Identification of human eosinophil lysophospholipase as the constituent of Charcot–Leyden crystals

(PHOSPHOLIPASE B)

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ABSTRACT Since the initial descriptions of Charcot–Leyden crystals more than 100 years ago, the presence of these slender, dipyrimal crystals in human tissues and biologic fluids has become a hallmark of eosinophilic leukocyte infiltration, especially in association with allergic and helminthic diseases. The formation of these crystals in vitro after disruption of human eosinophils, but not of other cell types, in hypotonic saline or detergent established the eosinophil as the unique cellular source of the crystalline protein. Charcot–Leyden crystals have now been found to express lysophospholipase activity (lysolecithin acylhydrolase, EC 3.1.1.5), and the solubilized Charcot–Leyden crystal protein presents a single stained protein band that is coincident with the lysophospholipase activity eluted from replicate gels on alkaline polyacrylamide gel electrophoresis. On sodium dodecyl sulfate/polyacrylamide gel electrophoresis, the solubilized Charcot–Leyden crystal protein migrates with a molecular weight of 17,400, which is comparable to that of eosinophil lysophospholipase purified chromatographically to homogeneity; further, on combination, the two proteins comigrate as a single staining band. Finally, the chromatographically purified eosinophil lysophospholipase in hypotonic buffer forms dipyridal crystals morphologically identical to Charcot–Leyden crystals. The findings that chromatographically purified, homogeneous eosinophil lysophospholipase and Charcot–Leyden crystal protein express the same enzymatic activity, are of the same size and charge, and form crystals of identical morphology indicate that human eosinophil lysophospholipase is the constituent of Charcot–Leyden crystals.

Prominent blood or tissue accumulations of eosinophilic leukocytes occur in many allergic reactions and helminthic infections as well as in the course of some neoplastic, inflammatory, and immunodeficiency diseases (1). Charcot–Leyden crystals (CLC), which are characteristically long, slender, dipyridal crystals, were initially described in the mid-nineteenth century (2, 3) and are hallmarks of eosinophil involvement in some tissue reactions. CLC may be prominent in spumum of patients with asthma (3), pulmonary ascarisiasis (4), and tropical eosinophilia (5), in the feces of patients with amebic, Trichuris, or ulcerative colitis (6), and in tissues containing eosinophil granulomas of bone (7) and granulomas associated with tissue-invading helminths (8). Disruption of eosinophils either by detergents (9) or by homogenization and suspension in hypotonic saline (5) allows for the formation of CLC in vitro. Similar disruption of other classes of leukocytes or of eosinophils derived from nonprimate mammalian species fails to result in CLC formation (10). Thus, eosinophils, specifically those from primate species, have been established as the unique cellular source of this crystalline material (11). Human CLC are composed of a single protein, which differs from the major basic protein, the predominant protein in the large granule of the eosinophil (12). The intracellular source of the eosinophil CLC protein remains uncertain, although nuclear (9), granular (10), and cytoplasmic (12) origins have been proposed. Further, no biochemical activity or biologic function has been established for the protein composing the crystals (11). The findings that chromatographically purified, homogeneous eosinophil lysophospholipase (lysolecithin acylhydrolase, EC 3.1.1.5) and CLC protein express a common enzymatic activity, exhibit the same physicochemical characteristics of size and charge, and form crystals of identical morphology establish human eosinophil lysophospholipase as the constituent of CLC.

MATERIALS AND METHODS Materials. Reagents and equipment were obtained from the sources indicated: L-1-[1-14C]Palmitoyl lysophosphatidylcholine (specific activity about 50 mCi/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) and Aquasol scintillation fluid from New England Nuclear; Sephadex G-100, heparin-Sepharose, gel filtration and electrophoresis molecular weight calibration proteins, 6% dextran 70 in normal saline (Macrodx), and diatrizoate-Ficoll (Ficoll-Paque) from Pharmacia; synthetic palmitoyl lysophosphatidylcholine, egg-yolk phosphatidylcholine, iodacetamide, and glycine from Sigma; organonemicurial agarose (Affi-Gel 501), NaDodSO₄, acrylamide, N,N' -methylenebisacrylamide, N,N',N',N'-tetramethylrhodamine, urea, dithiothreitol, ammonium persulfate, Coomassie brilliant blue stain, and Bio-Rad protein assay from Bio-Rad; Na₂EDTA, chloroform, methanol, heptane, isopropanol, Spectrophotodiodes, and Gelman SA ITLC thin-layer chromatography sheets from Fisher; calcium and magnesium-free Hanks' balanced salt solution from Microbiological Associates (Walkersville, MD); 10,000 M₇, retentionollection bags from Schleicher & Schuell; Branson model 350 sonifier from Branson Sonic Power; and U/M-05 Diaflo membranes from Amicon.

Blood from normal donors provided neutrophils, mononuclear cells, platelets, and erythrocytes (13), and donors with blood eosinophilias due to trichinosis, filariasis, drug allergies, and a hyper eosinophilic syndrome provided granulocytes enriched in eosinophils (14). Eosinophils from a donor with hyper eosinophilia (leucocyte count of 28,000–36,000/mm³ of blood with 87–94% eosinophils) were used without further purification as a source of protein for formation of CLC and for isolation of enzyme.

Assay of Lysophospholipase. The cell preparations were disrupted by sonication and dialyzed against 0.1 M NaCl overnight before assay of cellular lysophospholipase. A 100 to 200-μl portion of cell extract or chromatographic fraction was added to a reaction mixture with a final volume of 1 ml, con-
taining 250 nmol of synthetic palmitoyl lysophosphatidylcholine and 0.96 nmol of [14C]palmitoyl lysophosphatidylcholine, both of which had been sonicated for 2 min at 4°C at setting 5 with a microtip (Branson model 350 sonifier) in 0.1 M Tris-HCl, pH 7.5/2 mM EDTA. Five hundred micrograms of phosphatidylcholine, sonicated in buffer for 9 min as above, was included in the assay of chromatographic fractions. The reaction mixture was incubated at 37°C for 1 hr and was then extracted with acidified isopropanol/heptane (15) to partition the released fatty acid into the upper organic layer, from which a sample was taken and its radioactivity was measured in Aquasol (14). The identification and quantitation of the released fatty acid was confirmed by extracting replicate incubation mixtures with chloroform/methanol/water (10:10:3, vol/vol) and resolving the extracted lipids by thin-layer chromatography on silicic acid with a neutral lipid solvent system in comparison with purified standards (14). One unit of enzyme activity represents 1 nmol of fatty acid liberated per hour at 37°C, at pH 7.5.

Purification of Eosinophil Lysoosphospholipase. Approximately 1 × 10⁹ leukocytes (94% eosinophils, 6% neutrophils) were disrupted by sonication in 0.05 M Tris-HCl, pH 8.5/2 mM EDTA. After the cells were centrifuged at 750 × g for 10 min, the supernatant fluid was subjected to gel filtration on Sephadex G-100 in 0.05 M Tris-HCl, pH 8.5/2 mM EDTA. The fractions containing the peak of enzyme activity, which eluted at about 66% of bed volume, were pooled and applied to a column of organonemeric agarose equilibrated in 0.1 M Tris-HCl, pH 7.5/2 mM EDTA. Lysoosphospholipase eluted early with a gradient from 0 to 0.35 M dithiothreitol in 0.1 M Tris-HCl, pH 7.5/2 mM EDTA/0.15 M NaCl. The peak lysoosphospholipase-containing fractions were adjusted to a conductivity of 4 mS and applied to a heparin-Sepharose column equilibrated with 60 mM Tris-HCl, pH 7.5/1.2 mM EDTA. Lysoosphospholipase activity, which appeared in the fell-through fractions, was homogeneous, demonstrating a single Coomassie brilliant blue staining band on both NaDodSO₄/10% polyacrylamide gel electrophoresis and 7.5% polyacrylamide gel electrophoresis at pH 8.9.

Preparation of CLC. After dextran-induced sedimentation of erythrocytes, 1.64 × 10⁹ leukocytes from a patient with hypersesinophilic syndrome were washed twice in calcium and magnesium-free Hanks' balanced salt solution and residual erythrocytes were lysed by the addition of 0.15% (wt/vol) NaCl. The eosinophil-rich leukocytes were sedimented at 700 × g for 2 min at room temperature and disrupted by sonication on ice for 2 min at an output of 7 with a microtip in 45 ml of 0.15% NaCl. Particulate material was removed by sequential centrifugation at 2000 × g for 15 min at 4°C and then at 32,000 × g for 30 min at 4°C. The supernatant was held overnight at 4°C and then concentrated 4-fold by positive-pressure ultrafiltration with a UM-05 Diaflo membrane to facilitate crystal formation (5). Crystals were collected and washed once in cold distilled H₂O by centrifugation at 2000 × g for 20 min at 4°C. After microscopic confirmation of the characteristic crystalline morphology, the sample was lyophilized.

Analytical Methods. NaDodSO₄/polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gels (16). Samples in 8 M urea were treated with 10 mM dithiothreitol at 56°C for 5 min, followed by alkylation in 10 mM iodoacetamide at 56°C for 15 min. NaDodSO₄ was added to a concentration of 1% (wt/vol), and the samples were incubated at 100°C for 10 min. Protein standards included phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin. Analytical polyacrylamide disc gel electrophoresis was performed with pH 8.9, 7.5% acrylamide gels (17) with the modifying addition of 2 mM dithiothreitol to the electrode buffer. Gels were electrophoresed at 2.5 mA per gel for 3 hr before use.

RESULTS

For the same numbers of disrupted human peripheral blood leukocytes, lysoosphospholipase activity in the eosinophil-enriched granulocytes was 8-fold greater than that in the neutrophil-enriched granulocytes and 3-fold greater than that of the mononuclear leukocytes (Fig. 1). Relatively trivial activity was present in platelets and erythrocytes.

In a preliminary experiment, 200 µl of pelleted CLC contained 184 units of lysoosphospholipase activity when assayed directly. To determine if this enzyme activity was a major component of the crystal, lyophilized CLC were solubilized in 0.1 M Tris-HCl, pH 7.5/2 mM EDTA, and 40-µg quantities of protein, as assessed by the Bio-Rad assay using human IgG as a reference standard, were subjected to alkaline disc gel electrophoresis in two replicate gels. In the gel stained with Coomassie brilliant blue, a single stained protein band was present. The parallel gel was sliced into 2-mm sections and each section was macerated in 500 µl of 0.1 M Tris-HCl, pH 7.5/2 mM EDTA/2 mM dithiothreitol and dialyzed overnight against the same buffer at 4°C with 6000-8000 Mr cut-off dialysis membranes. Of the lysoosphospholipase activity recovered from eluted slices of this gel, 85% was in a 6-mm region coincident with the stained protein band (Fig. 2).

Eosinophil lysoosphospholipase, purified to homogeneity as judged by presentation of a single stained protein band on alkaline disc gel electrophoresis in parallel and in concert with CLC protein. Each protein migrated with the same Rf and in combination they comigrated to give a single band (Fig. 3). The molecular weight of CLC (Fig. 4), using 10 µg of CLC for each of six determinations, was 17,200 ± 300 (SEM), which is the same as the molecular weight of human eosinophil lysoosphospholipase, 17,200 ± 300 (SEM) (n = 9).

A solution of 900 µg of purified eosinophil lysoosphospholipase in 500 µl of 0.1 M Tris-HCl, pH 7.5/2 mM EDTA/2 mM dithiothreitol, was made hypotonic by addition of 1 ml of 0.15% NaCl and concentrated at 4°C to original volume by negative-pressure ultrafiltration on a 10,000 Mr collodion bag. Crystals with a morphology identical to that of CLC formed within minutes of concentration (Fig. 5).
FIG. 2. Alkaline disc gel electrophoresis of solubilized CLC protein derived from human eosinophils. Protein was applied to parallel gels in 40-μg portions; one gel was stained with Coomassie brilliant blue and the other was sliced and eluted for assay of lysophospholipase activity. The anode was at the right and the dye front was marked by an ink stab.

DISCUSSION

The observation of prominent lysophospholipase activity associated with the eosinophil was made with rat leukocytes on the basis of the histochemical precipitation of (18) and the titration of (19) fatty acid liberated from the substrate lysocolithin. Human eosinophils manifest more lysophospholipase activity than other leukocytes in a sensitive and specific assay based on quantitating the release of radiolabeled fatty acid from [14C]palmitoyl lysophosphatidylcholine (14). Human eosinophils express 8-fold more lysophospholipase activity than do neutrophils and 3-fold more than mononuclear leukocytes (Fig. 1). Thus, in the human, and possibly in rats (19) and mice (20), the eosinophil is preferentially endowed with the enzymatic capability to deacylate lysophospholipids.

During the course of purifying lysophospholipase from the human eosinophil (14), the similarity of physicochemical properties between this enzyme and the protein composing CLC suggested that the two might be related. CLC prepared from human eosinophils by ultrasonic disruption of the cells in hypotonic saline were found to express prominent lysophospholipase activity. Alkaline disc gel electrophoresis of CLC protein yielded a single band of Coomassie brilliant blue staining protein that was coincident with lysophospholipase activity recovered from a parallel gel (Fig. 2), indicating that the activity was inherent to the CLC protein and not merely

FIG. 3. NaDODSO₄/polyacrylamide gel electrophoresis in 10% gels of 45 μg of purified eosinophil lysophospholipase (left), the combination of 15 μg of purified eosinophil lysophospholipase and 20 μg of eosinophil-derived CLC protein (center), and 40 μg of eosinophil-derived CLC protein (right). All samples were reduced and alkylated. The bromphenol blue dye front was marked with a stab of ink.

FIG. 4. NaDODSO₄/polyacrylamide gel electrophoresis in 10% gels of reduced and alkylated samples of CLC protein and standard proteins, including α-lactalbumin (LA), soybean trypsin inhibitor (TI), carbonic anhydrase (CA), and ovalbumin (OA). Relative migration values \( (R_p) \) are presented as a ratio of the distance migrated for each protein relative to the migration distance of bromphenol blue tracking dye.

FIG. 5. CLC prepared from human eosinophils disrupted by sonication in 0.15% NaCl (A) and from purified eosinophil lysophospholipase (B). (×1400.)
adsorbed to the crystal. Moreover, on electrophoresis under dissociating conditions, after reduction and alkylation in the presence of NaDodSO₄ detergent, CLC protein migrated as a single band with the same mobility as purified eosinophil lysophospholipase; indeed, the two proteins comigrated on a single gel (Fig. 3). Additional confirmation that eosinophil lysophospholipase was the sole protein constituent of CLC was obtained by the demonstration that purified enzyme in hypotonic buffer formed dipyramidal crystals morphologically identical to CLC (Fig. 5).

Chemical knowledge of CLC was limited until Gleich and his associates found CLC to consist of a 13,000 Mr, acidic protein whose molecular weight and amino acid composition distinguished it from the 9300 Mr, major basic protein, the principal constituent of eosinophil large granules (12, 21). In the present study, molecular weights of the CLC protein and the purified lysophospholipase are similar, being 17,400 and 17,300, respectively. The greater molecular weight of CLC in the present study as compared to the molecular weights in the literature (12, 21) is not due to aberrant migration of eosinophil lysophospholipase, because analysis of the electrophoretic mobility on NaDodSO₄/polyacrylamide gel electrophoresis performed at four different concentrations of acrylamide, as suggested by Ferguson (22), gave the same result. The introduction of additional molecular weight markers, the α and β chains of hemoglobin (about 15,000 Mr) (16), confirmed the larger size of CLC and lysophospholipase. Some (5, 23), but not all (24), studies of CLC have found increased quantities of zinc associated with CLC, and the sulfhydryl dependence of lysophospholipase (18) allows for the possible binding of zinc ions by sulfhydryl groups of the cysteine residues (12, 23). However, the purification of lysophospholipase in the presence of 2 mM EDTA and the ability of purified enzyme to form CLC in the presence of EDTA suggest that zinc is not an integral component of the crystals.

CLC persist in sputum, feces, and tissues of patients with eosinophil inflammatory processes and are resistant to proteolytic digestion by chymotrypsin, trypsin, and pepsin (25). The retention of active lysophospholipase activity by CLC derived from human eosinophils raises the possibility that, even after the disintegration of eosinophils in inflammatory foci, the resulting CLC that are formed may represent an enzymatically active legacy that continues to function to degrade lysophospholipids. Because lysophospholipids are generated in inflammatory foci (26, 27) and may have a range of concentration-dependent cytotoxic and noncytotoxic effects on diverse cells (28-32), CLC formation and persistence in human tissues and fluids may represent more than a curious crystalline artifact and may have biological function.

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