Isolation and Preliminary Characterization of a Monoclonal Antibody That Interacts Preferentially with the Liver Isoenzyme of Human Alkaline Phosphatase

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We have prepared murine monoclonal antibodies against isolated human bone alkaline phosphatase (ALP, EC 3.1.3.1). Hybridoma supernatants were separately screened for reactivity against both human liver and bone ALP. Although most antibody-positive hybridomas showed similar reactivity against both isoenzymes, one hybridoma produced an antibody that interacted preferentially with liver ALP. This antibody was purified and used to establish an immunoassay to differentiate liver ALP from bone ALP. When equal activities of the two isoenzymes (as determined by a conventional enzymatic assay) were measured by the immunoassay, a fivefold greater response was obtained with liver than with bone ALP. The immunoassay can be used to measure the proportions of the bone and liver isoenzymes in mixtures of them. Cross reactivity with human placental and intestinal ALP is less than 3% relative to liver ALP. These findings support the feasibility of developing immunological methods to differentiate these isoenzymes in the clinical laboratory.

Additional Keyphrase: enzyme immunoassay

Quantification of serum alkaline phosphatase (ALP) is a well-established test in clinical diagnosis (1-3), but interpretation is complicated by the multiplicity of ALP isoenzymes, originating from different organs. The ALP isoenzymes from liver and bone have been particularly difficult to distinguish from one another. Although methods based on subtle differences in electrophoretic mobility or stability to heat or urea denaturation have been developed (4, 5), limited resolution and the necessity for precisely controlled conditions have limited their acceptance for routine use. Attempts to develop polyclonal antisera specific to human liver or bone ALP have not yet been successful (6, 7).

An alternative approach is to develop assays involving monoclonal antibodies directed against epitopes not common to both isoenzymes. Recently, several investigators have applied hybridoma technology to produce assays that distinguish between the placental and intestinal isoenzymes of human ALP (8-11). Attempts to duplicate these results for human liver and bone ALP have produced only cross-reacting antibodies (12). We report here the production and preliminary characterization of a monoclonal antibody that binds to the liver and bone isoenzymes of ALP but interacts preferentially with liver ALP. The specificity is such that the two isoenzymes can be separately measured.

Materials and Methods

Purification of human bone ALP. Human bone ALP was isolated as described elsewhere (13, 14). All steps were carried out at 4 °C. The isolation buffer used throughout consisted of, per liter, 10 mmol of Tris, 2 mmol of MgCl₂, and 30 μmol of ZnCl₂ (pH 7.5), and all columns were equilibrated and eluted with this buffer unless specified otherwise.

In brief, ribs and vertebrae obtained at autopsy from a four-year-old boy were trimmed free of soft tissue and pulverized into small fragments. Fragments totalling 116 g were first homogenized in six volumes of isolation buffer in a Waring Blender. n-Butanol was then added, to a final concentration of 300 mL/L, and the mixture was again homogenized. The homogenate was stirred gently for 16 h, to allow extraction of ALP into the aqueous phase, then centrifuged to separate the phases. The organic (upper) phase was discarded; the aqueous phase was aspirated and saved. The pellet was re-extracted by homogenization in 200 mL of the butanol/buffer mixture as above.

The aqueous phases were combined and fractionated with acetone. That fraction precipitating between acetone concentrations of 330 and 500 mL/L was retained. The precipitate was dissolved in 300 mL of isolation buffer and further fractionated by adding solid ammonium sulfate; the fraction precipitating between 50% and 70% saturation was retained. The precipitate was redissolved, dialyzed for 16 h against isolation buffer, and applied to a diethylaminoethyltrisacryl (Pharmacia, Piscataway, NJ 08854) column. The columnwas eluted with a linear gradient of NaCl, 20-120 mmol/L, and those fractions containing ALP activity, as detected by the conventional enzymic assay (see below), were pooled and concentrated by ultrafiltration.

This preparation was further purified by gel filtration over Sephacryl-G200 (Pharmacia), followed by affinity chromatography on concanavalin A-Sepharose (Sigma Chemical Co., St. Louis, MO 63178). In the latter procedure, bound ALP was eluted with 0.1 mol/L α-methyl-D-glucoside (Sigma). The eluted material was dialyzed against isolation buffer, concentrated by ultrafiltration, and stored at -20 °C. Protein was assayed by the method of Lowry et al. (15), and purity was assessed by electrophoresis in 100 μL polyacrylamide gels containing sodium dodecyl sulfate (Bio-Rad Laboratories, Richmond, CA 94804).

Conventional assay for human ALP activity. The method we compared with the present determination of ALP isoenzymes is essentially as previously described (16). To determine total ALP activity, we combined 25 μL of sample with 650 μL of reagent containing, per liter, 1.0 mol of diethanolamine, 0.5 mmol of MgCl₂, and 16 mmol of p-nitrophenyl phosphate (pH 10.15), and monitored the reaction rate by centrifugal analysis (Centrifichem; Baker Instruments, Allentown, PA 18011), measuring the absorbance at 405 nm and using a reaction temperature of 30 °C. One unit (U) of activity is defined as the quantity of enzyme that catalyzes the hydrolysis of 1 μmol of substrate per minute under these conditions.

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* Nonstandard abbreviations: ALP, alkaline phosphatase (orthophosphoric monoester phosphorylase, EC 3.1.3.1); pNPP, p-nitrophenyl phosphate.

Received September 28, 1984; accepted December 19, 1984.
The intestinal isoenzyme was estimated from the rate of inactivation in 10 mmol/L phenylalanine. The fractional contributions of liver and bone isoenzymes were calculated from the rate of inactivation in 3.2 mol/L urea, as compared with the relative rates of inactivation of standard preparations assayed at the same time. We modified the data handling method for this assay to account for the nonlinear rate of inactivation of bone ALP in the presence of urea, the method (R.F. Foraman, personal communication) used routinely in our laboratory.

**Immunization and production of hybridomas.** BALB/c mice were injected intraperitoneally with 50 to 100 μg of purified human bone ALP emulsified in Freund's complete adjuvant. Booster injections were administered at two-week intervals in Freund's incomplete adjuvant. Mice were bled from their tail veins, and the serum collected was tested for the presence of anti-ALP antibodies by a solid-phase immunoassay (see below). We removed the mice's spleens and fused 10⁵ spleen cells with 10⁶ murine NS-1 myeloma cells in polyethylene glycol as previously described (17). Fused cells were distributed into four 24-well cluster plates (Costar, Cambridge, MA 02139) and cultured in RPMI medium (KC Biologicals, Lenexa, KS 66215) containing fetal calf serum, 100 mL/L, and supplemented with hypoxanthine, aminopterin, and thymidine. Usually, hybridomas were detectable within 10 days after the fusion in approximately 95% of the wells.

**Detection and purification of anti-ALP monoclonal antibodies.** Supernatants of the culture media from fusion wells were screened for anti-ALP antibodies with a solid-phase immunoassay. We used 12 × 75 mm plastic tubes (Sarstedt, Princeton, NJ 08540) coated with 200 μL of rabbit IgG anti-mouse Ig (10 mg/L in 50 mmol/L Na₂CO₃, pH 9.5) by overnight incubation at 4 °C. All further incubations were performed at 4 °C for 16 h unless specified otherwise. All reagents were added in a total volume of 200 μL, and the tubes were washed three times with Tris–siline buffer (per liter, 50 mmol of Tris, 0.1 mol of NaCl, 1 g of bovine serum albumin, and 200 mg of Na₂CO₃, pH 7.3) between reagent additions. Tubes were incubated sequentially with undiluted hybridoma supernates, then with diluted human serum (see below) containing predominantly liver or bone ALP isoenzyme. These enzyme "probes" were obtained from individual serum specimens in which the total ALP activity exceeded 2000 U/L (reference interval for adult males, 90–239 U/L) by the conventional assay, and for which the percentage of the desired isoenzyme (liver or bone) exceeded 90% as determined by urea inhibition. Before addition to the assay tubes, all sera were diluted with Tris–siline buffer to a total ALP activity of 50 U/L. We then added substrate reagent (per liter, 1.0 mol of diethanolamine, 50 μmol of MgCl₂, and 16 mmol of pNPβ, pH 10.15) at room temperature. After color developed (usually 15 to 60 min), we transferred 100-μL aliquots to 96-well microtiter plates (Costar), and determined their absorbance at 410 nm with a Dynatech MR 600 (Dynatech, Alexandria, VA 22314) microplate reader. Immune and nonimmune mouse sera were used in place of hybridoma supernates as positive and negative controls, respectively. After screening the first few fusions, we found that we could perform the assay entirely in microtiter plates, thus obviating the tube-to-plate transfer before reading the sample absorbance.

The desired hybridomas were subcloned by the method of limiting dilution, and monoclonal antibodies were obtained in quantity from mouse ascites fluid after intraperitoneal injection of the hybridoma cells as previously described (18). Antibodies were purified from ascites fluid by precipitation with 50% saturated ammonium sulfate, followed by gel filtration over Ultragel ACA-34 (LKB, Gaithersburg, MD 20877).

**Assay of serum ALP with purified monoclonal antibodies.** Again, we used 96-well microtiter plates (Costar), adding all reagents in a total volume of 100 μL, and washing the wells between reagent additions with sodium phosphate buffer (10 mmol/L, pH 7.4) containing 0.14 mol of NaCl, 500 mg of Tween-20, and 200 mg of NaN₃ per liter. First we coated the microtiter wells with purified monoclonal antibody (10 mg/L in 50 mmol/L Na₂CO₃, pH 9.5), incubating for 16 h at 4 °C. The antibody-coated wells were then incubated with serum samples containing high activities of liver or bone ALP. The amount of bound ALP was detected by addition of substrate, essentially as described above. Variations in incubation conditions are specified in the Figure legends.

**Miscellaneous.** Human placental ALP was obtained commercially (Sigma). A clarified homogenate of human jejunal mucosa served as source of intestinal ALP.

**Results**

**Purification of human bone ALP.** Isolation of ALP from human bone yielded a highly purified preparation as assessed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Figure 1). Although a more sensitive silver stain indicated the presence of additional components (data not shown), we estimate that more than 90% of the protein migrated as a single band of molecular mass approximately 75 000 Da, a size consistent with that reported by others for the subunit of human liver and bone ALP (7, 13). The yield of ALP from 160 g of human bone (ribs and vertebrae) was 5.8 mg of protein, having a specific ALP activity of 1180 kU/g. Analysis by the urea inhibition assay indicated that the purified ALP was 95% bone isozyme, which suggests a similarity between the ALP purified from bone and its circulating counterpart in serum.

We applied the same purification procedure to human liver. The overall yield and degree of purification were considerably less than from bone (data not shown). Moreover, several independent isolates showed variable amounts of nonliver isoenzyme activity by urea inhibition, suggesting that the form isolated from hepatic parenchyma may differ in some respect from the circulating isoenzyme, an
observation noted previously by others (18). In all immunizations described below, we used human bone ALP as the antigen.

Production and detection of anti-ALP monoclonal antibodies. Of 181 supernates screened, 15 were positive for anti-ALP activity as defined by absorbance at 410 nm \( (A_{410}) \) at least fivefold that of the negative control. All of these supernates were positive for both anti-liver and anti-bone ALP activity. Although the majority developed roughly equal \( A_{410} \) when tested against either isoenzyme, for two the rate of absorbance increase was much more rapid with liver ALP. When one of them, designated B4-50, was tested in greater detail against equal activities of liver and bone ALP (50 U/L), its response in the screening immunoassay was about 3.5-fold greater for liver ALP than bone ALP (Figure 2).

Because the serum liver- and bone-ALP "probes" used for initial screening of hybridoma supernates were obtained from only two patients, one with liver disease and one with bone disease, it was conceivable that the differential response of B4-50 may have resulted from an individual polymorphism unrelated to that responsible for organ-specificity. To rule out this possibility, we tested the B4-50 supernate against additional serum specimens containing predominantly liver ALP (eight different patients) or bone ALP (seven different patients), having diluted all samples to 50 U/L ALP activity. The specimens containing liver ALP all produced greater responses in the present immunoassay than those containing bone ALP, with an average ratio of \( A_{410}(\text{liver})/A_{410}(\text{bone}) \) of 3.1 (Figure 3).

Further characterization of monoclonal antibody B4-50. After isolating antibody B4-50 in quantity from ascites we used it to coat microtiter plates directly, and tested various assay conditions in an attempt to enhance the ability of the immobilized antibody to discriminate between liver and bone ALP (Figure 4). Binding was maximum after approximately 20 h. The ability of the present immunoassay to discriminate between liver ALP and bone ALP increased as the temperature of incubation increased from 4 \(^\circ\)C to 30 \(^\circ\)C, with antibody B4-50 showing a fivefold greater response to liver ALP than to bone ALP at 30 \(^\circ\)C (Figure 4C). There was no further improvement in relative discrimination at incubation temperatures above 30 \(^\circ\)C (data not shown).

Figure 4(D-F) shows the results of parallel assays with a second purified, immobilized monoclonal antibody, B4-78, originally selected because during initial screening it reacted equally with bone isoenzymes, a characteristic considered potentially useful in establishing a clinical assay. Bone ALP gave a slightly greater response than liver ALP when incubated with immobilized B4-78 at each temperature. This markedly different behavior by antibodies B4-50 and B4-78 against the liver and bone isoenzymes under identical conditions emphasizes that the observed preferential response of liver ALP in this immunoassay in fact depends on the antibody and is not attributable to some systematic artifact in the behavior of bone and liver ALP.

Cross reactivity of immobilized antibody B4-50 with human placental and intestinal ALP. The potential cross reactivity of antibody B4-50 with other human ALP isoenzymes of clinical significance was investigated by incubating immobilized B4-50 with increasing amounts of placental, intestinal, liver, and bone ALP. Antibody B4-50 gave a fivefold greater response with liver ALP as compared with
bone ALP over a range of enzyme activities from 1 to 128 U/L (Figure 5A). In additional experiments, this fivefold greater response was maintained for activities up to 500 U/L (data not shown). A slight degree of cross reactivity with human placental and intestinal ALP was detected at activities greater than 50 U/L, but the response with these two isoenzymes was less than 3% of that observed for an equal activity concentration of liver ALP. Antibody B4-78 responded equally to liver and bone ALP over activities ranging from 1 to 128 U/L (Figure 5B); it showed no cross reactivity with human placental or intestinal ALP over this range.

**Discrimination of known mixtures of human liver and bone ALP.** These results suggested that antibody B4-50 might be useful in a clinical assay for quantifying liver and bone ALP isoenzymes in human serum. Because these experiments were conducted with dilutions of sera containing predominantly one or the other isoenzyme (i.e., greater than 90%), we wanted to determine whether antibody B4-50 could discriminate predetermined mixtures of the isoenzymes. Serum specimens containing predominantly liver or bone ALP were diluted to a total activity of 50 U/L (as determined by conventional assay) and then combined in appropriate volumes to produce a series of mixtures with various percentages of the two isoenzymes while maintaining a constant total activity of 50 U/L. We then assayed the mixtures by immunossays with immobilized antibodies B4-50 and B4-78 (Figure 6). With immobilized B4-50, development of $A_{410}$ was proportional to the percentage of liver ALP as the latter was increased in 10% increments from 0% to 100%. In contrast, when the same mixtures were assayed with immobilized B4-78, there was only a slight increase in $A_{410}$ development with increasing percentage of bone ALP—a finding consistent with B4-78’s slight preference for bone ALP (Figure 4). Thus monocalonal antibody B4-50, although not entirely specific for the liver isoenzyme, could quantitatively discriminate various mixtures of liver and bone ALP in human serum.

**Discussion**

Evidence presented in recent years supports the existence of three separate gene loci for human ALP (7, 19). Two of these genes code for placental and intestinal ALP, whereas a third code for the isoenzyme found in liver, bone, and kidney. Both polyclonal (6) and monoclonal (9–12) antibod-

![Graph showing cross reactivity of purified monocalonal antibodies B4-50 (A) and B4-78 (B) with human liver (●), bone (○), placental (△), or intestinal (△) ALP for 20 h at 30°C](image)

**Fig. 5.** Cross reactivity of purified monocalonal antibodies B4-50 (A) and B4-78 (B) with human liver (●), bone (○), placental (△), or intestinal (△) ALP for 20 h at 30°C

![Graph showing discrimination by monoclonal antibody B4-50 between human liver and bone ALP in pre-defined mixtures](image)

**Fig. 6.** Discrimination by monoclonal antibody B4-50 between human liver and bone ALP in pre-defined mixtures

Human serum specimens containing predominantly either liver or bone ALP were diluted and combined to generate mixtures of known composition while maintaining a total ALP activity of 50 U/L by conventional assay. Mixtures were assayed by solid-phase immunonassay in microtiter wells coated with antibody B4-50 (6) or B4-78 (A). These specific for ALP coded from each of these three loci have been described. Among the third group of isoenzymes, the subtle structural differences that result in altered electrophoretic mobility as well as altered stability to heat or urea are presumably attributable to different post-translational modifications. Previous attempts to exploit these structural variations to develop a specific immunological reagent have not been successful—polyclonal antibodies produced against liver ALP cross-react with bone ALP and vice versa. Attempts to generate specificity by adsorbing polyclonal antisera with one or the other isoenzyme have also failed (6). These data suggest that all the antigenic sites on one isoenzyme may also be present on the other.

Efforts to obtain specific immunological reagents for serum liver and bone ALP are confounded by suggestions that the isoenzyme purified from hepatocytes may not be identical to the circulating enzyme observed in cholestatic disease (18). Such reports raise the possibility that the enzymes isolated from tissue may differ somehow from their circulating counterparts because of modifications in vivo or alterations during purification. To assure that all hybridoma supernatants positive for anti-ALP activity were, in fact, directed against the desired circulating isoenzymes, we used serum specimens from patients with liver or bone disease to develop the enzymatic probes for screening and subsequent characterization of monoclonal antibodies by solid-phase immunonassay.

Our data are consistent with the concept that all antigenic sites on human liver and bone ALP may be shared. In addition to the immunizations with bone ALP described above, several hundred hybridomas have been screened after fusions in which human liver ALP was used as antigen (data not shown). To date, all positive supernatants have always shown at least some degree of cross reactivity with each isoenzyme. Although these results cannot exclude the possibility that a totally specific antibody will be found in some as-yet-untried fusions, they do suggest that such
antibodies, if they exist, are rarely represented among the polyclonal population, or that their affinity is below the threshold of sensitivity of our screening assay. In such a case, the prospect of isolating a monoclonal antibody absolutely specific for human liver or bone ALP may be unlikely by conventional methods of immunization (20). The existence of nonidentical but structurally homologous epitopes may limit the potential repertoire to antibodies having only partial specificity and exhibiting some degree of cross-reactivity, e.g., antibody B4-50. This conclusion is supported by the paradoxical observation that B4-50 interacts preferentially with liver ALP although bone ALP was used as immunogen. Alternatively, B4-50 could be a cross-reacting antibody that inhibits the activity of the bone ALP to a greater extent than that of the liver form.

Antibody B4-50 is currently being evaluated to ascertain whether its specificity is sufficient to allow reliable quantitative determination of serum liver ALP and bone ALP in the routine clinical laboratory.

References