
<td>3.2</td>
<td>100.0</td>
</tr>
</tbody>
</table>

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stimulated by the discovery of a placental-like alkaline phosphatase in the serum of a male patient with lung cancer (1). Since then various tumors have been found to produce this enzyme. Together with α-fetoprotein, carcinoembryonic antigen, and chorionadotropin, PLAP has inspired the coined of the term “oncodevelopmental proteins.” The first three have proved helpful in the management of tumors such as hepatoma, gastrointestinal cancer, and embryonal genital tumors.

It is within this last category of tumors that preliminary evidence shows the potential usefulness of PLAP determinations. Soon after the discovery of the presence of heat-stable placental ALP in normal testis (22), the possibility that PLAP might be expressed in seminomas was explored and encouraging results were obtained (23, 24). The insufficient sensitivity of the assays used precluded further evaluation of this tumor marker. Obviously, a more highly sensitive immunoassay was needed for monitoring PLAP in the serum of patients.

The present method is simple in principle, can be completed in one day, and allows convenient determination of many samples. The reagents used and the design of the assay were carefully selected to maximize sensitivity. The assay is highly specific for PLAP, interference by rheumatoid factor has been eliminated, and the cross reaction of our antibodies to intestinal alkaline phosphatase was managed by rendering them monospecific through exhaustive absorption on an intestinal extract-Sepharose column. We used the Titertek Multiskan apparatus for reading the final color reaction, to save time; however, the assay can be performed without it, by transferring the 250 μL of developed substrate–buffer–acid mixture, appropriately diluted, to a cuvette for regular spectrophotometry.

A prerequisite for the effective exploitation of a tumor marker is to establish basal values for a healthy reference population. Even though our assay has not allowed us to determine the complete distribution of normal values, we have established the normal upper limit. This value, 1.85 μg/L, agrees well with that reported recently for radioimmunoassay.

Discussion

Study of the different PLAP phenotypes was greatly
(25), which defined the upper limit as 2 μg/L, under which 95% of the normal values were found to be distributed. Our normal values also agree well with those obtained with a highly sensitive catalytic assay (26), with which the normal range was defined as 0.3 (SD 0.4) μg/L.

Heat stability has long been regarded as a unique feature of the placental-type alkaline phosphatase and several catalytic assays have been based on this property. We found an excellent correlation between the results of two such assays and sandwich ELISA, although a few discrepant samples deserve some attention. Thus, significant activity of alkaline phosphatase were measured in two ascitic-fluid samples that showed no PLAP by our sandwich ELISA. The conditions of heat inactivation may have been insufficient to completely eliminate non-placental alkaline phosphatase activity, which is often concomitantly increased in cancer patients (27). On the other hand, four samples had high concentrations of PLAP by sandwich ELISA with no detectable catalytic activity. This discrepancy could arise from the presence of enzyme inhibitors in the fluid of these patients, loss of Zn from the active site, or the detection of catalytically defective tumor-derived enzyme. Or it could indicate the presence of the heat-labile PLAP recently demonstrated in some malignant conditions (11). These cases exemplify the desirability to use immunological assays for the measurement of PLAP in establishing its role as a tumor marker.

An extensive study on the value of PLAP determinations in seminomas by use of sandwich ELISA is under way in our laboratory, and the results will soon be published.

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References