Prevalence and Properties of the Intestinal Alkaline Phosphatase Identified in Serum by Cellulose Acetate Electrophoresis

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We investigated the prevalence and characteristics of intestinal alkaline phosphatase (ALP; EC 3.1.3.1) identified in human serum by cellulose acetate electrophoresis in 8% of fasting serum samples from hospital patients (n = 500) and in 35% of fasting serum samples from patients with diabetes mellitus (n = 106; not differentiated between types 1 and 2). The intestinal ALP electrophoretic band was usually heterogeneous and contained two major subtypes of ALP. Isoelectric focusing of intestinal-ALP-positive serum treated with levanosiole and neuraminidase (EC 3.2.1.18) revealed two distinct regions of enzymatic activity that comigrated with ALP extracted from small intestinal and colonic mucosa. Anodic intestinal ALP was resistant to treatment with levanosiole and neuraminidase and comigrated with ALP from small intestinal mucosa. The more-cathodic intestinal ALP, which comigrated with ALP from colonic mucosa, was completely inhibited by levanosiole and converted by neuraminidase to a species with a more basic pI than that of neuraminidase-digested tissue-nonspecific form. This component of intestinal ALP may be of vascular origin.

Additional Keyphrases: isoelectric focusing · isoenzymes

The existence of four isoenzymes of human alkaline phosphatase [ALP; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1], each encoded by its own gene locus, is now generally accepted (1–4). These isoenzymes, identified by their putative primary tissue source, are termed placental, intestinal, ovary and testicular germ cell, and tissue-nonspecific (including liver, lung, bone, leukocytes, and kidney) ALP. At least three of these four isoenzymes are found in multiple forms in both their tissues of origin and in serum (1, 2).

The concentration of intestinal ALP in the serum of an individual is related to the individuals ABO blood group and secretor status and increases after dietary fat intake (5). The serum ALP concentration is higher in BO secretors than in ABO nonsecretors or A secretors. The concentration of intestinal ALP in the serum of BO secretors is further increased by cirrhosis (3, 6) and chronic renal failure (7). Cellulose acetate electrophoresis indicates an increased prevalence of this isoenzyme in fasting serum from diabetic patients (8, 9), with no difference between types 1 and 2 diabetes mellitus (9). Measurements of intestinal ALP in serum seem to be of little or no use in diagnosing or monitoring intestinal diseases (3).

Successful laboratory methods for distinguishing the intestinal ALP activity in serum from the other sources of activity include selective L-phenylalanine inhibition of the intestinal enzyme activity (10), levamisole inhibition of the other ALP sources (11), cellulose acetate electrophoresis and other forms of zone electrophoresis (12), and isoelectric focusing (2). Inactivation of specific isoenzymes by monoclonal antibodies has also been demonstrated (13); users of this technique report that at least trace amounts of the placental, intestinal, and tissue-nonspecific ALP isoenzymes are produced in most tissues (3, 13).

Reportedly, human intestinal ALP is completely resistant to digestion by neuraminidase (sialidase; EC 3.2.1.18), whereas the other forms usually found in human serum are converted by this treatment to variants that migrate more cathodically on cellulose acetate electrophoresis (14). However, our laboratory demonstrated that at least one component of enzyme extracted from intestinal mucosa is converted by neuraminidase (15); although this variant is a minor fraction of the small intestinal mixture, it is predominant in the colon. Muira et al. (16) showed that the ALPs extracted from ileum differ from those from duodenum with respect to N-terminal amino acid sequence but are similar antigenically and have nearly identical electrophoretic mobilities. Neuraminidase was not used to distinguish these forms.

Although cellulose acetate electrophoresis is probably the most widely used method for distinguishing the isoenzymes of ALP in serum, it is not sensitive enough to detect the small amount of intestinal isoenzyme reportedly present in most individuals. Moreover, when present in serum, this intestinal band demonstrates a considerable subject-to-subject variability in electrophoretic behavior. The migration rate varies slightly, and the shape of the band (i.e., sharpness, or area of membrane occupied by a band of given activity) has an even greater range of variation (Figure 1). Although this could be due to the well-documented heterogeneity of the enzyme in and from the intestinal mucosa, another possibility is that a variable portion of the enzyme undergoes some transformation after leaving the cell of origin (e.g., loss or gain of carbohydrate residue). Less likely, the observed isoenzyme could be wholly or partially from a source other than the intestine.

Using electrophoretic and chemical techniques, we studied the intestinal ALP identified in human serum by cellulose acetate electrophoresis to learn about its
composition and source. We also studied the prevalence of intestinal isoenzyme in a group of patients with diagnosed diabetes mellitus. We compared findings in serum samples with characteristic ALP isoenzymes extracted from tissues.

Materials and Methods
To characterize the frequency of occurrence of detectable intestinal ALP in serum of hospital patients, we obtained serum specimens from 500 patients at Roger Williams Hospital in the morning after an overnight fast. We chose fasting specimens to minimize the effect of eating on the serum concentration of the intestinal isoenzyme (5). Specimens were chosen without conscious bias.

We likewise obtained fasting serum specimens from 106 patients at Roger Williams Hospital with a primary or secondary diagnosis of diabetes mellitus (types 1 and 2) (9). All specimens were analyzed for ALP activity and isoenzymes and were tested for ABO status.

Qualitative and Quantitative Determination of ALP and Isoenzymes
We measured total ALP activity by a modified Bowers and McComb method (17), utilizing p-nitrophenyl phosphate as the substrate in a diethanolamine/sucinate buffer. The assay was performed with a Micro KDA (American Monitor, Indianapolis, IN). The upper limit of normal for ALP in our laboratory is 260 U/L.

We used cellulose acetate electrophoresis (Helena Labs., Beaumont, TX) and stained the bands by coupling Fast Violet B salt with β-naphthol acid liberated by the hydrolysis of β-naphthol phosphoric acid sodium salt. Intestinal bands were identified by their isoenzyme pattern; the electrophoretic patterns from patients' specimens were compared with those of control specimens, containing intestinal ALP, that were included with each analytical run. Electrophoretic patterns were scanned with the Corning 760 fluorometer/densitometer (Corning Medical and Scientific, Medfield, MA).

We performed isoelectric focusing with the Resolve Isofocus Electrophoresis System (Isolab Inc., Akron, OH). Briefly, serum was applied to an agarose gel and electrophoresed at 15 W (constant power) for 35–50 min, over a cooling unit (5 °C without load). We then stained the isoenzymes at their isoelectric point by adding α-naphthyl phosphate and 4-aminodiphenylamine diaminon sulfate and then heating at 37 °C for 15 min. We destained the gel in glacial acetic acid until the background color was removed and then soaked the gel for 1 h in distilled water to remove ampholytes and destaining solution.

Serum and Tissue Studies
Serum samples showing various ALP isoenzymes on cellulose acetate, including any of the heterogeneous types of intestinal isoenzyme, were subjected to chemical inhibition of ALP.

For the chemical-inhibition experiments, we divided patients' sera into four 0.5-mL aliquots. To the aliquots we added 10 μL of 5 mmol/L levamisole reagent, 20 μL of 150 mmol/L L-phenylalanine reagent, 20 μL of 300 mmol/L L-phenylalanine reagent, or no reagent. Levamisole and L-phenylalanine were purchased from Sigma Chemical Co. (St. Louis, MO). All four groups of samples were incubated at room temperature for 18 h, then analyzed for total ALP activity and electrophoresed on cellulose acetate as described above. When levamisole-inactivated specimens were electrophoresed on cellulose acetate or subjected to isoelectric focusing, the staining reagent was modified to include levamisole at a final concentration of 12 mmol/L.

Serum samples having an intestinal band were also subjected to neuraminidase (Sigma Type VI, from Clostridium perfringens) digestion and ABO grouping. We incubated 100 μL of serum with 100 μL of neuraminidase in acetate buffer, pH 6.5 (150 U/L, final concentration in serum), at 37 °C for 18 h. Treated and untreated samples were then electrophoresed on cellulose acetate and subjected to isoelectric focusing. A series of samples was also incubated with twice the original concentration of neuraminidase (i.e., 300 U/L final concentration) to determine whether the observed differences in isoenzyme migration after neuraminidase digestion were solely a product of the concentration of the enzyme used.

ABO grouping was performed according to the protocol specified by the American Association of Blood Banks (18). Abdominal and breast adipose tissue, kidney cortex and medulla, and large and small intestinal mucosa were obtained, prepared, and extracted as previously reported (15).

Results
With cellulose acetate separation of ALP, a band was clearly visible in the range usually attributed to the intestinal isoenzyme (low limit of detectability = 13 U/L) in 38 (8%) of the 500 fasting hospital patients'
samples. The mean total ALP activity of the 500 serum specimens was 359 U/L (range 69–3944 U/L); that of the 38 intestinal-band-positive samples was 308 U/L (range 155–688 U/L).

Of the intestinal-band-positive group, 79% had blood type O, 11% type B, 7% type A, and 3% type AB; this agrees with the reported relationship of this band to ABO status (5). Twenty-six of the 38 patients (68%) had a primary or secondary diagnosis of diabetes mellitus or lung disease or showed gastrointestinal bleeding. This is in contrast to a combined prevalence of 14% for these conditions in the total population of patients seen at our institution during the same period.

Of the 106 patients preselected because of a diagnosis of diabetes mellitus, 37 (35%) showed an intestinal ALP band after cellulose acetate electrophoresis. The mean total serum ALP activity of this sample (n = 106) was 276 U/L (range 119–393 U/L). Of this group, 75% had blood type O, 11% type B, 11% type A, and 3% type AB.

The cellulose acetate electrophoretic mobility and width of the intestinal band varied among specimens (Figure 1). As determined by densitometry, the percent of the total ALP activity represented by the intestinal band varied from 22% to 74%, with one sample showing 100% intestinal ALP.

With isoelectric focusing, the intestinal band was usually separated into two regions, each composed of three or four bands (Figure 2). The more acidic (anodic) were focused in the pI range of 4.65–4.73; the more basic (cathodic) were focused in the range 4.85–4.95. The more-cathodically focused band was not always visible. The resolution of these two regions is more complete when the focusing is stopped after 35 min. If the separation proceeds for the full 50 min, the bands are closer and may overlap. This probably explains the failure to observe the more-cathodically migrating enzyme in reported studies (2, 4).

We found no relationship between a patient’s diagnosis and the relative quantity of intestinal band on cellulose acetate or the presence of the cathodically focused band from isoelectric focusing.

![Fig. 2. Isoelectric focusing of serum specimens, demonstrating the two distinct regions of intestinal ALP activity](image)

Each pair of lanes represents serum specimens before (odd nos.) and after (even nos.) neuraminidase treatment. Lanes 1, 2, and 5–8: the more-anodically focused, neuraminidase-resistant, intestinal ALP bands; lanes 3 and 4: the cathodically focused, neuraminidase-sensitive intestinal ALP bands; C: control sera; *: pI scheme according to Griffiths and Black (2, 4)

Treatment of the specimens with neuraminidase before electrophoresis permitted the classification of the population showing the intestinal band into two groups. In the first group, there was no change in electrophoretic behavior of the intestinal band after neuraminidase treatment and, upon isoelectric focusing, most of the intestinal activity was in the more-anodic region. In the second group, the enzyme in the intestinal band was partially or completely altered by neuraminidase (Figure 3); total ALP in the intestinal band, calculated from densitometric scanning of cellulose acetate, decreased by 20–100% from the pretreatment values. This decrease was not changed when we doubled the neuraminidase concentration in the digestion mixture.

With isoelectric focusing, the more cathodically focused band was no longer present after neuraminidase treatment, but a band appeared that migrated at a more basic pI than that of the neuraminidase-converted tissue-nonspecific enzyme (Figure 2). Levamisole treatment completely inactivated this cathodic band, whereas the more anodically focused intestinal enzyme was completely resistant to levamisole.

We compared the results obtained from cellulose acetate electrophoresis of serum samples with the behavior of ALP from small intestinal and colonic mucosa extracts (Figure 4). ALP extracted from colonic mucosa was sensitive to neuraminidase and levamisole, whereas ALP from small intestinal mucosa was completely resistant to both.

We were also interested in enzyme extracted from kidney cortex and medulla and particularly from adipose tissue, where the ALP is reportedly from capillaries and not adipocytes (19). The kidney and adipose capillary enzymes are putatively tissue nonspecific but may show electrophoretic mobility similar to that of the intestinal variant. The result of isoelectric focusing of the various tissue extracts, before and after neuraminidase treatment, is shown in Figure 5.

The colonic and small intestinal mucosal extracts each showed the two regions of ALP (18), but the relative concentration of the neuraminidase- and levamisole-resistant, anodically migrating band was much greater in the small intestinal sample. This is consistent with the zone-electrophoretic results previ-
adipose vascular enzyme. All tissue-extracted ALPs except the more-anodically focused intestinal enzyme were completely inactivated by levamisole treatment.

Phenylalanine treatment of ALP extracted from small intestine reduced total activity by 26%, whereas treatment of a colonic extract resulted in no loss of ALP activity.

Discussion

Komoda et al. (14) first reported that ALP extracted from small intestinal mucosa contains two major components. This was later confirmed and extended to the colonic mucosal enzyme by our laboratory (15). Apparently, both forms are also present in most serum samples that show electrophoretically detectable concentrations of intestinal isoenzyme. The varying ratio of the serum concentrations of the two forms, which do not separate with zone electrophoresis, probably cause the difference in migration rate and shape of the intestinal band. The heterogeneity of the two major forms may also contribute to this effect.

Neither component is the intestinal variant ALP recently reported in plasma (20–22). This variant migrates with the bone isoform of the tissue-nonspecific enzyme in zone electrophoresis, is completely resistant to neuraminidase digestion, and is postulated to be intestinal enzyme with attached membrane-binding domain (20).

Isoelectric focusing results for serum intestinal ALP (as defined by cellulose acetate electrophoresis) are similar to those for the intestinal mucosal extract. This similarity increases when isoelectric focusing follows treatment with neuraminidase or levamisole. ALP from some serum samples behaves similarly to the small intestinal isoenzyme or colonic isoenzyme, with most samples showing characteristics of both.

We believe that the neuraminidase- and levamisole-sensitive component of the serum intestinal ALP (de-
fined by zone electrophoresis) is of vascular origin. It may be derived from the intestinal mucosa or from other tissues, e.g., adipose tissue (19). The presence of a vascular ALP, as distinct from brush border ALP, in cells from the small and large intestinal epithelia was reported by Lev and Griffiths (23), who used histochemical techniques, and has also been identified histochemically in various other tissues. This is most probably the neuraminidase- and levamisole-sensitive component of the enzyme in the intestinal extracts. Presumably, the vascular enzyme is a variant of the tissue-nonspecific gene product, although this is not proven.

The cause of the increased prevalence of serum intestinal ALP in diabetic patients is not known. Fishman (24) showed that the membrane anchoring of the enzyme is through a phosphatidylinositol chain end. Unakami et al. (25) postulated that the increased release of ALP might be through increased activity of phosphatidylinositol lipase; however, they failed to demonstrate this phenomenon in small intestinal preparations from streptozotocin-induced rats. Perhaps diabetes-related changes in regulation of amino acid and carbohydrate transport across the intestine involve ALP activity (26).

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References
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