The preparation of monoclonal antibodies to human bone and liver alkaline phosphatase and their use in immunoaffinity purification and in studying these enzymes when present in serum

Elaine M. BAILYES, Richard N. SEABROOK, Jacqueline CALVIN, Gerald A. MAGUIRE,*
Christopher P. PRICE, Kenneth SIDDLE and J. Paul LUZIO

Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road,
Cambridge CB2 2QR, U.K.

1. Liver and bone alkaline phosphatase isoenzymes were solubilized with the zwitterionic detergent sulphobetaine 14, and purified to homogeneity by using a monoclonal antibody previously raised against a partially-purified preparation of the liver isoenzyme. Both purified isoenzymes had a specific activity in the range 1100–1400 $\mu\text{mol}/\text{min}$ per mg of protein with a subunit M_r of 80000 determined by SDS/polyacrylamide gel electrophoresis. Butanol extraction instead of detergent solubilization, before immunoaffinity purification of the liver enzyme, resulted in the same specific activity and subunit M_r . The native M_r of the sulphobetaine 14-solubilized enzyme was consistent with the enzyme being a dimer of two identical subunits and was higher than that of the butanol-extracted enzyme, presumably due to the binding of the detergent micelle. 2. Pure bone and liver alkaline phosphatase were used to raise further antibodies to the two isoenzymes. Altogether, 27 antibody-producing cell lines were cloned from 12 mice. Several of these antibodies showed a > 2 -fold preference for bone alkaline phosphatase in the binding assay used for screening. No antibodies showing a preference for liver alkaline phosphatase were successfully cloned. None of the antibodies showed significant cross-reaction with placental or intestinal alkaline phosphatase. Epitope analysis of the 27 antibodies using liver alkaline phosphatase as antigen gave rise to six groupings, with four antibodies unclassified. The six major epitope groups were also observed using bone alkaline phosphatase as antigen. 3. Serum from patients with cholestasis contains soluble and particulate forms of alkaline phosphatase. The soluble serum enzyme had the same size and charge as butanol-extracted liver enzyme on native polyacrylamide-gel electrophoresis. Cellulose acetate electrophoresis separated the soluble and particulate serum alkaline phosphatases as slow- and fast-moving forms respectively. In the presence of sulphobetaine 14 all the serum enzyme migrated as the slow-moving form on cellulose acetate electrophoresis. Monoclonal anti-(alkaline phosphatase) immunoadsorbents did not bind the particulate form of alkaline phosphatase in cholestatic serum but bound the soluble form. In the presence of sulphobetaine 14 all the cholestatic serum alkaline phosphatase bound to the immunoadsorbents. 4. The electrophoretic and immunological data are consistent with both particulate and soluble forms of alkaline phosphatase in cholestatic serum being derived from the hepatocyte membrane.

INTRODUCTION

The alkaline phosphatases (EC 3.1.3.1) are intrinsic plasma membrane enzymes found in many tissues. Structural and genetic studies divide the isoenzymes of man into three distinct groups: placental, intestinal (fetal and adult), and hepatic/renal/skeletal (McComb *et al.*, 1979; Seargeant & Stinson, 1979; Moss, 1982). Liver and bone alkaline phosphatase are thought to be products of the same gene locus but they can be qualitatively distinguished by their different electrophoretic mobility on charge-separation gels and by their different stability to denaturation with heat or urea. These differences in properties are probably due to tissue-specific post-translational modifications such as glycosylation (Moss & King, 1962; Stinson & Seargeant, 1981; Moss, 1984a). Most reported polyclonal antisera raised to either liver or bone isoenzymes show cross-reactivity (McComb *et al.*, 1979). Soluble forms of liver and bone isoenzymes occur

in normal serum, and elevated levels in serum are a feature of certain liver and bone diseases (Moss, 1982, 1984b). Part of the serum alkaline phosphatase of patients with hepatobiliary disease is of high M_r and may be complexed with other plasma membrane enzymes and lipids. High- M_r alkaline phosphatase is diagnostic of obstructive liver diseases, particularly extra-hepatic obstruction (De Broe *et al.*, 1975; Price & Sammons, 1976; Moss, 1982; Siede & Seiffert, 1983). The origin of high- M_r alkaline phosphatase and its relationship with soluble serum enzyme has generated considerable controversy (Price & Sammons, 1974; Crofton & Smith, 1981; De Broe *et al.*, 1985; Kaplan, 1986; Wulkan & Leijnse, 1986; Maguire *et al.*, 1986).

The aim of the present study was to prepare and use monoclonal antibodies against liver and bone alkaline phosphatase in investigating the nature and extent of differences between these isoenzymes and between tissue and serum forms. In addition it was hoped that the

* Present address: Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K.

potential high specificity of monoclonal antibodies could be used in establishing clinically useful assays for particular tissue or serum forms. Initially, six mouse monoclonal antibodies were raised using partially-purified human liver alkaline phosphatase as antigen (Bailyes *et al.*, 1985). They all reacted with the same epitope on the enzyme, and cross-reacted with bone alkaline phosphatase. One of the antibodies was chosen for immunoaffinity purification of liver and bone alkaline phosphatases to provide pure antigen for preparing further monoclonal antibodies.

EXPERIMENTAL

Materials

Sulphobetaine 14 [Zwittergent TM 3-14; 3-(*NN*-dimethyl-*N*-tetradecyl)ammoniopropane-1-sulphonate] was obtained from Calbiochem. Titan III cellulose acetate was purchased from Helena Lab (U.K.), Unit 2B, Airport Industrial Estate, Kenton, Newcastle-upon-Tyne, U.K.

Assays

Alkaline phosphatase activity was measured at 37 °C using 10 mM-4-nitrophenyl phosphate in 0.75 M-2-amino-2-methylpropan-1-ol/HCl buffer, pH 10.3; 1 unit of enzyme activity = 1 μ mol of substrate hydrolysed/min. Enzyme activity after polyacrylamide-gel electrophoresis was determined by incubating the gels in 1 mg of α -naphthyl acid phosphate (Sigma N-7000)/ml in 0.38 M-Tris/0.5 mM-MgCl₂ for 30 min, followed by 1.33 mg of Fast Blue BB (Sigma F-0250)/ml in the above buffer to stain the activity bands. Enzyme activity after cellulose acetate electrophoresis was also determined by this method using an overlay technique, but with 2-amino-2-methylpropan-1-ol/HCl, pH 10.3, as the buffer. 5'-Nucleotidase activity was measured by a continuous monitoring colorimetric method with AMP as substrate (Maguire, 1985). Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Monoclonal antibodies

Balb/c mice were immunized with liver or bone alkaline phosphatase and monoclonal antibodies to these enzymes were produced as described for the production of antibody 5N4-2 (Siddle *et al.*, 1981). Culture supernatants were screened for anti-(alkaline phosphatase) antibodies by the enzyme-antigen immunoassay of Jemmerson & Fishman (1982). Polyvinyl chloride 96-well flexible assay plates (Falcon 3912) were coated with sheep anti-(mouse IgG) antibodies (Siddle *et al.*, 1981) by incubating the wells overnight at 4 °C with 150 μ l of 50 mM-potassium phosphate buffer, pH 6.5, containing 0.1 mg of sheep IgG/ml. After washing with 50 mM-Tris/HCl (pH 7.5)/1 mM-MgCl₂/0.1% sulphobetaine 14/0.1% bovine serum albumin (wash buffer), the wells were incubated for 1 h with 100 μ l of spent culture supernatants and 50 μ l of wash buffer. After washing, wells were incubated for 1 h with 100 μ l of liver or bone alkaline phosphatase at 0.1 unit/ml in wash buffer. After further washing, 100 μ l of enzyme assay cocktail was added and the plates were incubated at 37 °C. The reaction was stopped by the addition of 50 μ l of 2 M-NaOH and the A_{405} of the wells was determined

with an automatic plate-reader (Flow Laboratories; Titertek Multiscan MC).

Purification of liver and bone alkaline phosphatase

Microsomal membranes were prepared from human liver obtained < 12 h post-mortem and from fetal limb bones < 48 h post mortem. The enzymes were solubilized with 2% (w/v) sulphobetaine 14 and purified to homogeneity on an anti-(alkaline phosphatase) monoclonal antibody immunoabsorbent prepared by coupling the antibody to aminocellulose by diazotization (Hales & Woodhead, 1980). The enzymes were eluted with fresh 50 mM-diethylamine at 0 °C, neutralized with 2 M-Tris/HCl, pH 6.8, passed down an ACA34 gel filtration column and concentrated using an Amicon PM30 membrane (Bailyes *et al.*, 1982). Butanol-extracted liver alkaline phosphatase was partially purified by the first three steps of Meyer *et al.* (1982) before application to the monoclonal antibody immunoabsorbent. The liver was extracted with a 2:1 mix of 10 mM-Tris/HCl (pH 7)/1 mM-MgCl₂/0.025 mM-ZnCl₂ and butan-1-ol and the aqueous phase was applied batchwise to DE-52 cellulose equilibrated in the above buffer. The enzyme was eluted with 0.75 M-NaCl and bound to concanavalin A-Sepharose (Pharmacia). After elution with α -methylmannoside the enzyme was purified to homogeneity as described for the detergent-solubilized enzyme, except that no detergent was added to the buffers used.

Liver membrane preparation

A human liver membrane fraction was also prepared by the method described by Cuatrecasas (1972) for rat liver and used to study the binding of membrane-bound and detergent-solubilized liver alkaline phosphatase to the antibody immunoabsorbents.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was conducted as previously described (Bailyes *et al.*, 1982). Gels were stained for protein by the silver stain method of Ansorge (1982). Non-denaturing polyacrylamide gels were prepared as described previously (Newby *et al.*, 1978), using buffer system 4229 of Jovin *et al.* (1970). The final concentrations of the buffer solutions used were: cathode buffer, 40 mM-4-aminobutyric acid/NH₃ solution, pH 9.9; stacking and resolving gel buffer, 34 mM-H₂SO₄/NH₃ solution, pH 7.4; anode buffer, 62.5 mM-NH₃ solution/50 mM-HCl. Electrophoresis was conducted at room temperature. Serum forms of alkaline phosphatase were separated by electrophoresis on cellulose acetate in 0.08 M-barbitone/0.04 M-Tris, pH 8.8, at 180 V for 25 min.

Serum alkaline phosphatase

Serum samples were selected from those received in the routine National Health Service Laboratory. Only samples with alkaline phosphatase activity > 500 units/l (normal range 30–135 units/l) were selected for further analysis. The binding of serum alkaline phosphatase (or high- and low- M_r fractions isolated by gel filtration on Sephacryl S-300) to solid phase antibody was assessed by incubating 50 μ l samples with 50 μ l of washed immunoabsorbent (2 mg of cellulose/ml in 50 mM-Tris/HCl, pH 7.4) for 5 min at 20 °C, then centrifuging for 1 min in an Eppendorf 5432 centrifuge and assaying the supernatant for enzyme activity. Detergent (sulpho-

betaine 14; 2%, w/v) when present was added to the sample for 1 h at 20 °C prior to immunoadsorbent treatment.

Epitope analysis

The relationship between the binding sites of different antibodies was investigated by testing the effect of preincubation of alkaline phosphatase with one antibody in solution on subsequent binding to a second immobilized antibody, in the standard screening assay (Soos & Siddle, 1983; Soos *et al.*, 1986). The preincubation mixture consisted of spent culture medium or IgG (50 µg of protein/ml) with an equal volume of alkaline phosphatase at 0.1 unit/ml. The antibodies were immobilized by incubating sheep anti-(mouse IgG)-coated wells with either spent culture medium or purified IgG (50 µg of protein/ml) and then blocking any excess binding sites with non-specific mouse IgG.

RESULTS

Immunoaffinity purification and properties of liver and bone alkaline phosphatase

Microsomal membranes from human liver and fetal bones were prepared, extracted with sulphobetaine 14 and the alkaline phosphatases immunoaffinity-purified using a previously isolated monoclonal antibody (coded LAP 1/7) to the liver isoenzyme (Bailes *et al.*, 1985), as described in the Experimental section. A typical preparation of the liver isoenzyme is shown in Table 1. After the immunoaffinity step, gel filtration removed minor contaminants, principally immunoglobulin light chains. Omitting gel filtration resulted in a lower specific activity (960 units/mg of protein). Bone alkaline phosphatase was more difficult to prepare than liver alkaline phosphatase. After diethylamine elution and neutralization it was important to change the buffer quickly by using a Sephadex G-25 column, otherwise the activity was unstable. The specific activity obtained in a typical preparation of the pure bone isoenzyme (1091 units/mg of protein) was similar to that of pure liver alkaline phosphatase (1357 units/mg of protein) in agreement with previous reports (Stinson & Seargeant, 1981; Meyer *et al.*, 1982). Both isoenzymes had an M_r of 80000 determined by SDS/polyacrylamide-gel electrophoresis (Fig. 1). On non-denaturing polyacrylamide gels the mobility of the bone isoenzyme was slightly lower than that of the liver isoenzyme (Fig. 2).

Detergent solubilization or butanol extraction of human liver made no difference to the SDS-gel M_r of the

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified bone and liver alkaline phosphatase

Sulphobetaine 14-extracted (track a), butanol-extracted liver alkaline phosphatase (track b) and sulphobetaine 14-extracted bone alkaline phosphatase (track c) (2 units of each) were reduced and electrophoresed on a 10% (w/v acrylamide monomer concentration) gel. Track (d), M_r marker proteins (Bio-Rad). The gel was stained for protein by the silver stain method (Ansorge, 1982).

Table 1. Immunoaffinity purification of liver alkaline phosphatase

Step	Units	Specific activity (units/mg)	Yield (%)
Homogenate	509	0.06	100
Membranes	173	0.2	34
Detergent extract	200	4	39
Immunoabsorbent binding, elution, gel filtration and Amicon concentration	152	1357	30

subsequently immunoaffinity-purified enzyme; both preparations, made from the same liver, gave a single band of M_r 80000 (Fig. 1). Upon gel filtration on Ultrogel AcA 34 (the final purification step after elution from the immunoabsorbent), the butanol-extracted enzyme had a smaller apparent M_r . On non-denaturing polyacrylamide gels the butanol-extracted enzyme had a higher mobility, and did not require detergent to enter the restrictive gels. The mobility (R_F) of the butanol- and sulphobetaine 14-solubilized liver enzymes over a range of gel concentrations from 7 to 12% (total monomer, w/v) was determined and a Ferguson plot ($\log R_F$ versus gel concentration) was constructed (Fig. 3). The slopes of this plot (K_r , the retardation coefficient, a measure of molecular size; Rodbard & Chrambach, 1974) were -0.098 for the butanol-extracted enzyme and -0.11 for the detergent-extracted enzyme. These K_r values can be translated into geometric mean radii of 3.7 nm for the butanol-extracted enzyme ($\equiv M_r$ 168000) and 4.0 nm for the sulphobetaine 14-extracted enzyme ($\equiv M_r$ 210000) by linear interpolation from a standard curve as described by Rodbard & Chrambach (1974). Thus the difference in electrophoretic mobility between the two forms is due to size, not charge, and can probably be accounted for by the binding of the detergent micelle which has an M_r of approx. 30000 (Hjelmeland & Chrambach, 1984). Neither treatment of the sulphobetaine 14-extracted purified enzyme with 25% (v/v) butanol for 45 min at 0 °C nor treatment of the

Fig. 2. Non-denaturing polyacrylamide gel electrophoresis of purified bone and liver alkaline phosphatase

Purified bone (tracks *a* and *c*) and liver (tracks *b* and *d*) isoenzymes (18 m-unit of each) were electrophoresed in 0.1% sulphobetaine 14 on a 10% T (w/v total monomer concentration) polyacrylamide gel. The gel was stained for alkaline phosphatase activity.

Fig. 3. Ferguson plot analysis of sulphobetaine 14-extracted and butanol-extracted liver alkaline phosphatase

Purified sulphobetaine 14-extracted (●) and butanol-extracted (○) liver alkaline phosphatase (30 m-unit each) were electrophoresed on non-denaturing tube gels over the concentration range 7–12% T and stained for alkaline phosphatase activity. The slopes of the two plots were calculated by least-squares linear regression. The mobility of the low- M_r form of the serum enzyme from a patient with cholestasis is also shown (△).

butanol-extracted purified enzyme with sulphobetaine 14 altered their respective electrophoretic mobilities. However, butanol extraction of liver membranes solubilized with sulphobetaine 14 did convert part of the activity to a mobility corresponding to that of the butanol-extracted purified enzyme (results not shown). In one preparation of sulphobetaine 14-solubilized liver alkaline phosphatase, a small but significant proportion of the purified enzyme had the mobility of the butanol-extracted enzyme on non-denaturing polyacrylamide gels (Fig. 2). In all other preparations, including the portion of liver that was split to provide the sulphobetaine 14-solubilized and butanol-extracted enzyme preparations, only one activity band was seen after detergent extraction.

Monoclonal antibodies to alkaline phosphatase

Including the previous preparations of six monoclonal antibodies to liver alkaline phosphatase (Bailyes *et al.*, 1985), three sets of fusions for liver alkaline phosphatase and two sets of fusions for bone alkaline phosphatase were performed, using 12 mice with positive serum titres. In the liver alkaline phosphatase fusions, 137 positive culture supernatants were detected, and in the bone isoenzyme fusions 178 positive culture supernatants. All

Fig. 4. Relative binding of bone:liver alkaline phosphatase in two separate fusions

Culture supernatants, from uncloned cells, that had been identified by the binding of liver alkaline phosphatase in the standard screening assay were tested for their ability to discriminate between bone and liver alkaline phosphatase. Both purified enzymes were used at 0.1 unit/ml. The mice were immunized with the liver isoenzyme in fusion (*a*) and with the bone isoenzyme in fusion (*b*). The ratio of binding of bone alkaline phosphatase:liver alkaline phosphatase was calculated for each antibody and the resulting ratios were sorted into classes of 0.3–0.49 up to 3.9–4.09 (*x* axis). The number in each class is shown on the *y* axis.

Fig. 5. Discrimination of bone and liver alkaline phosphatase by antibody BAP 1/9

The wells of the plates (Microwell module F-16 high binding capacity; Nunc) were incubated overnight at room temperature in the air flow of a laminar flow cabinet with 100 μ l of sheep anti-(mouse IgG) (50 μ g/ml) in 50 mM-potassium phosphate, pH 6.5. Excess binding sites were blocked with bovine serum albumin (5 mg/ml) and the wells were washed with wash buffer. The plates were then incubated with antibody BAP 1/9 at 50 μ g/ml for 1 h at 20 °C. After two washes, the wells were incubated with the bone isoenzyme (●) or the liver isoenzyme (○) over the concentration range 0.0075–0.2 unit/ml for 1 h. After two further washes, the wells were incubated with enzyme assay cocktail for 40 min at 37 °C and the A_{405} of the wells was determined on the plate-reader. Each point is the mean of at least four determinations. For all points S.E.M. < 0.008 A_{405} . The slopes of the two plots were calculated by least-squares linear regression and were 3.6 for the bone enzyme and 1.6 for the liver enzyme.

were incubated with equal amounts of enzyme activity of purified liver and bone alkaline phosphatase to determine their specificity. No antibodies totally specific for liver or bone alkaline phosphatase were observed. The profiles of the ratios of binding of bone:liver alkaline phosphatase obtained in a set of liver isoenzyme fusions and bone isoenzyme fusions prior to cloning is shown in Fig. 4. Most of the antibodies (70%) in the liver fusion (four spleens) displayed a binding ratio of 0.9–1.3, and 70% of the antibodies in the bone fusion (four spleens) displayed a binding ratio of 1.1–1.7. From all the fusions a selection of cells were cloned, providing 27 antibodies together, 15 raised to the liver isoenzyme and 12 raised to the bone isoenzyme. None of these

antibodies affected enzyme activity singly or in pairs (results not shown). None showed significant cross-reaction with placental or intestinal alkaline phosphatase in the binding assay. Several of the antibodies retained a preference for bone alkaline phosphatase after cloning (nine of them showing at least a 2-fold preference; results not shown). The specificity was tested by coating plates with 5-fold dilutions of culture supernatant (undiluted to 1:3125) and incubating with equal amounts of liver and bone alkaline phosphatase. For some antibodies the apparent preference for bone alkaline phosphatase increased at lower antibody concentrations, e.g. antibodies coded BAP 7B3 and BAP 4A5 increased from a ratio of 1.8 to 3.0 from undiluted to 1:3125 dilution. Other antibodies maintained a fairly constant ratio of bone:liver enzyme binding over the entire concentration range. Ten antibodies (BAP 1/8, 1/9, 1/10, 1/15, 4A5, 4D1, 5A5, 5D4, 7B3; LAP 1/5) were subsequently purified from ascites fluid. All of these antibodies were found to be IgG₁, except for BAP 4DI which was IgG_{2a}, using a dot immunoblotting assay (Beyer, 1984). The binding of bone and liver alkaline phosphatase over the concentration range 0.0075–0.2 unit/ml to antibody BAP 1/9 is shown in Fig. 5. In two separate experiments the ratio of the slope of the bone enzyme plot to that of the liver enzyme plot was 2.25 and 2.1.

Epitope analysis of the monoclonal antibodies (Soos & Siddle, 1983; Soos *et al.*, 1986) revealed a complex pattern. Twenty-three antibodies were analysed in one experiment and the binding of liver alkaline phosphatase to monoclonal antibody-coated plates in the presence of soluble antibody, expressed relative to the binding obtained in medium alone, is shown for each antibody (Fig. 6). Nineteen of these twenty-three antibodies were assigned to six epitope groupings on the basis of their patterns of binding. The remaining four antibodies were difficult to classify, i.e. antibodies coded 5D4, 5A5, LAP 2/35 and LAP 2/50. Antibody LAP 1/7 was classified in other similar experiments into epitope group I and antibodies BAP 1/8, 1/9 and 1/10 similarly into epitope group V. Using bone enzyme for epitope analysis the antibodies could be assigned to the same epitope groupings as with liver enzyme (results not shown). From the epitope analysis it is possible to identify antibodies from different epitope groups that will simultaneously bind to the enzyme, particularly antibodies from groups I and V. However, it should be noted that not all pairs of antibodies from different groups were capable of simultaneous binding to liver alkaline phosphatase. Some antibodies in solution stimulated the binding of enzyme to the solid phase up to 388% of the binding found in medium alone; antibodies coded BAP 1/15, LAP 2/22, LAP 2/49, BAP 3C1, BAP 4D1, were particularly effective at this, giving 50% or more stimulation with six or more different antibodies. The Group I antibodies BAP 4B3, LAP 1/1, LAP 1/5, LAP 1/9, LAP 1/12 and LAP 1/13 were particularly affected when present as the solid phase. This effect was also observed when purified antibodies were used instead of antibodies from culture supernatants.

Serum alkaline phosphatase

The reaction with monoclonal antibodies of both high- and low- M_r forms of alkaline phosphatase found in serum from cholestatic patients was investigated by

Fig. 6. Epitope analysis of monoclonal antibodies to alkaline phosphatase

Sheep anti-(mouse IgG)-coated plates were incubated with the spent culture supernatants for 90 min at 20 °C. After two washes, the wells were incubated for 1 h with liver alkaline phosphatase at 0.1 unit/ml, that had been preincubated with spent culture supernatants and mouse monoclonal antibody X63 (0.5 mg/ml). After two further washes the bound enzyme was assayed as described in the standard screening assay. Binding is expressed as a ratio of the binding obtained in the absence of preincubation of the enzyme with a second antibody. Each histogram is labelled with the antibody used on the solid phase; antibodies in solution

Fig. 7. Gel filtration of serum alkaline phosphatase

A serum sample (0.5 ml) from a patient with obstructive liver disease was applied to a column (60 cm $\times$ 0.5 cm) of Sephacryl S-300 and eluted with 50 mM-sodium phosphate pH 7.4, containing 0.1% bovine serum albumin at 10 ml/h. Fractions (0.5 ml) were collected and assayed for alkaline phosphatase activity and for comparison 5'-nucleotidase activity. The arrow marks the void volume. In the sample shown, 16% of alkaline phosphatase and 62% of 5'-nucleotidase eluted in the void volume.

measuring uptake onto monoclonal antibody immuno-adsorbents. The high- and low- M_r forms of serum alkaline phosphatase were separated by gel filtration (Fig. 7). In serum samples from four cholestatic patients $25 \pm 7\%$ of alkaline phosphatase and $68 \pm 5\%$ of 5'-nucleotidase eluted in the void volume. High- and low- M_r forms of serum alkaline phosphatase were also distinguished as fast-moving and slow-moving forms respectively by cellulose acetate electrophoresis (Fig. 8) in agreement with De Broe *et al.* (1985). When high- M_r serum alkaline phosphatase was incubated with monoclonal antibody LAP 1/12 immuno-adsorbent, binding of enzyme activity was $20 \pm 6\%$ ($n = 6$) in the absence of detergent and $78 \pm 6\%$ ($n = 6$) in the presence of 2% (w/v) sulphobetaine 14. Similar data were observed for the binding of liver membrane fractions; $12 \pm 8\%$ ($n = 6$) alkaline phosphatase being bound in the absence of detergent and $82 \pm 9\%$ ($n = 6$) in its presence. In contrast, most of the serum low- M_r alkaline phosphatase bound to the immuno-adsorbent with $[87 \pm 2\%$ ($n = 5$)], or without $[76 \pm 5\%$ ($n = 5$)], detergent treatment. In agreement with the immuno-adsorbent binding data,

Fig. 8. Cellulose acetate electrophoresis of serum alkaline phosphatase

Tracks (a) and (h) show alkaline phosphatase in serum samples from a patient with obstructive liver disease (fast, F, and slow, S, bands are observed). Tracks (d) and (g) are samples from the same patient treated with butanol (1:1 v/v, 5 min, 20 °C) or 2% (w/v) sulphobetaine 14 respectively. Tracks (e) and (f) show samples from the same patient treated with monoclonal antibody LAP 1/12 immuno-adsorbent; in (f) the sample was pretreated with 2% sulphobetaine 14. For comparison track (c) shows a serum sample from a jaundiced patient without bile duct obstruction (no fast band F is seen), and track (b) an equivalent sample treated with LAP 1/12 immuno-adsorbent. The origin of sample loading for each track is marked O.

when serum from a patient with obstructive liver disease was reacted with antibody LAP 1/12 immuno-adsorbent, alkaline phosphatase that was slow-moving during electrophoresis on cellulose acetate was selectively bound relative to the fast-moving form (Fig. 8).

Further analysis of the binding of high- and low- M_r forms of serum alkaline phosphatase to monoclonal antibodies showed that binding to LAP 3/60 (Group I) and BAP 4A5 (Group II) immuno-adsorbents was similar to that observed to the LAP 1/12 immuno-adsorbent (Group I) and to an immuno-adsorbent prepared from a polyclonal rabbit antiserum to liver alkaline phosphatase (Maguire *et al.*, 1986). In an experiment with a serum sample from a patient with Paget's disease in which the alkaline phosphatase activity (presumed to be of bone origin) was 2000 units/l, $> 99\%$ of the enzyme activity bound to a LAP 1/12 immuno-adsorbent.

Investigation of the mobility in polyacrylamide-gel electrophoresis of low- M_r soluble alkaline phosphatase from the sera of patients with obstructive liver disease showed that this enzyme did not require detergent to

are in the same order for each histogram, and follow the order of the antibodies on the solid phase, top to bottom, left to right. Group I, LAP/1 to LAP 3/60; Group II, LAP 2/49 to BAP 4A5; Group III, LAP 3/51 to LAP 2/52; Group IV, LAP 3/24 and BAP 4D1; Group V, LAP 2/22 and BAP 1/15; Group VI, BAP 5D2 and BAP 3C1. *Marks the same antibody in both phases; ND, binding not determined. The data shown are from a single experiment; each bar represents the mean of two determinations.

enter restriction polyacrylamide gels and had a mobility similar to that of butanol-extracted liver membrane enzyme (Fig. 3).

DISCUSSION

In the present experiments to prepare monoclonal antibodies to alkaline phosphatase, five sets of cell fusions (three for liver enzyme and two for bone) produced 315 positive culture supernatants which cross-reacted with both isoenzymes. Subsequently 27 antibody-producing cell lines were successfully cloned and after preparing ascites fluid nine antibodies were shown to retain at least 2-fold specificity for bone relative to liver alkaline phosphatase. No antibodies specific for liver isoenzyme were obtained. Reaction of the monoclonal antibodies with both isoenzymes was in agreement with previous observations that polyclonal antisera to these isoenzymes usually cross-react (McComb *et al.*, 1979), although there is a single report of the preparation of a bone-specific polyclonal antiserum using enzyme from a cultured cell line as antigen (Singh & Tsang, 1975). Nevertheless, it might have been expected that monoclonal antibodies would show greater specificity in view of the observation that alleles of placental alkaline phosphatase having very similar physical properties can be distinguished by monoclonal antibodies (Harris, 1983). Using bone alkaline phosphatase as antigen a larger percentage of culture supernatants showed some specificity for the bone enzyme compared with using liver alkaline phosphatase as antigen (Fig. 4). Epitope analysis of the 27 monoclonal antibodies assessed by competitive binding showed a complex pattern of at least six epitope groups. Certain antibody pairs used in solution or solid phase did not show reciprocal effects. A similar situation was observed with monoclonal antibodies prepared to the human insulin receptor (Soos *et al.*, 1986). In the present study, immunoaffinity-purified liver and bone alkaline phosphatase isoenzymes appeared to be homodimers of M_r -80000 subunits. It is possible that binding of an antibody to one of the subunits may cause conformational changes allowing greater binding of other antibodies, thus accounting for the observation that some antibodies stimulated the binding of the enzyme to others. Epitopes defined by antibody binding to liver alkaline phosphatase appear to be present on bone alkaline phosphatase and antibodies showing some specificity for the bone isoenzyme are present in each epitope group. Recent experiments on the liver isoenzyme using endoglycosidase F suggest that approximately one-third of the apparent M_r can be accounted for by carbohydrate side chains (Garattini *et al.*, 1986). Differences in the carbohydrate side chains are known to explain some of the different properties of liver and bone alkaline phosphatase isoenzymes, and it is known that neuraminidase treatment abolishes the difference in isoelectric point between them (McKenna *et al.*, 1979) without affecting thermostability (Stinson & Seargeant, 1981). Tryptic peptide maps of purified bone and liver isoenzymes are very similar. Clearly, differences in carbohydrate side chains may account for differences in the binding of monoclonal antibodies from several epitope groups to the two isoenzymes. In previous studies monoclonal antibodies raised to liver alkaline phosphatase have been reported, 16 showing cross-

reactivity with bone isoenzyme (Harris, 1983), one showing some specificity to the bone isoenzyme (Meyer *et al.*, 1982), and one showing some specificity to the liver isoenzyme (Lawson *et al.*, 1985). In the latter case a 3-fold preference for liver alkaline phosphatase observed at room temperature increased to a 5-fold preference after incubation for 20 h at 30 °C. Clearly the prospect of an isoenzyme-specific immunoassay for bone or liver alkaline phosphatase based on any of the 27 antibodies in the present study requires careful investigation of binding conditions.

Liver alkaline phosphatase is an intrinsic membrane enzyme and solubilization with organic solvents or detergents is required for purification. Whilst the size difference observed in the present study between the native enzyme purified after solubilization in butanol or sulphobetaine 14 was consistent with the size of the detergent micelle, neither agent altered the properties of enzyme purified in the other. The present analysis of immunoaffinity-purified liver alkaline phosphatase confirms that it is a homodimer of two M_r -80000 subunits. Although the primary structure is unknown, there is some primary sequence homology between liver alkaline phosphatase and the placental isoenzyme (Garattini *et al.*, 1986), of which the complete amino acid sequence has been inferred from molecular cloning (Millan, 1986). The primary sequence data suggests that the placental isoenzyme has a large extracellular *N*-terminal portion, a hydrophobic membrane-anchoring domain and lacks a cytoplasmic tail. Phosphatidylinositol-specific phospholipase C can release alkaline phosphatase from liver cell membranes (Shukla *et al.*, 1980; Chakrabartty & Stinson, 1983) and it has been suggested that this may result from a covalent attachment of phosphatidylinositol to provide the enzyme's hydrophobic membrane anchor (Low & Zilversmit, 1980; Low *et al.*, 1986). An endogenous phospholipase C which can be activated by butanol has been proposed to be responsible both for the butanol-solubilized form of liver alkaline phosphatase and the soluble serum form (Miki *et al.*, 1985). Our data are consistent with this, since butanol extraction had no effect on the detergent-purified enzyme which does not contain phospholipase C. Also it is of interest to note that a much larger proportion of alkaline phosphatase in the serum of cholestatic patients is present as a soluble rather than a particulate form compared with the enzyme 5'-nucleotidase (Fig. 7; Chuang *et al.*, 1984). This enzyme, like alkaline phosphatase, is an ectoenzyme with no apparent cytoplasmic tail (Baron *et al.*, 1986) but is less easily released from the plasma membrane by phosphatidylinositol-specific phospholipase C (Shukla *et al.*, 1980).

There has been controversy over the origin of the high- M_r particulate form of alkaline phosphatase observed in the serum of patients with obstructive liver disease (Kaplan, 1986; Maguire *et al.*, 1986; Wulkan & Leijnse, 1986). The present experiments show that, when solubilized with the detergent sulphobetaine 14, the enzyme behaves like the soluble serum enzyme both on migration in cellulose acetate electrophoresis and in binding to a variety of immunoabsorbents which also bind to the detergent-solubilized or butanol-extracted purified liver membrane alkaline phosphatase. The data are consistent with both the soluble serum enzyme and particulate enzyme deriving from liver plasma membrane. Whilst we cannot rule out direct shedding from the

hepatocyte blood sinusoidal surface (De Broe *et al.*, 1985), bile regurgitation (Binns *et al.*, 1962), reverse transcytosis of enzyme released from the bile canaliculi surface or enzyme released as a result of disrupted endocytic pathways (Maguire *et al.*, 1986), it is unlikely that the particulate serum enzyme is a specific bile form (Price & Sammons, 1974). The inability of the particulate serum enzyme to bind to immunoadsorbents except in the presence of detergent was not unexpected, since the particulate alkaline phosphatase may be present as membrane fragments or micelles in association with other enzymes (De Broe *et al.*, 1975). It is known that membrane vesicles and fragments can only bind efficiently to immunoadsorbents when indirect techniques are used, in which a membrane-antibody complex is captured on an anti-immunoglobulin immunoadsorbent (Luzio *et al.*, 1976; Richardson & Luzio, 1986). The selective binding of soluble serum alkaline phosphatase to a monoclonal antibody immunoadsorbent may allow the development of an assay to measure the unbound particulate form of the enzyme in serum. At present there is no simple quantitative method of doing this, yet it has been proposed that the presence of the high- M_r particulate form of alkaline phosphatase in serum is a good indicator of obstructive liver disease (Price & Sammons, 1976; Siede & Seiffert, 1983).

This work was supported by IQ (Bio) Ltd., Cambridge. K. S. is a Wellcome Trust Senior Lecturer.

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