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Case report

A macro-enzyme cause of an isolated increase of alkaline phosphatase

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ABSTRACT

Background: Macroenzyme complexes of serum enzymes and antibody can increase the circulating enzymatic activity and may lead to unnecessary additional testing and procedures. Laboratory physicians and scientists need to be aware of techniques to identify macroenzyme complexes when suspected.
Case report: To investigate the possibility of a macro-alkaline phosphatase in the serum of a 74 year old male with persistently increased alkaline phosphatase we coupled a protein A/G agarose affinity chromatography technique with isoenzyme electrophoresis to look for the presence of macro-alkaline phosphatase.
Results: The majority of the alkaline phosphatase activity in the patient's serum sample was bound to the column and only a minor fraction (25%) of alkaline phosphatase activity was present in the column flow-through. The alkaline phosphatase activity was also found to co-elute with the immunoglobulins in the patient sample. The alkaline phosphatase activity in a control serum sample concurrently treated in the same manner did not bind to the column and was found in the column flow-through.
Conclusion: The use of protein A/G agarose affinity chromatography is a rapid and simple method that can be applied to the investigation of other macro-enzyme complexes.

1. Introduction

The cause of spurious increases in the concentration or activity of clinical chemistry analytes that do not correlate with a known disease process can prove challenging for the clinician and laboratorian to elucidate. Many causes including heterophile antibodies and other interferences must be considered. Occasionally for reasons yet unknown a loss of immunological self-tolerance will result in the generation of antibodies that recognize one of the serum enzymes. The binding of a serum enzyme by anti-enzyme antibody prolongs the enzyme half-life resulting in an increased serum activity. The frequency of most macroenzyme complexes is unknown however macroamylase occurs in approximately 1% of an unselected population and in 2.6% of hyperamylasemia patients [1]. While benign the presence of a macroenzyme may lead to unnecessary testing and diagnostic procedures.

2. Case report

A 74 year old male had a history significant for prostate cancer, basal cell carcinoma, squamous cell carcinoma in situ, secondary hyperparathyroidism, actinic keratosis and gastroesophageal reflux disease (GERD) presented to his gastroenterologist for follow-up of his GERD and for persistently elevated alkaline phosphatase (ALP).

At presentation the patient's relevant laboratory values were: albumin 45 g/l (32–52 g/l); ALP 545 U/l (40–120 U/l); aspartate aminotransferase (AST) 34 U/l (0–39 U/l); alanine aminotransferase (ALT) 26 U/l (0–55 U/l); total bilirubin 4 mg/l (2–13 mg/l); direct bilirubin 1 mg/l (0–3 mg/l); and calcium 2.38 mmol/l (2.13–2.63 mmol/l). The ALP activity had been persistently elevated for four years and had varied from a peak of 1034 U/l in December of 2010 to a nadir of 402 U/l in June of 2012 and had since plateaued at approximately 600 U/l. The elevation in ALP was not accompanied by an elevation in gamma glutamyltransferase, peak activity equal to 32 U/l (reference interval: 8–61 U/l).

Approximately 2 years prior to presentation the patient underwent imaging of the head and chest via computed tomography due to the persistent elevation of ALP. These studies demonstrated no masses, lesions or other causes of elevated ALP. A limited ultrasound examination of the abdomen was also carried out with no abnormalities visualized. An ALP isoenzyme electrophoresis study was requested in order to determine the source of the ALP. The resulting pattern revealed an

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abnormally migrating band that accounted for 78% of the total ALP activity. Due to the long history of chronic ALP elevation and unidentified ALP isoenzyme pattern the laboratory was contacted for further testing suggestions. An abnormally migrating band such as this one could be consistent with the presence of a macro-ALP complex; however the electrophoretic study alone was not sufficient to confirm this possibility.

3. Materials and methods

3.1. Sebia ISO-PAL alkaline phosphatase isoenzyme electrophoresis

Patient samples were analyzed on Sebia ISO-PAL alkaline phosphatase isoenzyme electrophoresis gels according to manufacturer's directions. Briefly aliquots of the sample and column fractions were applied to the applicator comb and wheat germ lectin applied to half of the wells on a second comb in order to impede the migration of bone ALP.

3.2. Protein A/G agarose column purification

Columns containing protein A/G agarose resin with high affinity for all 4 subclasses of human IgG and weak affinity for IgA and IgM were from Thermo Scientific and were equilibrated with phosphate buffered saline (PBS) pH 7.2, prior to use. Patient and control serum samples (control patient also had high ALP due to cholestasis) were diluted in PBS to a final IgG concentration of 3.5 mg/ml, this material was labeled as starting material. Two milliliters of PBS buffered samples was applied to the pre-equilibrated protein A/G columns, and incubated at room temperature for 60 min on a rocker to suspend the agarose beads. Following incubation the columns were centrifuged and the resulting filtrates saved. The columns were washed 3× with 2 ml of PBS, and filtrates saved. The columns were then placed in new 15 ml conical tubes containing 100 μl of 1 mol/l Tris–HCl, pH 8.0. The retained immunoglobulins were eluted from the columns into the Tris buffer via centrifugation with 1 ml of elution buffer, pH 2.8. The activities of ALP, AST, ALT, GGT and IgG concentration in all fractions were determined on a Roche Cobas 6000 analyzer according to standard procedures.

3.3. Heat inactivation study

To elucidate the isoenzyme(s) bound by the immunoglobulin the patient serum and column fractions were subjected to a heat inactivation protocol as described previously [2]. Briefly, patient serum and protein A/G column fractions were incubated at 56 °C for 10 min in order to inactivate bone ALP isoenzyme, or at 65 °C for 30 min in order to inactivate the bone and liver ALP isoenzymes. Heat treated serum samples and the column eluates were then subjected to electrophoresis as detailed above.

4. Results

As demonstrated in Table 1 the IgG concentration in the flow-through from both control and test patients was below the lower limit of detection of the assay. The control patient and suspected macro-ALP flow-through fractions contained 93% and 25% of the starting ALP activity, respectively. The wash fractions for the suspected macro-ALP serum also contained small amounts of ALP likely demonstrating some non-specific binding of ALP to the column or that the column was overloaded.

The elution fractions for both control and suspected macro-ALP samples contained IgG as expected. The suspected macro-ALP sample also demonstrated considerable ALP activity while the control serum yielded none (Table 1). As the ALP activity in the suspected macro-ALP sample paralleled the IgG concentration we demonstrated that the increased ALP activity in this patient's serum was due to the presence of a macro-ALP complex.

To further characterize the macro-ALP, aliquots of the starting material, flow-through and the eluates of both control and macro-ALP patients were applied to a Sebia ISO-PAL alkaline phosphatase isoenzyme electrophoresis gel and electrophoresed according to the package insert without the addition of wheat germ lectin (Fig. 1). A typical migration pattern of serum ALP is represented in lane 1. The diffuse anodal band is composed of bone and liver 1 isoenzymes and placental ALP would migrate in this region as well if present. Separation of the liver 1 and bone isoenzymes is accomplished by the addition of wheat germ lectin in duplicate lanes, however this was not done for this study. The discrete band immediately cathodal to the diffuse band is the liver 2 isoenzyme.

The serum of the macro-ALP patient differs substantially from this control serum by the presence of a large slowly migrating band cathodal to the liver 2 band (lane 2). This dark staining band was greatly diminished in the flow-through (lane 3) revealing 2 bands that could represent the intestinal isoenzymes, however we were unable to verify this in the absence of appropriately characterized controls. The eluate (lane 4) again demonstrated the slowly migrating band and a noticeable lack of liver and bone isoenzymes indicating that some ALP in the sample was not bound to an immunoglobulin.

To define which isoenzyme(s) were immunoglobulin bound, aliquots of the starting material and column eluates were subjected to a heat inactivation protocol as described elsewhere and electrophoresed as above or quantified by colorimetric assay [2]. Incubation of the starting material and column eluate at 56 °C for 10 min in order to inactivate the bone ALP isoenzyme failed to produce a reduction in staining intensity in either the patient serum or column eluate as determined by gel densitometry. Incubation of additional aliquots at 65 °C for 30 min in order to inactivate the bone and liver ALP isoenzymes did however produce a decrease in signal intensity of the starting material and column eluate to 71.9% and 73% of their respective controls held at 4 °C (data not shown). The reduction in staining due to incubation at 65 °C suggested that the macro-ALP complex was composed at least in part by the liver isoenzymes.

In order to better define the composition of the macro-ALP complex the heat inactivation protocol was repeated on the remaining aliquots of serum and column eluate. Starting material and eluate were again incubated at 65 °C for 30 min followed by quantitation via a colorimetric assay on a Roche Cobas 6000 analyzer using standard procedures. Incubation of the starting material and column eluate at 65 °C produced a

Table 1
Enzyme activity and IgG concentration of column fractions.

	Control patient sample					Suspected macro-ALP sample				
	ALP (U/l)	IgG (mg/dl)	AST (U/l)	ALT (U/l)	GGT (U/l)	ALP (U/l)	IgG (mg/dl)	AST (U/l)	ALT (U/l)	GGT (U/l)
Column load	68	355	9	8	84	212	450	9	4	10
Flow-through	63	<40	7	<5	76	54	<40	7	<5	9
Wash 1	5	<40	1	<5	5	10	<40	<5	<5	<5
Wash 2	<5	<40	<5	<5	<5	5	<40	<5	<5	<5
Wash 3	<5	<40	<5	<5	<5	6	<40	<5	<5	<5
Elution 1	<5	215	<5	<5	<5	132	305	<5	<5	<5
Elution 2	<5	209	<5	<5	<5	66	117	<5	<5	<5
Elution 3	<5	71	<5	<5	<5	<5	62	<5	<5	<5

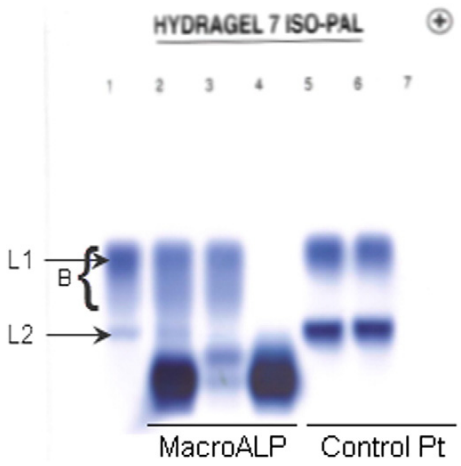

Fig. 1. ALP isoenzyme electrophoresis of column fractions. 10 μl of Sebia ISO PAL control or column fractions was loaded onto the gel. Lane 1, Sebia control sera; lane 2, macro-ALP starting material; lane 3, macro-ALP flow-through; lane 4, macro-ALP pooled eluates; lane 5, control patient starting material; lane 6, control patient flow-through; lane 7, control patient pooled eluates. L1, liver 1 isoenzyme; L2, liver 2 isoenzyme; B, bone isoenzymes.

reduction in activity to 35% and 27% of their respective controls held at 4 °C. Combinining these heat inactivation experiments demonstrates that the liver ALP isoenzyme was the predominant target of the anti-ALP antibody in this patient.

5. Discussion

While widely expressed the majority of serum alkaline phosphatase (ALP) comes from the expression of tissue-nonspecific ALP in the bone and liver. Bone ALP and liver ALP, while not true isoenzymes, differ in glycosylation. The product of a separate gene, intestinal ALP is also detectable in approximately 40% of patient sera [1]. Serum ALP activity may be increased due to appropriate or inappropriate expression of additional isoforms of ALP. These isoforms including placental, germ cell (Nagao), and fetal intestinal (Kasahara) are expressed appropriately during pregnancy (placental) or inappropriately (placental/Regan, germ cell/Nagao, fetal intestinal/Kasahara) in association with cancer [2].

ALP elevations are frequently due to cholestasis and in the absence of overt symptoms determination of gamma-glutamyltransferase (GGT) or 5′-nucleotidase (5′-NT) may clarify the source of the increased ALP activity as being from the liver or bone. Both GGT and 5′-NT are also widely expressed in various tissues, however the majority of circulating enzyme activity is again liver derived. Concomitant elevations in ALP and GGT or 5′NT suggest liver as the source of increased ALP. However GGT may also be increased in the absence of cholestasis due to heavy alcohol use, use of anticonvulsant drugs or the ingestion of large doses of acetaminophen [2]. As our patient did not demonstrate elevated GGT activity or symptoms of cholestasis another source of ALP was sought. The additional methods that could be used to characterize serum ALP are numerous, and include heat inactivation or chemical inactivation, isoform specific immunoassays and isoenzyme electrophoresis.

ALP isoforms differ in sensitivity to heat and bone ALP and will degrade at temperatures exceeding 56 °C. Conversely the placental isoform is heat stable and will withstand incubation at 65 °C for 30 min [2]. By incubating aliquots of the serum at various temperatures the laboratorian can determine if the source of the ALP is bone, liver or placental in origin. Validated heat inactivation testing is available at some reference laboratories.

Commercially available methods useful in determining the source of increased ALP activity include a bone specific ALP immunoassay marketed by Beckman-Coulter and an ALP isoenzyme gel electrophoresis kit from Sebia. However the bone ALP immunoassay is of limited utility as the immunoassay is not completely specific for the bone isoform and displays approximately 16% cross-reactivity to the liver isoform [3]. Consequently we chose to use the Sebia ISO-PAL alkaline phosphatase isoenzyme gel electrophoresis kit as it fractionates the various ALP isoforms via differences in the charge of the ALP isoenzymes.

Isoenzyme electrophoresis is useful for the identification of bone, liver and placental isoforms however complete separation of isoforms is not achieved without the addition of wheat-germ agglutinin (lectin) which preferentially binds sialic acid residues present on bone-ALP and impedes its migration. In the absence of lectin, the bone, placental and liver isoforms co-migrate, preventing accurate quantification. The downfall of this method is the same as the heat inactivation protocol as neither method can readily identify the atypical ALP isoforms associated with cancer (Nagao, Regan, Kasahara) from the benign macro-ALP.

Macroenzymes, while benign, may lead to unnecessary testing and diagnostic procedures. Consequently it is of value to conclusively demonstrate the presence of macro-ALP. A number of strategies to identify macroenzymes have been proposed including polyethylene glycol precipitation, counter-immunoelectrophoresis, gel electrophoresis, or the use of protein A or G agarose immunoprecipitation [4–9]. Our strategy of pairing an affinity chromatography purification technique using protein A/G agarose with characterization via isoenzyme analysis and heat inactivation techniques conclusively demonstrated that a macro-ALP complex was responsible for the persistent elevation in this patient's serum.

While we were able to demonstrate that the elevation of ALP activity was associated with the presence of macro-ALP and that the predominant isoenzyme bound by the antibody was the liver isoenzyme we were unable to ascertain a reason for the fluctuating ALP activity. The finding of macro-ALP was however significant as we were able to indicate to the physician that the elevation in ALP was not reflective of a disease process. We also counseled the physician to evaluate symptoms consistent with cholestasis with alternative canalicular enzyme assays such as GGT or 5′-NT.

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