Brief Definitive Report

SPECIFIC PROTEASE DEFICIENCY IN POLYMORPHONUCLEAR LEUKOCYTES OF CHÉDIAK-HIGASHI SYNDROME AND BEIGE MICE*

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The Chédiak-Higashi syndrome (CHS) is a rare disorder, characterized by giant granules in most granule-containing cells. Its major clinical expression is a marked susceptibility to pyogenic infections which, in the absence of defects in humoral or cellular immunity, has directed attention to polymorphonuclear leukocytes (PMNs). The properties of CHS PMNs include abnormally large azurophile granules (1), somewhat reduced levels of β-glucuronidase and myeloperoxidase (35–50% of normal) (2), defects in chemotactic responses (3), and low bactericidal activity against some gram-positive microorganisms (4). Many of these defects, including those in PMNs, have also been found in animal models such as the beige mouse (bg/bg) (5), mink, cattle (6), cats (7), and a killer whale (8), but no specific molecular defect that could account for the enhanced sensitivity to bacterial infections has been identified.

We recently found that fibrinolysis by human PMNs is due to the combined effects of plasminogen activator and elastase (9). Since elastase is localized in azurophile granules (10), the abnormality of these organelles in CHS leukocytes prompted us to assay for this enzyme. The results show that leukocyte elastase is either very low or undetectable in CHS patients, and the corresponding murine protease is profoundly decreased in leukocytes of beige mice.

Materials and Methods

Materials. NCS mice were obtained from the stock maintained at The Rockefeller University; BALB/cJ and C57 BL/6J from The Jackson Laboratory, Bar Harbor, Maine; and C57 BL/6J bg/bg were the generous gift of Dr. J. Oliver at the University of Connecticut Health Center, Farmington, Conn. Plasmagel was obtained from Roger Bellon Laboratories, Paris, France. The sources of all other materials were as described previously (9).

Case Histories

The patients described below have been studied at the Hôpital des Enfants Malades, Paris, and were in the care of one of the authors (C. G.).

CHS CASE NO. 1. 5-yr-old female, with frequent *Staphylococcus aureus* infections. Leukocytes: 3,200 per mm^3; 50% neutrophils, 99% with abnormal granules; bactericidal activity (11) decreased against *S. aureus*, normal against *Serratia marcescens*. Not in accelerated phase of the disease, no treatment.

CHS CASE NO. 2. 4-yr-old female, rare *S. aureus* infections. Leukocytes: 4,200 per mm^3; 55%

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neutrophils, 66% with abnormal granules; bactericidal activity decreased against *S. aureus* and *Escherichia coli*. Not in accelerated phase, no treatment.

CHS CASE NO. 3. 5-yr-old male, frequent *Neisseria* and *Candida albicans* infections. Leukocytes: 3,300 per mm^3, 55% neutrophils, 100% abnormal granules; bactericidal activity decreased against *S. aureus*, normal against *S. marcescens*. Not in accelerated phase of the disease, no treatment.

Preparation of Cells

HUMAN. Human leukocytes were prepared by dextran or plasmagel sedimentation (1 h, 37°C) of heparinized (2 U/ml) venous blood. The plasma containing leukocytes was collected, the cells were washed twice by centrifugation (300 *g*, 5 min) and resuspended in phosphate-buffered saline (PBS). Cells were counted in Türk's solution, and also stained with Giemsa for differential counting. After final centrifugation, the pellet was frozen, stored at −80°C, and transported in dry ice. The cells were thawed and resuspended in PBS containing 0.5% Triton X-100, at 1.5×10^7 PMNs/ml, the lysate was vortexed, centrifuged (1,000 *g*, 10 min), and the supernate was assayed for fibrinolytic activity.

MOUSE. Peritoneal cells were collected by lavage with PBS 16 h after intraperitoneal injection of 30-μg endotoxin (S. Minnesota LPS MR 595-S391) in 1 ml PBS. After washing in PBS, the cells were stained and counted. The yield for all strains tested was $6–10 \times 10^6$ cells per mouse; 80–85% were PMNs, 10% were macrophages, and 5–10% were lymphocytes. The cells (10^6/ml) were lysed in PBS containing 0.25% Triton X-100, and the lysate was assayed directly, or after dilution in PBS. Alternatively, cells (10^7/ml) were lysed in PBS-0.25% Triton X-100, the lysate was centrifuged (1,000 *g*, 10 min), and the activity of the supernate was analyzed by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) (9).

Assay for Fibrinolytic Activity. ^{125}I-fibrin-coated wells of Linbro plates (Linbro Scientific Inc., Hamden, Conn.) were prepared as described elsewhere (9). Each well contained ≅20 μg ^{125}I-fibrin (5,000 cpm/μg). The fibrinolytic activity of mouse cells was measured by adding 1 ml of lysates into ^{125}I-fibrin coated wells and measuring the percentage of total radioactivity solubilized during 2 h of incubation at 37°C.

Elastase activity was determined as follows: aliquots (5–50 μl) of human leukocyte lysate or purified porcine pancreatic elastase (0.2 U/ml; 72.7 U/mg, Worthington Biochemical Corp., Freehold, N. J.) were added to ^{125}I-fibrin-coated wells containing 250 μl 0.1 M Tris-Cl, pH 8.1; the rate of fibrinolysis was determined by measuring the radioactivity solubilized at 37°C as a function of time. Under these conditions, the rate of fibrinolysis was linear for up to 2 h, but was not quite linear with concentration of pure elastase or leukocyte extracts. We have therefore used the 2-h values for comparing different samples, and have related these to a standard curve obtained by measuring the activity of known amounts of pure elastase under identical conditions. We note that the specific enzymatic activity of leukocyte preparations may vary, depending upon the purity of PMNs and erythrocyte contamination, and this accounts for the difference in specific activity of PMNs from normal subjects in the present work, compared with that reported previously (9). Finally, crude leukocyte lysates inhibit somewhat the activity of pure elastase, so that the addition of pure enzyme to cell extracts yields values that are not fully additive; the shortfall being ≅30%. We do not know whether this is due to inactivating enzymes, to specific elastase inhibitors, or to substrate competition between leukocyte protein and ^{125}I-fibrin.

Results

Human PMNs contain several proteases that are catalytically active at neutral pH, including collagenase (12), plasminogen activator (9), a chymotrypsin-like enzyme (13), and elastase (14). When human PMNs are lysed and assayed with ^{125}I-fibrin as substrate in the absence of plasminogen, the main source of proteolysis is elastase. In fact, the plasminogen-independent fibrinolytic activity of human leukocyte lysates is a measure of their elastase (9), as suggested by the following evidence: (*a*) the activity is resistant to trypsin and chymotrypsin inhibitors and it is inhibited more than 85% by *N*-acetyl-$(Ala)_4$-chloromethylketone (5×10^{-3} M), a specific inhibitor of elastase; (*b*) the activity migrates in SDS-PAGE as three bands with apparent mol wt in the range

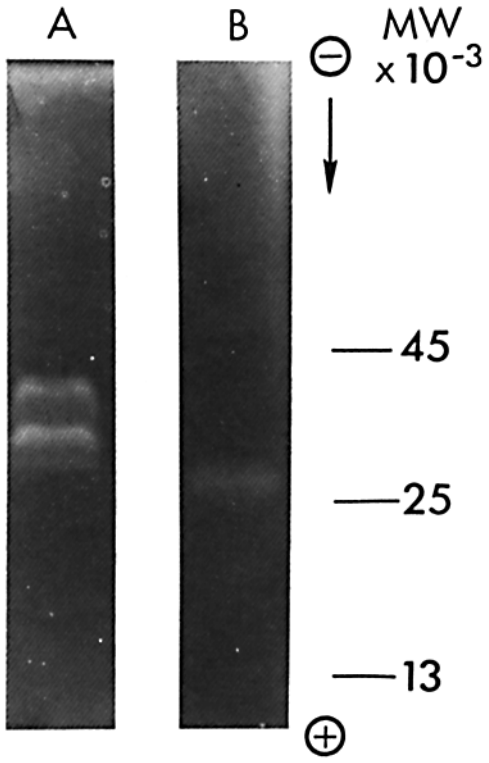

FIG. 1. Identification of proteases after SDS-PAGE of lysates of: (A) human blood leukocytes, and (B) mouse peritoneal exudate leukocytes. Lysates were prepared as described in Materials and Methods, electrophoresed in SDS-11% polyacrylamide slab gels, and the gels were processed for detection of fibrinolytic activity using a fibrin-agar gel (9), in the absence of plasminogen. The figure is a photograph of the amido-black-stained fibrin-agar layer; the clear areas are due to lysis of fibrin in the agar gel by proteases diffusing out of the polyacrylamide gels. Parallel lanes containing lysates of CHS blood leukocytes or beige mouse peritoneal exudate leukocytes did not show any zones of lysis.

28,000–35,000, corresponding to those reported for purified human leukocyte elastase (14); (*c*) when assayed using fibrin-agar gels (9), elastase is the major neutral protease detected in lysates of unfractionated leukocytes from blood (Fig. 1 A).

Murine leukocyte lysates also contain a single major neutral protease when assayed on fibrin-agar gels (Fig. 1 B). In mol wt ≅27,000 this enzyme is close to human elastase, and, like elastase, it is a serine protease inhibited 92% by di-isopropyl-fluorophosphate (10^{-4} M) and 87% by soybean trypsin inhibitor (100 μg/ml). However, unlike elastase, it is resistant to *N*-acetyl-$(Ala)_4$-chloromethylketone; it is also resistant to the trypsin inhibitors *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (10^{-3} M) and nitrophenyl-*p*-guanidinobenzoate (10^{-4} M). The chymotrypsin inhibitors L-1-tosylamide-2-phenylethyl-chloromethyl ketone (10^{-3} M) and *N*-carbobenzoxy-L-phenylalanine chloromethyl ketone (10^{-3} M) reduced this activity by somewhat less than 50%. Thus, this enzyme is a serine protease of the pancreatic type, but its precise substrate specificity, while in part chymotryptic, remains incompletely defined.

We have tested lysates of buffy coats from CHS patients, and of peritoneal

TABLE I
Elastase in Lysates of Human Leukocytes

CHS case no.	Elastase (units per 10^6 PMNs × 10^3)
1	<0.1
2	0.2
3	<0.1
Normal (range)	3.2 (1.5–5.6)

Human leukocytes were prepared, lysed, and assayed for fibrinolytic activity, and the values obtained were related to standard preparations of purified elastase, as described in Methods. The elastase content of normal leukocytes was determined by assaying cells from six control subjects (two adults and four children).

exudates from beige mice both by the gel method (Fig. 1), and by measuring the solubilization of ^{125}I-fibrin. In the gel system elastase was undetectable, and no other plasminogen-independent activity was present in human CHS lysates; likewise, the corresponding murine protease (of Fig. 1 B) was absent in lysates of beige mouse peritoneal leukocytes. When assayed with ^{125}I-fibrin as substrate, plasminogen-independent fibrinolysis was undetectable in lysates from two CHS patients, whereas the third patient, clinically the least affected, contained less than 10% of the normal level (Table I). Comparable results for mice are presented in Table II, and these show that lysates from beige mice contain at most 3% of the neutral protease activity found in control strains.

It is unlikely that the reduced activity in either of the affected cell extracts is due to excess inhibitors. Mixtures of inactive and active extracts gave some reduction of the expected level, but this was slight (35%). More importantly, however, we have found that all catalytically inactive complexes formed between proteases and macromolecular inhibitors so far tested are dissociated during SDS-PAGE, allowing the protease to be visualized by the gel method of Fig. 1 (A. Granelli-Piperno and E. Reich, unpublished observations). Were an inactive enzyme-inhibitor complex the explanation for our findings, some active bands should have been detected in the gels. Although we cannot rigorously exclude excess inhibitor as the basis of our observations, such a mechanism is not the most probable one.

Discussion

Despite the limitations of the procedures noted in Materials and Methods, our results leave little doubt that CHS leukocytes are profoundly deficient in elastase, and that an analogous deficiency occurs in beige mice. Moreover, since elastase is localized in azurophile granules, our findings provide a satisfying correlate for the morphological abnormality of these granules both in CHS and in beige mice. While no cause and effect relationships between the enzymatic and morphological changes can be deduced at present, it will be of interest to determine whether or not specific enzyme deficiencies are associated with the granule alterations in other tissues. Since the enzyme deficiency we have observed could arise from failure to activate proenzymes, or from irreversible enzyme inactivation during granule formation, it would also be useful to assay CHS and beige mouse leukocytes for substances that might be immunologically cross-reactive with their respective missing enzymes.

Of the three CHS patients examined, two (case nos. 1 and 3) were severely

TABLE II
Proteolytic Activity of Lysates of Mouse Peritoneal Cells

Strain	Substrate solubilized by lysates from:	
	3×10^4 Cells	1×10^6 Cells
	%	
BALB/cJ	4.7	37
NCS	1.9	31
C57 BL/6J, +/+	2.4	33
C57 BL/6J, bg/bg	0.1	2.3

Peritoneal cells from endotoxin-injected mice were lysed and assayed for fibrinolytic activity as described in Materials and Methods.

affected and experienced numerous infections; all of their PMNs had abnormal granules, and their leukocyte elastase was undetectable. A third patient (case no. 2) had a clinically milder form of the disease; abnormal granules were present only in a fraction of her PMNs, and her level of elastase was very low, but detectable. Hence, severity of disease, proportion of morphologically affected PMNs, and levels of leukocyte elastase appear to be correlated; it will be useful to test this correlation in a larger series of CHS patients.

The increased susceptibility of CHS patients to infections has been linked to the impairment of leukocyte function, and the reported defects in bactericidal capacity of PMNs from CHS patients (4) and beige mice (5) might be significant pathogenetic factors. Since normal phagocytic rate and capacity of PMNs are retained in these disorders (15, 5), the reduced bactericidal activity is more likely due to a defective intracellular killing mechanism. The possibility that elastase deficiency might introduce a rate limitation in the killing of some bacteria is attractive, because the peptidoglycan structure of organisms such as *S. aureus* contains an elastase-sensitive bond (16), and because this enzyme can lyse the cell walls of autoclaved *S. aureus* (16), a pathogen that CHS PMNs kill inefficiently (4), and to which these patients are specially susceptible (17). While not by itself bactericidal in vitro (16), elastase potentiates the activity of granulocyte microbicidal systems against *E. coli* and *S. aureus* (18), and participates in the digestion of *E. coli* proteins in human PMNs (19); elastase might therefore be essential for normal rates of intracellular killing.

Summary

Peripheral blood leukocytes of three patients with Chédiak-Higashi syndrome (CHS) contained very low or undetectable levels of elastase, the major neutral protease in these cells. Likewise, peritoneal exudate leukocytes of beige mice (the murine counterpart of CHS) contained correspondingly reduced levels of their major neutral protease, a serine enzyme of mol wt 27,000. The elastase deficiency in CHS polymorphonuclear leukocytes might account in part for the high incidence of infections in these patients.

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