Enzyme–Antigen Immunoassay for Human Placental Alkaline Phosphatase in Serum and Tissue Extracts, and Its Application as a Tumor Marker

Dirk E. Pollet,1 Etienne J. Nouwen,1 Jacques B. Schelstraete,1 Jean Renard,2 Andre Van de Voorde,3 and Marc E. De Broe1,4

In this enzyme–antigen immunoassay for human placental alkaline phosphatase (hPLAP; EC 3.1.3.1.) in serum and tissue extracts, polyclonal rabbit antisera to mouse IgG2b is adsorbed to the wells of a microtiter plate, its excess binding sites are blocked, then it is incubated with murine monoclonal anti-hPLAP and mixed with serially diluted standard or sample antigen. The amount of antigen bound is determined by measuring its enzymic activity. The standard curve is linear for hPLAP concentrations of 0.2 to 1 U/L. The mean within-assay CV was 3.8% (SD 0.9%) for a serum sample and 6.1% (SD 3.0%) for a tissue extract. The respective mean between-assay CVs were: 6.7% (SD 2.0%), and 7.0% (SD 2.0%). Serum hPLAP concentrations, determined in four different dilutions, had a CV of 5.5%. We evaluated the method by standard additions and by comparing dilution curves for purified hPLAP, hPLAP in serum, and hPLAP in tissue extracts. The upper limit of activity in normal subjects was 0.1 U/L for serum samples, and 1.0 mU/g wet weight of tissue for tissue extracts. hPLAP activity was increased in 9.8% of all cancer patients, and in 40% of ovarian cancer patients. Almost half of the tumor biopsies were positive for hPLAP activity, and 94% of the biopsies from ovarian neoplasia had an increased activity of this isoenzyme. Of the nonmalignant tissues examined, normal lung tissue had the highest hPLAP activity.

Additional Keyphrases: isoenzymes • monoclonal antibodies • cutoff value • ovarian cancer • reference interval • smoking • lung cancer

Human placental alkaline phosphatase, hPLAP (EC 3.1.3.1.), is ordinarily expressed on the plasma membrane of the microvilli of the syncytiotrophoblast, but has also been identified as a oncodiagnostic protein in various cancer tissues (1–5). Since the discovery of the so-called Regan isozyme of alkaline phosphatase in serum and tumor tissue of a patient with carcinoma of the lung (6), several investigators (7–9) have confirmed the potential of this molecule as a tumor marker. However, a major difficulty in detecting hPLAP with polyclonal antisera has always been the cross reactivity of these antibodies with the common epitopes of intestinal alkaline phosphatase (10–15). The biochemical and physical properties of the alkaline phosphatase isoenzymes vary, but are not sufficiently different to allow detection of small amounts (<1 U/L) of one of them in a complex mixture (16). Immunochemical quantification of hPLAP has become possible by the isolation of a hybridoma cell line that produces a hPLAP-specific monoclonal antibody (17,18). By using this antibody we have established a monospecific enzyme–antigen immunoassay capable of detecting the low activity concentrations of hPLAP in serum (range 0.02–1 U/L) and of quantifying hPLAP in butanol extracts of tumor tissue obtained at biopsy.

Materials and Methods

Materials

For all enzyme–antigen immunoassay measurements we used a Model DU-8 spectrophotometer (Beckman Instruments, Fullerton, CA 92634) equipped with "microplate" accessories.

Flat-bottomed micro-immunoplates I were from Nunc, Roskilde, Denmark (cat. no. 239454); rabbit antisera to mouse IgG2b, was from Nordic, Tilburg, the Netherlands; bovine serum albumin was from Sigma Chemical Co., St. Louis, MO (cat. no. A-8022); p-nitrophenyl phosphate from Baker Chemicals, Phillipsburg, NJ (cat. no. 3351); ethylaminoethanol from Merck, Darmstadt, F.R.G. (cat. no. 801392); Tween 20 polyoxyethylene sorbitan monolaurate from ICI Americas Inc., Wilmington, DE; and fetal calf serum from GIBCO, Paisley, Scotland.

The murine monoclonal antibody, "E6," against hPLAP was produced as described (18). It reacts specifically with hPLAP but with no other isoenzymic forms of alkaline phosphatase. The antibody-containing culture fluid of this hybridoma was used without any further purification.

The samples, taken from hospital patients selected without obvious bias, consisted of serum or heparinized plasma routinely provided for biochemical analysis in the clinical laboratory of the University Hospital Antwerp. Hepatitis-negative sera were kept at 4 °C for no longer than 48 h or stored for several weeks at −20 °C and thawed only once, immediately before use. The sampling period covered nine months.

Surgical biopsies were obtained from several cancer patients. In most cases a biopsy sample was taken both from the tumor and from adjacent tissue 10 cm away. All specimens were immediately frozen in liquid nitrogen and stored at −20 °C until they were extracted with butanol.

Methods

We prepared and purified hPLAP as described by Lehmann (19). The absence of alkaline phosphatase isoenzymes of liver, bone, or kidney origin was verified by reaction with one of our monoclonal antibodies to these isoenzymes. The hPLAP standard (250 U/L) was stored as 1-mL aliquots in liquid nitrogen in a solution of phosphate-buffered isotonic saline/bovine serum albumin (per liter: 0.2 g of KCl, 0.2 g of KH2PO4, 8 g of NaCl, 1.44 g of Na2HPO4 · 2H2O, and 5 g of albumin).

On request, the hybridoma (E6) culture fluid is available for research purposes.

---

1 Departments of 1Nephrology-Hypertension and 2Gynecology, University Hospital Antwerp, Wilrijkstraat 10, B-2610 Edegem, Belgium.
2 Department of Molecular Biology, State University Ghent, B-9000 Gent, Belgium.
3 Author to whom correspondence should be addressed.
4 Received July 5, 1984; accepted September 20, 1984.
For the hPLAP enzyme–antigen immunoassay, dilute the rabbit antiserum to mouse IgG<sub>2A</sub>, 1000-fold with, per liter, 10 mmol each of Tris (pH 8.5), NaCl, and NaN<sub>3</sub>. To each well of a 96-well immunoplate add 200 μL of diluted antiserum, incubate for 1 h at 37 °C, then remove the antibody solution and fill the wells with 250 μL of blocking solution (per liter: 10 mL of fetal calf serum, 0.2 g of KCl, 0.2 g of KH<sub>2</sub>PO<sub>4</sub>, 8 g of NaCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 10 g of albumin, and 50 mmol of NaN<sub>3</sub>). After another hour at 37 °C, remove the blocking solution and wash the plate three times with phosphate-buffered saline containing 0.5 mL of Tween 20 and 50 mmol of NaN<sub>3</sub> per liter. Next, dilute the culture medium of the hybridoma (E6) producing the monoclonal anti-hPLAP antibody 100-fold with phosphate-buffered saline, add 200 μL of this solution to each well of the microplate, and incubate the mixture for 3 h at room temperature. Then wash the plate three times with washing buffer (phosphate-buffered saline containing 50 mmol of NaN<sub>3</sub> per liter). Then add a known amount of hPLAP serially diluted with phosphate-buffered saline (200 μL of 40 to 0.02 U/L) to the microtiter plate, add 200 μL of the undiluted serum sample to the wells, and incubate at 4 °C for 16 h. Before developing the plate, wash it three times with the washing buffer. Add 200 μL of substrate (p-nitrophenyl phosphate in N-ethylaminoethanol, 5 mmol/L, pH 10.2) (20) to each well and let the reaction proceed at 37 °C for 4 h.

Stop the reaction by adding 50 μL of NaOH (3 mol/L), and measure the absorbance at 405 nm of each well on the plate. We use a spline function to compute the concentrations for the standard curve and for the samples. We assayed all samples in duplicate.

**Butanol extracts of tumor biopsies.** After homogenizing tissue samples of about 500 mg in a Potter–Elvehjem homogenizer, in 4.5 mL of Tris HCl (50 mmol/L, pH 6.8), we slowly added 2 mL of n-butanol to the homogenate while stirring with a magnetic stirrer during 20 min at room temperature. We then centrifuged for 20 min at 300 × g, and applied 1.5 mL of the aqueous phase to a 1.3 × 6.5 cm column of Sephadex G-25, eluting with the Tris HCl buffer at a flow rate of 1 mL/min. We collected 1-mL fractions, and pooled the fractions containing alkaline phosphatase activity. These were stored at −20 °C and thawed only once, just before analysis.

One unit (U) of enzyme activity is defined as the activity liberating 1 μmol of 4-nitrophenol (ε<sub>405</sub> = 14 600 L mol<sup>−1</sup> cm<sup>−1</sup>) from 4-nitrophenyl phosphate per minute at 37 °C when incubated in a solution containing 5 mmol of 4-nitrophenyl phosphate per liter of N-ethylaminoethanol buffer (0.1 mol/L, pH 10.2) (20).

In each assay, we used several control samples from normal subjects and a standard containing purified hPLAP, 0.6 U/L.

**Results**

**Analytical Variables**

*Assay optimization.* We examined several variables to determine optimum conditions for the assay, including type of microtiter plate, concentrations of reagents, and incubation time. Coating the microtiter wells with 1000-fold diluted rabbit antiserum to mouse IgG<sub>2A</sub> gave the best results. The monoclonal antibody in culture medium containing 100 mL of fetal calf serum per liter, diluted 100-fold with phosphate-buffered saline, yielded a maximum absorbance of 2.1 ± 0.1 A after incubation with alkaline phosphatase (1 U/L, 37 °C, 4 h).

**Standard curve.** Figure 1 shows standard curves for dilutions of a purified hPLAP standard (21 U/L), a representative normal serum (10.5 U/L) from a pregnant woman, and a representative butanol extract (5.3 U/L) of an ovarian tumor biopsy. All butanol extracts from tumor tissues examined (lung, ovary, stomach) had the same dilution curve. The linear portion of the curve covers the activity concentration range of 0.2 to 1 U/L. The assay sensitivity—defined as the least amount of hPLAP that differs significantly (Student’s t-test, n = 12) from zero at the 95% confidence limit—is 0.020 U/L. The half-saturating concentration of alkaline phosphatase for a 4-h incubation was 0.6 U/L.

*Reproducibility.* The within-assay precision was tested on different plates in the same assay run by parallel measurements of five dilution curves. The mean CV for determinations of purified hPLAP was 6.8% (SD 2.1%), for tissue extracts 6.1% (SD 3.0%) and for serum 3.8% (SD 0.9%). The within-assay CV of the upper reference limit in serum (0.1 U/L) was 2.8%. The precision between assays was tested on five different days for serum (CV, mean ± SD, 6.7 ± 2.0%), tissue extracts (7.0 ± 2.0%), and purified hPLAP (7.0 ± 2.1%). The interassay CV for the upper reference limit in serum (0.1 U/L) was 7.1%.

**Dilution curves.** Four serum samples were tested in four serial dilutions, each in duplicate, corresponding to antigen-binding values between 3% and 90%. The mean CV was 5.5% (SD 1.22%).

**Analytical recovery.** Analytical recovery was assessed by assaying increasing amounts of purified hPLAP added to a pooled specimen of normal human serum. The activities of the different dilutions were assayed in a single plate. We could account for more than 95% of the added hPLAP in the range 0.1 to 1 U/L. Samples containing more than this were diluted accordingly and re-assayed.

**Sampling and storage.** The enzyme–antigen immunoassay was developed for routine use in serum and in heparinized plasma. We determined the influence of storage temperature on results of the assay: 4, −20, and −70 °C. Storage

---

Fig. 1. Dilution curve of (21 U/L) purified hPLAP (○), hPLAP in a normal serum (10.5 U/L) from a pregnant woman (□), and hPLAP in a butanol extract (5.3 U/L) of ovarian-tumor tissue (■). All samples diluted with phosphate-buffered saline. Bars indicate ±1 SD (intra-assay variability).
at 4 °C for not longer than one week, or one cycle of freezing/thawing, did not affect immunological determinants of hPLAP, but repeated freezing and thawing decreased hPLAP activity. Samples to be stored for longer than one week were stored at -20 °C.

Clinical Applications

To assess this assay for the detection and quantification of hPLAP in serum and tissue extracts, we collected serum samples from normal blood donors (blood-transfusion center), hospital patients selected without regard to their disorders (hospital biochemical laboratory), cancer patients with a verified diagnosis, and patients with hematological disorders. Values for samples from pregnant women were not included when the 98th percentile value was calculated. Butanol extracts were prepared from surgical biopsies of neoplastic and normal tissues.

We used the values for a control population of healthy nonpregnant blood donors to establish the normal cutoff values for serum hPLAP. The range of values measured was 0.020–0.102 U/L; the 98th percentile value was 0.05 U/L.

To determine values for serum in clinical practice, we assayed 1650 sera from an overall hospital population without regard to pathology. The 98th percentile value was 0.096 U/L (range 0.020–3.730). For convenience we used 0.1 U/L as a cutoff value. All 21 patients having a value of 0.1 U/L or more for serum were identified and their value for circulating hPLAP was checked with use of a second blood sample. Results of determinations on consecutive samples from the same patients were internally consistent. The diagnoses of patients with serum hPLAP values between 0.1 and 0.2 U/L were as follows: one epilepsy (receiving antiepileptic therapy), one cerebrovascular accident (aspiration pneumonia), five smokers without any disorders obvious on thorough examination, one non-Hodgkin lymphoma, one ovarian carcinoma (metastasized), one breast carcinoma, two bronchial carcinomas, one prostatic carcinoma (metastasized). The diagnoses of patients with serum hPLAP >0.2 U/L were as follows: one metastasized cancer of unknown origin, two bronchial carcinomas (both metastasized), three ovarian carcinomas (all metastasized), and one endometrial carcinoma. We also analyzed 123 pre-operative serum samples from patients with well-documented tumors. Only 12 had a hPLAP value >0.1 U/L: two breast (5.9%), one endometrial (20.0%), four bronchial (22.2%), and four ovarian (40%) carcinomas. hPLAP activity was within the normal reference interval in the patients who had cancers of the bowel (three), cerebral (six), bladder (three), cervix (two), kidney (three), larynx (three), esophagus (one), stomach (four), vulva (four), adrenal glands (one), prostate (one), thyroid (four), acute leukemia (eight), myeloproliferative disorders (six), chronic lymphatic leukemia (three), and myeloma (one).

The normal reference interval for butanol extracts was determined on extracts of 23 fragments of healthy tissue (six stomachs, five ovaries, two colons, two lymph nodes, two rectums, two breasts, one kidney, one esophagus, one spleen, and one liver). The 98th percentile value was 1.00 mU/g of tissue (range 0 to 1.60 mU/g). Tissue extracts with hPLAP concentrations exceeding 1.00 mU/g were considered to exceed the normal reference interval.

For several patients we compared hPLAP values of normal tumor and normal tissue samples of the same organ (Figure 2). The activity in about 50% (51/103) of all tumor tissue extracts exceeded the reference interval, in contrast to that in 9.8% of the serum samples. The highest incidence of supranormal values was found for lung tissue (96%, with values as high as 176 mU/g) and ovarian tissue (94%, with up to 557 mU/g). Moreover, normal lung tissue contained a higher concentration of hPLAP than did any other nonmalignant tissues examined. Total alkaline phosphatase activity in tumor tissue was significantly higher (p <0.01, Student’s t-test) than in normal tissue. However, we found no correlation between total alkaline phosphatase and hPLAP activity: the highest concentrations of hPLAP occurring in tissues other than those containing the most total alkaline phosphatase.

Discussion

Because many tumors express this onco-developmental enzyme (21, 22), hPLAP—together with α-fetoprotein, carcinoembryonic antigen, and chorionic gonadotropin—has been useful in the management of hepatoma, gastrointestinal cancer, and embryonal genital tumors (10, 23–26).

By combining the specificity of both monoclonal and monoclonal antibodies (E6) with the sensitivity of the endogenous enzyme reaction, we developed a simple, reproducible, and highly sensitive enzyme–antigen immunoassay, suited for determination of hPLAP in serum, plasma, or butanol extracts of tissues. Because unpurified antibodies can be used, this method is easy, inexpensive, and applicable for mass-screening programs. Dilution curves for serum, butanol extracts, and standard antigen are similar. The range of hPLAP detected by this assay (0.02–1 U/L) is sufficiently broad and accurate to measure neoplasia-associated concentrations in serum and tissue extracts. The use of a highly
specific monoclonal antibody obviates those inaccuracies inherent in inactivation analysis and cross-reactivity of polyclonal antisera. The latter, raised against hPLAP, always cross react with intestinal alkaline phosphatase, and this cannot be completely obviated by heating for 10 min at 65 °C as some have suggested (27). Furthermore, serum intestinal alkaline phosphatase can be increased to 100 U/l in several clinical situations (28), its activity being dependent on ABO blood group, secretory status, and oral fat intake (21).

The sensitivity of our method was found to be 50 ng/l when a standard with a specific activity of 380 kU/g was used.

A first requirement for effective use of a tumor marker is to establish basal values for serum from a healthy reference population. Second, one must determine if an unselected hospital reference population shows a different distribution of serum values, owing to some specific or unspecific effects. The difference in mean hPLAP concentration we observed between blood donors and hospital patients was probably related to nonspecific antigen–antibody adsorption. The mean total alkaline phosphatase concentration in our hospital patients significantly exceeded the mean total alkaline phosphatase concentration for blood donors, being in some patients more than 1000 U/l; a nonspecific adsorption of 0.01% would result in an apparent hPLAP of more than 0.1 U/l in these sera. We noted no significant difference between hPLAP concentrations in serum and heparinized plasma, in contrast to earlier findings (22).

We could not establish the complete distribution of normal values, given the lower detection limit of 0.02 U/l. A serum hPLAP >0.2 U/l was associated in all cases with a malignant tumor. The group of patients whose serum hPLAP values were between 0.1 and 0.2 U/l had a 50% prevalence of malignancy (see Results). Increased activities of hPLAP suggested the presence of gynecological cancer (ovarian, endometrial, or breast) or a bronchial neoplasia. In close agreement with previous reports (3, 21, 22, 25), the overall increase in hPLAP activity in our group of cancer patients was 9.8% (12/123).

The normal reference interval for butanol extracts of tissue was calculated from data on extracts of healthy tissues, excluding lung tissue. hPLAP activity was increased in tumor tissue of lung (96%), ovary (94%), breast (22%), and stomach (25%). Normal lung tissue in most cases contained more hPLAP than did tumor tissue, in agreement with recent reports showing trace amounts of placental-like alkaline phosphatase in non-malignant lung tissue (26, 29, 30). This illustrates that a normal reference interval has to be determined separately for each different tissue. The histological localization of hPLAP in lung tissue remains to be determined.

The highest hPLAP activities were found in ovarian carcinomas (up to 557 mU/g), an organ subject to no interference by high activities in adjacent tissue. The high percentage of increased hPLAP activity in ovarian tumors correlates well with the high incidence of above-normal hPLAP values in the serum of these patients.

Although other specific and nonspecific tumor markers are already known for the detection and follow-up of ovarian carcinoma (31–33), the diagnostic difficulties and poor survival rate with this cancer make useful the addition of another marker to this battery of tests. Which tumor marker has the highest sensitivity and specificity remains to be determined.

We thank Dr. L. Lepoutre for supplying serum samples and Dra. P. Buytaert and C. Hanch for the surgical biopsies. The technical assistance of F. Coola, D. Bauwens, E. Martens, and D. Schellemans is gratefully acknowledged. This work was supported by grants from the "Fonds voor kankeronderzoek van de Algemene Spoel-er Lijfrente-kas, Nationale Loterij - FGWO" (grant 9.0005.84), the "National Program for Reinforcement of the Scientific Research" (PREST/UIA 04), and a research grant from the University of Antwerp.

References

23. McDicken IW, Stamp GH, McLaughlin PJ, Johnson PM. Expression of human placental-type alkaline phosphatase in pri-