Effect of Phospholipase C on High-Molecular-Mass Alkaline Phosphatase in Serum

Elizabeth Sykes, Frederick L. Klechie,1 and Emanuel Epstein

Electrophoresis of some serum samples on polyacrylamide gel, followed by staining for alkaline phosphatase (EC 3.1.3.1), produces a band of activity at the gel origin. This high-Mr band consists of liver membrane fragments containing alkaline phosphatase and other enzymes. Alkaline phosphatase is closely associated with phosphatidylinositol in liver plasma membranes, and we have found that phospholipase C (EC 3.1.4.3) from Bacillus cereus, known to possess some phosphatidylinositol specificity, was able to release liver alkaline phosphatase from the high-Mr band. Two preparations of phospholipase C from Clostridium perfringens, however, which has no phosphatidylinositol specificity, had no effect on the alkaline phosphatase activity in the high-Mr band.

Additional Keyphrases: isoenzymes; variation, source of; liver plasma-membrane; phosphatidylinositol; electrophoresis, polyacrylamide gel

Electrophoresis of certain serum samples on polyacrylamide gel, followed by staining for alkaline phosphatase, produces a band of activity at the gel origin (1). This fraction of activity has been termed high-Mr alkaline phosphatase (2) and is commonly associated with liver disease (1, 3, 4). The mobility of the high-Mr band (HMWB) is dependent on the method of electrophoresis; it migrates rapidly on agar gel and cellulose acetate (5) and appears in the void volume during filtration through Sepharose 4B gel (6). The HMWB is usually considered to represent plasma membrane fragments, probably of hepatocyte origin (5), that contain alkaline phosphatase and other enzymes. Alkaline phosphatase (EC 3.1.3.1; orthophosphoric-monoester phosphohydrolase [alkaline optimum]) is found at the outer surface of liver plasma membranes and is associated with phosphatidylinositol within the membrane. Phosphatidylinositol-specific phospholipase C (EC 3.1.4.3; phosphatidylcholine:phosphohydrolase) releases alkaline phosphatase from plasma membranes obtained from liver homogenates (7–9). We investigated the effect of Bacillus cereus phospholipase C (Type III; Sigma Chemical Co., St. Louis, MO 63178), known to contain some phosphatidylinositol specificity, and Clostridium perfringens phospholipase C (Sigma; Types I and IX), possessing no phosphatidylinositol specificity, on serum from six patients. All six serum samples contained a prominent HMWB and had normally migrating liver and bone isoenzymes of alkaline phosphatase.

Materials and Methods

Materials

Acrylamide, N,N'-methylenebisacrylamide (Bis), and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Eastman Organic Chemicals, Rochester, NY 14650. Tris, riboflavin, bromphenol blue (tracking dye), B. cereus phospholipase C (Type III, 160 kU/g protein), Cl. perfringens phospholipase C (Type I, 12.5 kU/g protein), and Cl. perfringens phospholipase C (Type IX, 120 kU/g protein) were from Sigma. J. T. Baker Chemical Co., Phillipsburg, NJ 08865, supplied ammonium persulfate and boric acid; United States Biochemical Corp., Cleveland, OH 44122, supplied the 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt).

Methods

Serum samples were obtained from six patients (Table 1), found on polyacrylamide gel electrophoresis (PAGE) to have both the HMWB and normally migrating bone and liver alkaline phosphatase isoenzymes. Total alkaline phosphatase activities in serum ranged from 134 to 237 U/L (mean 198 U/L). Patients 1 to 5 had evidence, either by biopsy, radiographic scans, or liver-function tests, of liver disease. The cause of the increased serum alkaline phosphatase in patient 6 is possibly attributable to passive congestion of the liver.

Preparations of phospholipase C or isotonic saline (0.9 g/L NaCl) were added to serum samples to give the following final concentrations of enzyme activity (kU/L); B. cereus 80, Cl. perfringens (Type I) 420, and Cl. perfringens (Type IX) 417. Controls of phospholipase C in saline were also prepared. The specimens were vortex-mixed for 30 s, then incubated at 37°C for 3 h. We then applied 25 μL of each

Table 1. Clinical Summary of Patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, yr</th>
<th>Total alkaline phosphatase, a U/L</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>233</td>
<td>Fever of unknown origin; results of liver-function tests above normal b</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>190</td>
<td>Carcinoma colon; hepatic metastases (liver scan)</td>
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<tr>
<td>3</td>
<td>33</td>
<td>183</td>
<td>Vasculitis; portal inflammation (by liver biopsy)</td>
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<tr>
<td>4</td>
<td>23</td>
<td>237</td>
<td>Diabetes mellitus; cholelithiasis</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>212</td>
<td>Atherosclerotic heart disease; hepatic hemangiomata (liver scan)</td>
</tr>
<tr>
<td>6</td>
<td>78</td>
<td>134</td>
<td>Congestive heart failure; temporal arteritis</td>
</tr>
</tbody>
</table>

a Normal range, 30–115 U/L.

b Aspartate aminotransferase 95 U/L (normal 0–41 U/L); lactate dehydrogenase 272 U/L (normal 60–200 U/L).
incubation mixture to a polyacrylamide gel and subjected the samples to electrophoresis for 45 min by a well-established method (10–12). Polyacrylamide 7% (acrylamide:Bis = 37.8, w/w), with ammonium persulfate and TEMED as catalyst, was used for the separating gel, and photopolymerized polyacrylamide 2.5% (acrylamide:Bis = 4, w/w) was used as the stacking gel. The constituents of the stacking and loading gels were the same. The substrate used for locating the alkaline phosphatase activity was 5-bromo-4-chloro-indolyl phosphate (p-toluidine salt) (12). Gels were stored in dilute (75 mL/L) acetic acid solution.

The remaining (nonelectrophoresed) portion of each incubation mixture was used to determine total serum alkaline phosphatase activity with an Abbott VP bichromatic analyzer (Abbott Laboratories, Diagnostics Division, Irving, TX 75015) and the Abbott "A-Gent" alkaline phosphatase test, which is based, with some modifications, on the method of Bowers and McComb (13). The reaction involves an amine salt of p-nitrophenyl phosphate as substrate and 2-aminomethyl-1,3-propanediol as buffer in the presence of magnesium ions; absorbance is measured at 415 nm (37 °C).

**Results**

Treatment with phospholipase C derived from *B. cereus* caused the alkaline phosphatase activity present within the HMWB to migrate from the gel origin to the position of liver isoenzyme (Figure 1). Consequently, these samples showed increased activity of liver alkaline phosphatase isoenzyme and markedly less HMWB than in the serum control. In contrast, neither preparation of phospholipase C from *C. perfringens* appeared to alter the mobility of the HMWB. The most impure preparation of *C. perfringens* (Type I, 12.5 kU/g protein) produced an overall decrease in alkaline phosphatase activity on PAGE and caused the liver and bone isoenzymes to migrate together only 58% as far as in the serum control. This preparation also caused the appearance of alkaline phosphatase activity on top of the stacking gel in two of the serum samples (Figure 1, no. 3 and 4). *C. perfringens* (Type IX, 120 kU/g protein) phospholipase C caused the following electrophoretic changes: an increase in liver alkaline phosphatase staining in three of the serum samples (Figure 1, no. 1, 2, and 4); a light, diffuse area of gel staining above the liver alkaline phosphatase in two of the serum samples (Figure 1, no. 3 and 4); and a new band of activity on top of the stacking gel in the same two samples. No alkaline phosphatase activity was demonstrated after electrophoresis of incubation mixtures containing each specific phospholipase C individually and saline instead of serum.

Incubating either the serum–saline controls or the serum–phospholipase C (*B. cereus* and *C. perfringens*, Type I) mixtures at 37 °C for 3 h caused the total alkaline phosphatase activity to increase by between 15 and 29% over that in the unincubated controls (Table 2). The difference between incubated controls and phospholipase C-treated samples was not significant.

**Discussion**

Alkaline phosphatase activity present at the origin of the separating gel after PAGE, or as a rapidly migrating band on cellulose acetate, has been found in most instances to represent liver plasma membrane in association with alkaline phosphatase and other enzymes, e.g., 5'-nucleotidase (5). It may be present in the serum of patients with various liver diseases, including viral hepatitis, alcoholic liver disease, and cirrhosis, and is considered by some investigators (4) to be a potential tool for diagnosis of metastatic liver disease. Low and Finean (7) have shown that alkaline phosphatase is located at the outer surface of the liver.

**Table 2. Effect of Phospholipase C (PLC) on Total Alkaline Phosphatase Activity**

<table>
<thead>
<tr>
<th>Patient</th>
<th>No.</th>
<th>Alk. phosphatase acty, U/L</th>
<th>B. cereus PLC</th>
<th>Cl. perf. PLC²</th>
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<tr>
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<tr>
<td></td>
<td>6</td>
<td>134</td>
<td>153</td>
<td>152</td>
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</table>

*1 isotonic saline (NaCl 0.9 g/L).
²*Cl. perfringens* (Type I) phospholipase C.
*N*insufficient sample for determination.

**Fig. 1. Polyacrylamide gel electrophoreses of serum from patients 1–4 (see Table 1)**

Each group of four gels is arranged from left to right as follows: serum control, serum plus *B. cereus* phospholipase C, serum plus *C. perfringens* (Type I) phospholipase C, and serum plus *C. perfringens* (Type IX) phospholipase C. H, high-M, band at origin of separating gel (sera from patients 3 and 4 also show activity on top of stacking gel when treated with *C. perfringens* phospholipase C, Types I and IX); B, bone isoenzyme of alkaline phosphatase; L, liver isoenzyme.
plasma membrane, is closely associated with phosphatidylinositol, and can be released by phosphatidylinositol-specific phospholipase C. More recently, Kominami et al. (9) have also shown that phosphatidylinositol-specific phospholipase C (B. cereus) is able to release alkaline phosphatase from rat liver membrane fragments, causing the alkaline phosphatase to migrate with the liver isoenzyme on PAGE. These findings are in contrast to those reported for intestinal alkaline phosphatase, which appears to have no interactions with membrane phosphatidylinositol (14).

In our investigations, B. cereus phospholipase C, known to possess some phosphatidylinositol specificity (7, 15), released liver alkaline phosphatase from the HMWB. This suggested that the interaction of phosphatidylinositol and alkaline phosphatase had been disrupted, allowing the previously membrane-associated alkaline phosphatase to migrate with normal liver isoenzyme. The more impure preparations of Cl. perfringens have previously been shown to contain a variety of contaminating enzymes, e.g., protease, alpha-glycosidase, neuraminidase (16, 17). Treatment of serum with the least pure preparation available from Sigma caused a decrease in the liver/bone isoenzyme mobility and an overall decrease in alkaline phosphatase activity on PAGE; however, total alkaline phosphatase activity (determined with the Abbott VP), as compared with the incubated control, was unchanged. In patients 3 and 4 (Figure 1) this phospholipase C preparation also caused the appearance of alkaline phosphatase activity on top of the stacking gel.

We suggest that the alteration in liver and bone isoenzyme mobility caused by Cl. perfringens phospholipase C (Type I) may be due to structural modifications by contaminating enzymes. Neuraminidase has been reported to be a contaminant in impure Cl. perfringens phospholipase preparations (16, 17) and is known to retard the mobility of liver and bone alkaline phosphatase (18). De Broe and Wieme (17), using serum containing high-M₆ alkaline phosphatase and normal liver isoenzyme, showed conversion of these two components into a single fraction by use of Cl. perfringens phospholipase solution (Type I; Sigma). The newly formed fraction had a mobility slower than that of normal liver isoenzyme and corresponded to the position of neuraminidase-treated liver alkaline phosphatase. These changes in electrophoretic mobilities were described for agar gel, but are very similar to our own findings with polyacrylamide gel. The reason for decreased substrate specificity for 5-bromo-4-chloro-3-indolyl phosphate on PAGE, but unchanged substrate specificity for p-nitrophenol phosphate, is unclear but may also be related to contaminants within the Type I Cl. perfringens phospholipase C preparation. We also suggest that alkaline phosphatase activity at the origin of the stacking gel consists of membrane complexes larger than those seen at the origin of the separating gel.

Use of the purer preparation of Cl. perfringens (Type IX) did not alter HMWB or liver isoenzyme mobility. However, it did cause a slight increase in the activity of the liver isoenzyme in three samples (Figure 1, no. 1, 2, and 4) and the appearance of a band of activity on top of the stacking gel in two samples (Figure 1, no. 3 and 4). The cause of these inconsistent findings is unclear. We saw no evidence of alkaline phosphatase activity after electrophoresis of the phospholipase C preparations, thereby excluding intrinsic alkaline phosphatase activity as a cause for changes in band staining and mobility. Alkaline phosphatase activity is known to increase on standing at room temperature (19). The reason for this observation (19) is not entirely clear, but it is in accord with our findings of an increase in total alkaline phosphatase activity after incubation at 37°C for 3 h.

In conclusion, our investigations showed that B. cereus phospholipase C (Type III) released liver alkaline phosphatase from high-M₆ alkaline phosphatase (HMWB), suggesting disruption of phosphatidylinositol interactions. However, neither phospholipase C preparation from Cl. perfringens appeared to release the membrane-associated alkaline phosphatase.

References