Use of Alkaline Phosphatase Isoenzyme Analysis in the Evaluation of Cholestatic Liver Disease

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ABSTRACT

A useful laboratory test for the differentiation of liver, bone, and intestinal alkaline phosphatase (ALP) isoenzymes in serum is presented. Electrophoresis in polyacrylamide gel is performed with untreated serum as well as with serum incubated at 56°C for 10 min. The heating step denatures bone isoenzyme which may obscure the liver ALP band when present in large amounts. Visualization of ALP activity is accomplished by the use of buffered p-toluidinium 5-bromo-4-chloro-indolyl phosphate and magnesium ions. In serum of patients with cholestatic liver disease, the occurrence of large molecular weight liver cell membrane fragments which contain ALP activity is postulated. These ALP-containing fragments occur at the origin of the electrophoretogram, unable to penetrate the small pore separation gel. Abnormalities involving ALP isoenzymes, such as bone isoenzyme arising from increased osteoblastic activity, may be detected. Intestinal isoenzyme, normally present in small amounts in some subjects of blood groups B or O, may be elevated in certain liver diseases, such as cirrhosis. By the use of this method the routine question of whether an ALP found to be increased in a screening procedure is due to liver or bone abnormality may be answered. In addition, the occurrence of abnormal ALP bands arising from cholestatic conditions and the occurrence of abnormal amounts of intestinal isoenzyme may also be detected.

Introduction

It has been suggested that selected chemical tests be used for the diagnostic laboratory workup of liver disease. The use for such a strategy would point to the site of injury rather than to the category "liver disease." For example, the
appearance of abnormal amounts of the transaminases, alanine and aspartate aminotransferases (ALT and AST), would indicate cytoplasmic injury and/or mitochondrial injury to the hepatocyte. Abnormal serum concentrations of the "classical" enzymes of obstructive liver disease would suggest cellular membrane injury in the sinusoids and/or the canaliculi. It has been suggested that a panel of liver tests be devised to test all sites of liver injury, the transaminases, protein-synthetic processes (albumin, serum cholinesterase, prothrombin time) to test for hepatocyte injury, and the enzymes alkaline phosphatase (ALP), γ-glutamyl transferase (γ-GT), leucine aminopeptidase (LAP), 5'-nucleotidase (5'-N), among others, to test for membrane damage of cells lining the canaliculi and sinusoids. No condition, states Burke, is entirely pure as to the serum analytes concentration, but strong inferences can often be drawn concerning the patient's primary illness. Recent attempts have been made to study liver function tests by discriminant analysis and by computer-assisted procedures.

The purpose of this paper is to draw attention to the laboratory tests for ALP isoenzymes which may be important in determining the existence of a cholestatic condition. Traditionally, the clinician has ordered laboratory tests to discriminate between liver or bone as the organ source of an elevated ALP. While this need is still a viable one in view of the frequent occurrence of an elevated ALP in multi-channel screening procedures, the emphasis often shifts when the clinician suspects a liver disorder even in the absence of or with only a slight elevation of ALP. The finding of abnormal high molecular weight ALP isoenzymes in serum of patients with liver disease seems to be a test for detection of cholestatic liver disease.

Methods

The required blood specimen is clotted blood for both total and fractionated ALP. Blood collected with calcium and magnesium binding anticoagulants will exhibit falsely low values for total ALP, but there will be no interference with electrophoretic separation methods. The electrophoretic method used by us is a disc polyacrylamide gel electrophoretic (PAGE) method which has been extensively published. The substrate used for localizing ALP isoenzyme activity is p-toluidinium 5-bromo-4-chloro-indolyl phosphate. Its use as a substrate reagent for ALP activity in electrophoresis was first reported by us.

The procedure used in our laboratory is to devote two gel columns to each patient. One column is sampled with untreated serum, the second with serum previously heated at 56°C for 10 min to deactivate bone isoenzyme. A 25 μl serum sample is used per PAGE tube when total ALP is less than 400 IU per L. At higher levels, 10 μl samples are used. Visual examination is made of each pair of gel tubes, and the existence of bone, liver, abnormal liver, intestinal, or other ALP isoenzyme is noted. Other methods for ALP fractionation using cellulose acetate electrophoresis are notable. A value for total alkaline phosphatase is included in our report as well as the visual interpretation of the electrophoretogram.

Results and Discussion

Normally, a prominent and compact liver ALP zone (to be referred to as band) is the predominant ALP isoenzyme in the electrophoretogram (figure 1-1). Immediately behind the liver band and somewhat overlapping it is the bone iso-
enzyme. Liver isoenzyme in the PAGE procedure is a little faster than transferrin (in about the same position as ceruloplasmin). The intestinal isoenzyme, when present, is immediately behind transferrin. Transferrin, of course, is not visualized in the ALP electrophoretogram. In order to do so, it must be stained with a total protein stain such as Amido Black B in a separate procedure. For an illustration of the protein bands found in serum protein PAGE, see reference 9.

Bone isoenzyme is elevated in cases of increased osteoblastic activity and is readily seen in children (figure 1-2). Slightly increased bone isoenzyme can also be detected in the serum of people in about the fifth decade of life or greater (figure 1-3a). A highly increased adult bone fraction with a normal liver isoenzyme is shown in figure 1-3b.

Intestinal isoenzyme may be seen as a minor fraction in the serum of people who are of blood types B and O and who are secretors. A somewhat elevated intestinal band is depicted in figure 1-4. Elevation of the intestinal band may be an indicator of some liver diseases, such as cirrhosis.

Placental isoenzyme exists in serum as a band, when present, on the slow edge of liver isoenzyme and ordinarily merges with the liver isoenzyme. Placental isoenzyme as well as some of the variants associated with tumors may be visualized by pre-treating serums by heat treatment at 56°C for two hrs or at 65°C for 15 min which inactivates all other ALP isoenzymes.

The “cholestatic pattern” is one which indicates a liver disease involving the canaliculi and sinusoids. These patterns show an increased liver isoenzyme in the usual pre-transferrin site as well as abnormally occurring origin ALP bands in the large pore spacer and sample gels (see references 2,17,18,19). These abnormal ALP bands do not readily migrate through the large pore gels and fail to penetrate the small pore separation gel presumably because of their large molecular weight. In a non-molecular sieving medium such as cellulose acetate, these abnormal isoenzymes migrate more rapidly than the normal liver component and are found in the α1-globulin area. The cholestatic-type PAGE electrophoretogram may exist in one of two characteristic patterns: (1) an abnormal band appears at the top of the spacer gel, and (2) an abnormal band appears at the top of the separation gel as well as on the top of the spacer gel. Griffiths17 refers to these bands as canalicular and sinusoidal ALP, respectively. These electrophoretic types are shown in figures 1-5 and 1-6. Finally, a “mixed” electrophoretic pattern may exist, figure 1-7 where abnormal liver bands are present along with an increased bone isoenzyme.

Interest in the high molecular weight ALP was stimulated by Akedo et al in 19671 who studied a high molecular weight multi-protein lipid-containing complex which was isolated by gel filtration. The high molecular weight material contained ALP as well as leucine aminopeptidase activity. These authors speculated that the high molecular weight material, obtainable from serums of subjects with hepatic disease and hepatic cancer, represented hepatic membrane fragments because of the occurrence of enzymes which are known to be associated with cellular membranes. In a later paper, Shinkai and Akedo27 reinvestigated the subject and found that the high molecular weight material and actual liver plasma membranes had similar proportions of the enzymes ALP, LAP, Mg++-ATPase, and 5’-N, as well as antigenic similarities. Fritsche et al in
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Figure 1. The eight pairs of polyacrylamide gel electrophoretograms pictured have been uniformly produced with untreated serum (left gel) and with serum incubated at 56°C for 10 min prior to electrophoresis (right gel). The origin, denoted by the letter O, is at the interface of large pore gel and the small pore separation gel. Reference to origin bands denotes this interface as well as the zone at the top of the large pore gel. The letters L, B, and I refer to the zones corresponding to liver, bone, and intestinal isoenzyme, respectively. 1. A normal ALP isoenzyme pattern. Heat treatment of serum has inactivated the bone isoenzyme completely, the liver isoenzyme is slightly denatured. The tubes have been capped with Parafilm®. 2. Children who are growing normally yield this pattern. A residue of bone isoenzyme remains in the heated specimen because of the high titer of bone isoenzyme. Liver isoenzyme either is low or inactivates more readily than adult liver ALP. 3a. A slightly elevated bone isoenzyme is often seen in older patients. 3b. An elevated bone isoenzyme in an adult subject is depicted. 4. In this pair, liver ALP and intestinal ALP occur together with a small amount of origin ALP. While this intestinal component is more prominent than normal, the intestinal band may occur in subjects who are of blood groups B or O and who are secretors. 5. A small amount of origin ALP is seen in this electrophoretogram which otherwise would present a normal appearance. Even small amounts of the abnormal origin bands are often accompanied by elevated γ-glutamyltransferase (γ-GT) values. 6. Origin ALP bands are intense in this electrophoretogram pair. An ALP band is seen at the top of the spacer gel; another band is seen at the interface of the large pore gel and the small pore separation gel. Bone isoenzyme is not apparent. A highly elevated γ-GT is found in serum of patients exhibiting this pattern. 7. A mixed pattern of an abnormal liver pattern with origin bands and elevated bone isoenzyme is shown. Bone isoenzyme merges with the main liver band indicating the necessity of differentiation by heat denaturation of the bone fraction.

1972 and 1974, using cellulose acetate electrophoresis recognized that a fast (pre-liver) ALP could be demonstrated in sera from subjects with hepatic cancer and hepatobiliary disease. Rhone et al in 1973 also called attention to the fast (α1) liver ALP isoenzyme occurring in hepatobiliary disease by the use of cellulose acetate electrophoresis.

DeBroe et al in 1975 and Brockle-
hurst in 1976 isolated the high molecular weight material from serum by ultracentrifugation and studied the particulate matter by various enzyme tests and by electron microscopy. Their observations led to the conclusion that the particulate matter was composed of cell membrane fragments which contained the enzymes commonly associated with the liver cell membrane, that is, ALP, LAP, 5'-N, and γ-GT. A publication in 1978 by us involving alteration of the particulate (or slow moving) ALP by detergents of the Triton-X group suggests that the bond between protein and lipid is easily disrupted and may be altered by detergent action. The effect of detergent on the particulate ALP was to increase ALP mobility in the PAGE procedure, indicating that the molecular weight was diminished.

It is thought that shedding of membrane fragments is a normal occurrence. This takes place slowly in health since the abnormal high molecular weight material is not revealed in a normal serum. It becomes accelerated in hepatobiliary disease, hepatic cancer, obstructive diseases, etc., and may be seen in serum in those cases. It has been proposed that bile salts accelerate liver cell membrane loss in cholestatic disorders. While the mechanism for the occurrence of high molecular weight ALP activity is still to be elucidated, the value of the laboratory demonstration of the phenomenon to the clinician seems to be assured.

Résumé of Clinical Interpretations

The greatest utility of ALP PAGE with the finding of abnormal ALP isoenzymes is in obstructive diseases of the liver, primary biliary cirrhosis, and secondary liver damage especially when combined with lipoprotein-X (LP-X) assay and an independent liver enzyme test such as 5'-N or γ-GT. Kaplan et al have shown that in experimental rats ligation of the bile ducts caused induction of ALP, γ-GT, and 5'-N. The authors concluded from their experiments that the increase of these enzymes in cholestasis was due to increased production and not from regurgitation of bile fluid, nor from bile duct proliferation.

Recent interest in the fast ALP isoenzyme (cellulose acetate electrophoresis) has been shown in the field of oncology in that the abnormal ALP seems to be a sensitive indicator of cancer and of liver metastases.

Bone isoenzyme, an indicator of increased osteoblastic activity, may be elevated in pre-maturity, in bone diseases such as Paget’s disease, in diseases involving the vascular system, in vitamin D deficiency, and in liver diseases where metabolism of vitamin D is impaired.

Intestinal isoenzyme, when increased, may be a valuable indicator of liver disease, such as cirrhosis. Intestinal isoenzyme normally may appear as a minor fraction in the serum of people with blood types 0 and B and who are secretors, but may be induced in all people following a fatty meal.

References

2. Ames Company. Quick-Disc ODA Reagent Kit insert directions. Publ. #3510, Elkhart, IN 46514. Information as to commercial kits are available from the current manufacturer of the disc-gel apparatus and alkaline phosphatase PAGE, California Immuno Diagnostics, San Marcos, CA.
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