Eur. J. Clin. Chem. Clin. Biochem.
Vol. 30, 1992, pp. 343–347

Magnetic Beads in Suspension Enable a Rapid and Sensitive Immunodetection of Human Placental Alkaline Phosphatase

By *Patricia G. Hendrix*[1,2], *M. F. Hoylaerts*[1], *E. J. Nouwen*[1], *A. Van De Voorde*[2] and *M. E. De Broe*[1]

[1] *Departement Nefrologie-Hypertensie, Universitaire Instelling Antwerpen, Antwerpen, België*
[2] *NV Innogenetics, Zwijnaarde/Gent, België*

(Received December 5, 1991/February 23, 1992)

Summary: The kinetics and efficiency of the interaction between placental alkaline phosphatase and a monoclonal antibody (laboratory number 327) were determined by immunoassay using microtitre plates or magnetic beads. While only up to 45% of placental alkaline phosphatase was bound to microwells precoated with this antibody, even after prolonged incubation, no less than 60% and 100% binding were reached using magnetic beads after 1 and 3 h incubations, respectively.

High-molecular-mass placental alkaline phosphatase and complexed placental alkaline phosphatase forms were also completely bound to magnetic beads in the presence of deoxycholate (up to 9 g/l for serum samples). The assay sensitivity was improved up to 4-fold. In addition, 100% binding of the antigen was achieved during simultaneous incubation of magnetic beads, monoclonal antibody (125 μg/l), and placental alkaline phosphatase. This one-step enzymatic assay, based on magnetic beads, is an attractive alternative to the classic assay performed in microtitre plates, enabling rapid, precise, and sensitive antigen detection, and only necessitating a minimum of laboratory equipment.

Introduction

Placental and germ cell alkaline phosphatase[1]) are useful tumour markers for ovarian or seminoma cancer (1). Additionally, due to the near monopoly of the lung in expressing placental alkaline phosphatase, this isoenzyme also shows an interesting potential as a marker for injury to the alveolar-capillary barrier. Placental alkaline phosphatase activity has been detected in some epithelial cells of the respiratory bronchioli and in type I pneumocytes. In addition, a substantial amount of the enzyme is liberated from the basal cell surface of the latter cells into the underlying membrane (2). Indeed, elevated placental alkaline phosphatase serum levels have been found in several conditions involving acute lung injury such as the adult respiratory distress syndrome (3). Preventive or very early treatment of adult respiratory distress syndrome is currently hindered due to the lack of sensitive markers (4). Based on a prospective study, it was concluded that serum placental alkaline phosphatase elevations almost invariably preceded adult respiratory distress syndrome, suggesting that frequent placental alkaline phosphatase determinations could increase the sensitivity for detection of developing adult respiratory distress syndrome. The observation that adult respiratory distress syndrome did not develop in subjects maintaining normal serum placental alkaline phosphatase levels throughout the first 48 h after admission (3) was particularly relevant.

In order to develop and optimize a fast placental alkaline phosphatase assay to measure small fluctuations in serum placental alkaline phosphatase levels, a magnetic enzyme activity immunoassay, based on specific isoenzyme capture followed by measurement

[1]) Enzyme: Alkaline phosphatase (EC 3.1.3.1)

of retained enzyme activity, was compared with the classic version of this assay which was performed in microtitre plates. We presently describe this immunomagnetic assay, which is based on an anti-placental alkaline phosphatase monoclonal antibody (laboratory number 327 (5)). Because of improved diffusion and larger sample volumes, this assay enables a rapid (within 2 h), accurate, and precise determination of low serum placental alkaline phosphatase levels.

Materials and Methods

Chemicals and supplies

Rabbit anti-mouse IgG was supplied by Dakopatts (Glostrup, Denmark). Deoxycholate and *p*-nitrophenyl phosphate were from Sigma Chemical Co. (St. Louis, MO). Ninety-six-well microtitre plates were from Nunc (Roskilde, Denmark). The magnetic beads (Dynabeads M-450; 4.5 μm diameter) were supplied by Dynal (Skoyen, Norway). These Dynabeads are uniform magnetic polystyrene beads with 5 μg of affinity purified sheep-anti-mouse IgG1 (Fc) covalently bound to the surface of one mg beads; they are sold as a suspension containing approximately 420 × 10^9 beads (30 g) per litre. Sephacryl S-300 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The EAR400 multi-well reader was from SLT-labinstruments (Gröding, Austria). All other regular reagents were of pro analysis quality from Merck (Darmstadt, Germany).

Placental alkaline phosphatase isoforms

Placental alkaline phosphatase was extracted from a fresh placenta and gel filtration on a 1.6 × 77 cm column of Sephacryl S-300 was performed as described (5), separating placental alkaline phosphatase into a dimeric enzyme (M_r = 130 000) and a high-molecular-mass placental alkaline phosphatase-containing peak, eluting just behind the void volume of the column (M_r 10^6). After affinity chromatography, anion exchange and size-exclusion HPLC, the specific activity of placental alkaline phosphatase (> 95% pure) was 250 U/mg (5).

Enzyme activity immunoassay in microtitre wells

Placental alkaline phosphatase was assayed as described previously (6), using the anti-placental alkaline phosphatase monoclonal antibody 327 (5). Placental alkaline phosphatase samples (0.25 U/l, 200 μl) were incubated at 37 °C for 3 h, or as specified, and bound enzyme was visualised with 10 mmol/l *p*-nitrophenyl phosphate in N-ethylaminoethanol HCl buffer (20 g/l, pH 10.2). The fraction of binding was calculated by comparison with the activity of known standards.

Enzyme activity immunoassay with immunomagnetic beads

In the two-step assay, sheep anti-mouse IgG1 (Fc) coated magnetic beads were preloaded with antibody 327 by incubating 12 μl of the bead suspension in 120 μl of antibody solution (0 to 16 mg/l), for 5 h at 37 °C, under continuous stirring. After magnetic phase separation, the beads were washed 3 times in 1 ml of phosphate buffered saline, containing 0.5 ml of Tween-80 per litre, then resuspended in 120 μl of blocking solution. These preloaded immuno-beads (50 μl) were added to one ml placental alkaline phosphatase samples (0.25 U/l) and incubated at 37 °C for 3 h under gentle stirring. After magnetic phase separation, the supernatants were removed and the unbound placental alkaline phosphatase was measured. Bound placental alkaline phosphatase activity was visualized by addition of 200 μl of the buffered substrate solution to the beads. Staining was arrested after 45 min by the addition of 30 μl of a concentrated NaOH (5 mol/l) solution. Then, 200 μl of supernatants or standards were transferred to a 96-well microtitre plate to measure the absorbance at 405 nm in an EAR 400 multi-well reader, followed by calculation of the percentages of bound placental alkaline phosphatase.

Alternatively, in the one-step assay, 5 μl of sheep anti-mouse IgG1 (Fc)-coated beads were adjusted to 25 μl with blocking solution and incubated simultaneously with 25 μl of antibody solution (0 to 16 mg/l, or at 125 μg/l, as specified) and a placental alkaline phosphatase-containing sample (0.25 U/l, 1 ml). Incubations were performed at 37 °C under continuous gentle stirring for various time intervals. The magnetic phase separation and determination of placental alkaline phosphatase binding was performed as described above.

The accuracy of the placental alkaline phosphatase determination in this simultaneous incubation protocol was tested for a progressively diluted series of placental alkaline phosphatase samples (1 to 0.001 U/l; 1 ml), choosing a fixed antibody concentration (125 μg/l), and two different incubation times, i.e. 1 or 3 h, in combination with two different colour development times of 1 and 3 h, respectively. Binding efficiencies of high- and low-molecular-mass placental alkaline phosphatase were studied in a similar way.

Recovery analysis in serum

The magnetic bead immunoassay was performed on fresh sera, containing 0 to 2.5 U/l placental alkaline phosphatase which were supplemented with 250 mU of placental alkaline phosphatase per litre in the presence or absence of deoxycholate (9 g/l). The recovery was expressed using the following formula:

$$\frac{(A_{405\,nm}\,(\text{supplemented serum}) - A_{405\,nm}\,(\text{non-supplemented serum})) \times 100}{A_{405\,nm}\,(\text{placental alkaline phosphatase supplement})}$$

Results

Binding of placental alkaline phosphatase by antibody 327 insolubilised on magnetic beads

Results from the preloading of 5 μl of the sheep anti-mouse IgG1 (Fc)-coated beads (i.e. 2.1 × 10^6 beads, coated with 750 ng sheep anti-mouse IgG1 (Fc)) with increasing concentrations of the anti-placental alkaline phosphatase monoclonal antibody 327, and subsequent incubation with placental alkaline phosphatase samples for 3 h at 37 °C, indicated that 100% of the antigen could be bound to the solid phase when the antibody concentration was above 150 ng antibody/120 μl during preloading (fig. 1). When magnetic beads, antibody, and placental alkaline phosphatase sample were incubated simultaneously, the percentage

of placental alkaline phosphatase bound to the solid phase rose with increasing antibody concentrations. Complete antigen binding was observed at antibody concentrations of 125 µg/l, corresponding to surface densities of 0.036 pg antibody 327 per bead, i.e. 56 ng/cm^2. At antibody concentrations exceeding 500 µg/l, increasing concentrations of free antibodies competed for placental alkaline phosphatase-binding with the bound antibodies, explaining the descending limb of figure 1. Complementary measurements of unbound placental alkaline phosphatase confirmed these findings.

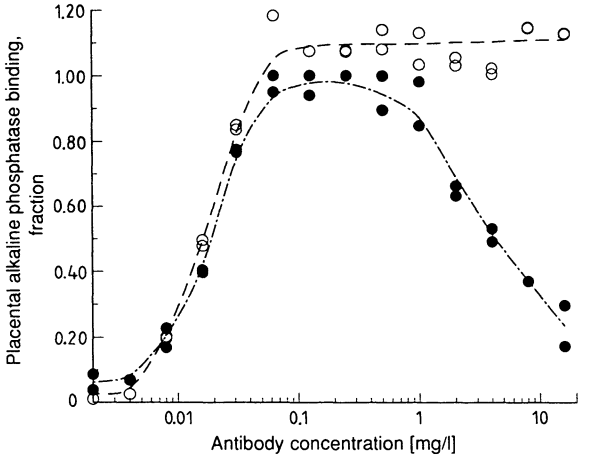

Fig. 1. Efficiency of placental alkaline phosphatase binding by increasing concentrations of monoclonal antibody 327 in a magnetic enzyme activity immunoassay. Placental alkaline phosphatase (0.25 U/l; 1 ml) was incubated with gentle stirring for 3 h at 37 °C with increasing concentrations of antibody 327 (0 to 16 mg/l), either attached on the magnetic sheep anti-mouse IgG1 (Fc)-coated beads beforehand, or incubated simultaneously with the antigen and the beads. The assays were continued (washing and staining) as described in *Materials and Methods*.
o, antibody concentrations during preloading of the sheep anti-mouse IgG1 (Fc)-coated magnetic beads;
●, antibody concentrations during the one-step magnetic bead immunoassay.

Kinetics of placental alkaline phosphatase binding in the magnetic bead assay

During the simultaneous incubation with beads (5 µl) and antibody (125 µg/l), the binding of placental alkaline phosphatase displayed first-order kinetics up to 1 h. An incubation time of 3 h was required to achieve complete antigen binding (fig. 2). During the immunoassay in microtitre wells, a much slower binding kinetic was observed, associated with a maximal binding efficiency of less than 45% (fig. 2).

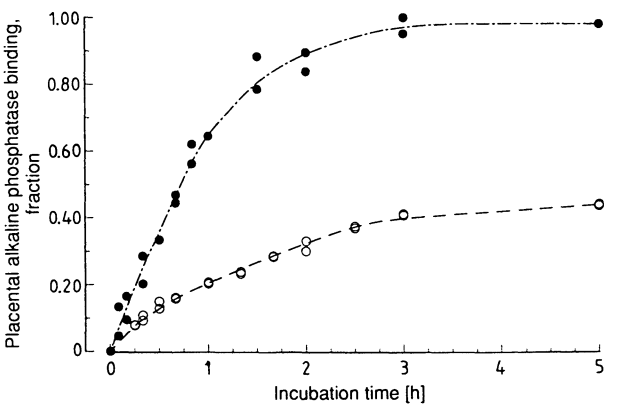

Fig. 2. Kinetics of placental alkaline phosphatase binding by antibody 327 in the EAIA performed on sheep anti-mouse coated magnetic beads or in microtitre plates. In the magnetic bead assay, a placental alkaline phosphatase-containing sample (0.25 U/l; 1 ml) was incubated for several time intervals with 25 µl antibody (final concentration 125 µg/l) and 5 µl immunobead suspension, adjusted to 25 µl beforehand.
In the classic immunoassay, 200 µl of the same placental alkaline phosphatase sample was incubated for different time intervals in microwells, preloaded with an excess of antibody.
Both assays were continued as described in *Materials and Methods*.
●, magnetic bead enzyme activity immunoassay;
o, classic enzyme activity immunoassay.

Standard curves, sensitivity and precision of the magnetic bead enzyme activity immunoassay

In the simultaneous incubation protocol, the fraction of bound placental alkaline phosphatase was constant, even for low placental alkaline phosphatase concentrations, i.e. about 60 and 100% for the short and prolonged assay procedures, respectively. The lower detection limit (defined as the amount of placental alkaline phosphatase corresponding to the upper limit of a 99% confidence interval of zero) corresponded to 0.006 U/l in the extended protocol (3 h incubation, 3 h colour development), whereas in the short assay (1 h incubation, 1 h colour development), it was 0.009 U/l. By contrast, the assay performed in microtitre plates showed a lower detection limit of 0.022 U/l placental alkaline phosphatase (3 h incubation, 3 h colour development).

Precision and accuracy of placental alkaline phosphatase measurement in serum using the magnetic bead enzyme activity immunoassay

This assay was satisfactory in terms of precision and accuracy. Analysis of two samples containing different amounts of placental alkaline phosphatase gave the following within-assay variation (n = 8 determina-

tions each): 1.4 ± 0.06 U/l (CV 4.0%) and 12.95 ± 0.44 µg/l (CV 3.4%). The between-assay variation calculated for the first sample on 4 consecutive days was 1.35 ± 0.06 µg/l (CV 4.2%). Using the magnetic immunoassay on fresh sera (containing 0 to 2.5 U/l placental alkaline phosphatase) which were supplemented with a known amount of placental alkaline phosphatase, only very low recoveries were obtained, varying from 4.3% to 57.0%. In view of the results obtained with deoxycholate in the microplate assay (4), this detergent (9 g/l final concentration) was also added in the present magnetic bead assay; recovery of placental alkaline phosphatase (95.7 ± 3.4%, n = 10) was complete, allowing accurate and precise placental alkaline phosphatase measurements in human serum samples.

Influence of antigen size on binding efficiency

The need for detergent inclusion during antigen incubation was particularly evident for the detection of high-molecular-mass placental alkaline phosphatase in microtitre plates. Only 11% of these placental alkaline phosphatase forms could be bound in the absence of deoxycholate, whereas 43% of dimeric placental alkaline phosphatase was bound. When deoxycholate concentrations were increased to 1 g/l, high- and low-molecular-mass placental alkaline phosphatase bound with the same efficiency. Switching to the magnetic assay did not improve the binding of the high-molecular-mass placental alkaline phosphatase (< 15%), unless deoxycholate was added. The binding efficiency then returned to 100% at the optimal antibody concentration of 125 µg/l (fig. 3).

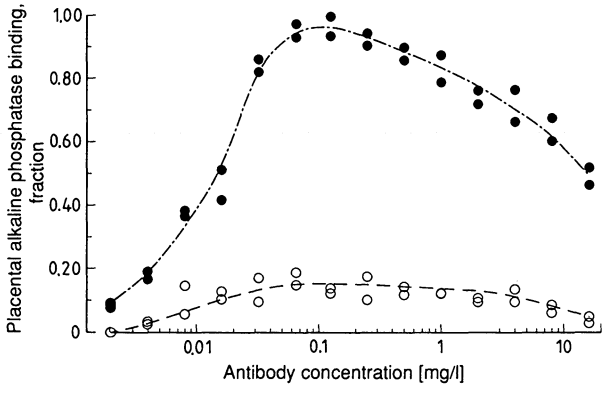

Fig. 3. Efficiency of binding of high-molecular-mass placental alkaline phosphatase by increasing concentrations of antibody 327, in the presence or absence of deoxycholate. High-molecular-mass placental alkaline phosphatase (0.25 U/l; 1 ml) and increasing concentrations of antibody 327 (0.002 to 16 mg/l) were incubated simultaneously for 3 h at 37 °C with 5 µl of a suspension of magnetic sheep anti-mouse IgG1 (Fc)-coated beads, both in the presence or absence of deoxycholate (1 g/l). The assay procedure was continued as described in *Materials and Methods*.
○, no deoxycholate;
●, with deoxycholate.

Discussion

The enzyme activity immunoassay principle, in which the enzyme activity of placental alkaline phosphatase is measured at the detection step (7), was developed for testing hybridoma cultures secreting anti-placental alkaline phosphatase monoclonal antibodies (8) and for the screening of placental alkaline phosphatase positivity in the serum of cancer patients (1). The present comparison of microplates and magnetic beads for use in an immunoassay, based on a specific anti-placental alkaline phosphatase monoclonal antibody, revealed a substantial increase, up to 100%, in the effectiveness of placental alkaline phosphatase binding in the assay, using magnetic beads. In addition to the advantage of using larger sample volumes (translated into higher sensitivities) and the easy phase separation, the simultaneous incubation of antigen, antibody, and magnetic beads made this an attractive assay. When the antibody concentration was raised in the one-step configuration, a plateau was reached between 0.1 and 0.5 mg/l, i.e. the ascending (antigen binding) and descending (competition) limbs of the curve were clearly separated. The high binding percentages obtained may be explained by a better orientation of antibody 327 (IgG1) when bound to the solid-phase, because the antibodies react exclusively with the Fc fragment of mouse IgG1 immunoglobulins, in contrast to the more random orientation of antibody 327 when bound to rabbit anti-mouse IgG-coated microwells. Furthermore, by gently stirring the beads homogeneously in the sample fluid, diffusional restrictions were reduced, leading to a more effective contact between the antibody and the antigen molecules than during incubations in microtitre wells. Finally, while total covered areas in both assay types were comparable, the amount of monoclonal antibody calculated per unit surface area was twice as high for the magnetic beads than for the microwells. The linearity of the standard curves indicated that the reaction rates were proportional to the amounts of placental alkaline phosphatase present, even for low concentrations of placental alkaline phosphatase. Placental alkaline phosphatase levels as low as 0.006 U/l could be accurately measured during the longer version of the protocol (3 h incubation, 3 h staining). When the total assay time was reduced to 2 h, the detection limit was reduced to 60%.

Although high binding efficiencies were observed for dimeric placental alkaline phosphatase, the recovery of high-molecular-mass placental alkaline phosphatase on the immunomagnetic beads was as poor as in the assay performed in microplates. However, upon dissociation of these placental alkaline phosphatase forms by deoxycholate, the magnetic immunoassay accurately reflected the total placental alkaline phosphatase levels. When measuring placental alkaline phosphatase levels in serum, even higher concentrations of deoxycholate were required due to interactions with other serum components, similarly to those seen in the enzyme activity immunoassay performed in microtitre plates (5).

When using a previously described solid-phase immunoassay for placental alkaline phosphatase based on small (3.3 μm) monodisperse polymer particles (non-magnetic) (9), complete placental alkaline phosphatase binding was achieved after only 10 min of incubation, probably linked to the use of a higher affinity antibody. Nevertheless, the magnetic bead assay has the advantage of being a simple, one-step procedure, and magnetic separation obviates the need for centrifugation. The availability of a properly graded colour scale would also eliminate the need for spectrophotometric reading. The magnetic bead enzyme activity immunoassay for placental alkaline phosphatase thus proposed meets all the criteria for a bedside placental alkaline phosphatase test during the follow-up of patients at risk of developing adult respiratory distress syndrome: it is a quick and simple method, without compromising assay sensitivity, accuracy, or precision.

Acknowledgements

This work was supported by grants from the "Fonds voor Kankeronderzoek van de Algemene Spaar- en Lijfrentekas" and the IWONL (VL 162).

References

1. De Broe, M. E. & Pollet, D. E. (1988) Multicenter evaluation of human placental alkaline phosphatase as a possible tumor-associated antigen in serum. Clin. Chem. *34*, 1995–1999.
2. Nouwen, E. J., Buyssens, N. & De Broe, M. E. (1990) Heat-stable alkaline phosphatase as a marker for human and monkey type-I pneumocytes. Cell Tissue Res. *260*, 321–335.
3. Eestermans, G., Demey, H. E. & De Broe, M. E. (1989) Serum markers in acute lung injury. Update in Intensive Care and Emergency Medicine *8*, 72–79.
4. Andreadis, N. & Petty, T. L. (1985) Adult respiratory distress syndrome: problems and progress. Am. Rev. Respir. Dis. *132*, 1344–1346.
5. Hendrix, P. G., Hoylaerts, M. F., Nouwen, E. J. & De Broe, M. E. (1990) Enzyme immunoassay of human placental and germ-cell alkaline phosphatase in serum. Clin. Chem. *36*, 1793–1799.
6. Pollet, D. E., Nouwen, E. J., Schelstraete, J. B., Renard, J., Van de Voorde, A. & De Broe, M. E. (1985) Enzyme-antigen immunoassay for human placental alkaline phosphatase in serum and tissue extracts and its application as a tumor marker. Clin. Chem. *31*, 41–45.
7. Usategui-Gomez, M., Yeager, F. M. & Fernandez De Castro, A. F. (1973) A sensitive immunochemical method for the determination of the Regan isoenzyme in serum. Cancer Res. *33*, 1574–1577.
8. Jemmerson, R. & Fishman, W. H: (1982) Convenient selection of monoclonal antibodies to isoenzymes of placental alkaline phosphatase using the catalytic activity of the antigen in enzyme antigen immunoassay. Anal. Biochem. *124*, 286–292.
9. Millán, J. L., Nustad, K. & Norgaard-Pedersen, B. (1985) Highly sensitive solid-phase immunoenzymometric assay for placental and placental-like alkaline phosphatases with a monoclonal antibody and monodisperse polymer particles. Clin. Chem. *31*, 54–59.

Patricia G. Hendrix
Nephrology-Hypertension
University Hospital Antwerp
Wilrijkstraat 10
B-2650 Edegem/Antwerpen