IN VITRO STIMULATION OF LYMPHOCYTES BY NEUTRAL PROTEINASES FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTE GRANULES

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Human polymorphonuclear leukocytes (PMN)[1] contain considerable amounts of neutral proteinases in their azurophil granules (1). Upon phagocytosis, these enzymes are released from the granules into phagocytic vacuoles and into the extracellular medium (2) where they may damage a large variety of tissue components (3).

Neutral proteinases such as trypsin, chymotrypsin, Pronase, and pancreatic elastase are known to induce lymphocyte transformation and proliferation (4–7). In chronic inflammatory processes, proteinases which are released from PMN may act on resident lymphocytes and modulate their behavior. In the present work, we have investigated the effects of purified elastase and cathepsin G[2] from human PMN on lymphocytes of different sources.

Materials and Methods

Isolation of Neutral Proteinases from Human PMN. PMN from pooled buffy coats of donor blood were purified to about 90%, as estimated by differential counts on Giemsa-stained smears. The cells were homogenized in 0.34 M sucrose (10), and the granules collected by centrifuging the postnuclear supernate at 3×10^6 *g*-min. The granules were disrupted and extracted in 0.2 M sodium acetate buffer pH 4.5 at 4°C. The solubilized material, which accounted on average for 35% of the granule protein and 70–90% of the total neutral proteolytic activity, was collected as a supernate after centrifugation at 3×10^6 *g*-min.

PMN elastase and cathepsin G, both of which are constituents of the azurophil granules (1), were separated by chromatography on ϵ-aminocaproic acid Sepharose. PMN elastase was further purified by gel filtration on Sephadex G-75, and cathepsin G by preparative polyacrylamide gel electrophoresis at acid pH. The purity of the fractions was assessed by analytical polyacrylamide gel electrophoresis according to Dewald et al. (1), except that no detergent was used. The complete procedure is described in detail in a separate paper.[3]

Biochemical Assays. Neutral proteolytic activity was determined by measuring the hydrolysis of histone and casein as described previously (1). Elastase-like activity was determined by

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[1] *Abbreviations used in this paper:* AS-D acetate, 2-(acetyl)-3-naphtoic acid *o*-toluidide; GGPNA, *N*-glutaryl-glycyl-glycyl-L-phenylalanine β-naphthylamide; LPS, bacterial lipopolysaccharide; NBA, *N*-tert-butoxycarbonyl-L-alanine *p*-nitrophenyl ester; PHA, phytohemagglutinin; PMN, polymorphonuclear leukocyte(s); PWM, pokeweed mitogen; SI, stimulation index.

[2] The name recommended for the chymotrypsin-like neutral proteinase from human PMN granules by A. J. Barrett (8, 9).

[3] Bretz et al., manuscript in preparation.

measuring either the rate of liberation of p-nitrophenol from N-tert-butoxycarbonyl-L-alanine p-nitrophenyl ester (NBA) (1) or the rate of hydrolysis of elastin. In the latter case, the following conditions were used: assay mixtures contained 0.5% (wt/vol) of elastin, the enzyme, and 0.05 M phosphate buffer pH 7.5, in a total volume of 0.4 ml. After incubation at 37°C for 60 min, the reaction was stopped by the addition of 0.1 ml of cold 25% TCA. TCA-soluble peptides were estimated as previously described (1). Chymotrypsin-like activity was determined by measuring β-naphthylamine liberated from N-glutaryl-glycyl-glycyl-L-phenylalanine β-naphthylamide (GGPNA), according to the method of Rinderknecht and Fleming (11). To 0.4 ml of 0.1 M Tris-maleate buffer pH 7.5, containing 2.5 mM $CaCl_2$ were added 2 μl of GGPNA (70 mg/ml or 6.7×10^{-7} M final concentration) in dimethylformamide and 20 μl of enzyme solution. Incubation was carried out at 37°C for 1–4 h. After stopping the reaction with 2.0 ml of 50 mM glycine-NaOH buffer pH 10.4, containing 5 mM EDTA, the fluorescence of β-naphthylamine was measured at 415 nm (excitation 335 nm) in a Hitachi-203 fluorimeter (Perkin-Elmer Corp. Instrument Div., Norwalk, Conn.). Standard assays contained 0.4 nmol of β-naphthylamine. Protein was determined according to Miller (12), using bovine serum albumin as a standard.

Lymphocyte Cultures. Human mononuclear cells were obtained from fresh heparinized blood by centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden and Winthrop Laboratories, Div. of Sterling Drug Inc., New York) (13). The cells (0.5×10^6) were cultured in the absence of serum. To RPMI 1640 medium were added 28 mM Hepes, antibiotics, and essential and nonessential amino acids and vitamins at the concentrations given by Eagle (14) for enrichment of his minimum essential medium. Balb/c/M mice were obtained from the Institut für biologisch-medizinische Forschung AG (Füllinsdorf, Switzerland), and homozygous nude mice (Balb/c/A/Bom/nu/nu) from Dr. Frijs, Bomholtgard (Ry, Denmark). Spleen and thymus cells were prepared from 8- to 12-wk-old male mice, and cultured at the concentration of 5×10^6 cells/ml in serum-free RPMI 1640 medium, as described previously (5). Animals treated with hydrocortisone received an intraperitoneal dose of 125 mg/kg 24 h before killing. Cultures were set up in 12 x 75-mm polystyrene tubes (Falcon Plastics, Inc., Oxnard, Calif.).

The following stimulants were used: bacterial lipopolysaccharide (LPS) from *Salmonella typhosa*, phytohemagglutinin P (PHA), pokeweed mitogen (PWM), trypsin from pig pancreas (3× crystallized, 4,500 NF U/mg), chymotrypsin from beef pancreas (3× crystallized, 1,200 NF U/mg), and elastase from pig pancreas (2× crystallized, 500 NF U/mg). These agents were added to the cultures in amounts which were found to be suitable on the basis of dose-response curves. PHA and PWM were dissolved according to the manufacturers' recommendations. In the case of PHA, this stock solution was diluted 10 times. Aprotinin (Trasylol) and soybean trypsin inhibitor were used as proteinase inhibitors.

For the measurement of thymidine incorporation, 1 μCi of [^{3}H]thymidine (5 μCi/mmol) was added to each culture 16 h before completion of the experiment. The cells were collected on filters and their radioactivity measured by scintillation counting as previously described (15). The results of these experiments are given as the mean counts per minute values of triplicate cultures. The stimulation index (SI) is the ratio between the average counts per minute values of stimulated and control cultures.

Immunofluorescence. Surface immunoglobulins were visualized on transformed cells from 48-h cultures by staining in suspension with a fluorescein-conjugated rabbit-antimouse immunoglobulin antibody, as described previously (16). This direct immunoglobulin detection assay was shown to be negative with transformed thymic cells and with macrophages even in the presence of B-cell culture supernates (16). Intracellular immunoglobulins were detected on methanol-fixed smears obtained by means of a cytocentrifuge from 96-h cultures, using the above antibody (15).

Reagents used in the present work were obtained from the following sources: Bovine serum albumin (BSA), from Armour Pharmaceutical Co. (Phoenix, Ariz.); N-glutaryl-glycyl-glycyl-L-phenylalanine β-naphthylamide (GGPNA), from Bachem AG (Liestal, Switzerland); Aprotinin (Trasylol; Delbay Pharmaceuticals, Inc., Kenilworth, N. J.), from Bayer AG (Leverkusen, GFR); bacterial lipopolysaccharide from *Salmonella typhosa* (LPS), and phytohemagglutinin P (PHA), from Difco Laboratories (Detroit, Mich.); β-naphthylamine, from Fluka AG (Buchs, Switzerland); antimycotic antibiotic mixture and pokeweed mitogen (PWM), from Grand Island Biological Co. (Grand Island, N. Y.); casein and hydrocortisone acetate, from Merck AG (Darmstadt, GFR); RPMI 1640 medium and mixtures of amino acids and vitamins (Eagle) from Microbiological Associates (Bethesda, Md.); trypsin, chymotrypsin, and elastase from pancreas, from Miles

TABLE I
*Specific Activities of Neutral Proteinases**

Enzyme	Substrate				
	Elastin	Casein	Histone	NBA	GGPNA
PMN elastase	0.236	2.10	16.26	9.76	0
Cathepsin G	<0.010	1.11	0.84	0.66	0.074
Elastase‡	0.208	4.88	2.46	3.63	0
Chymotrypsin‡	0.020	8.86	3.95	0.96	0.097
Trypsin‡	0.032	8.90	10.20	0.15	<0.001

* Mean values from two to six determinations, in units per milligram of protein. One unit of activity is defined as the amount of enzyme that liberates 1 μmol of product/min, i.e., 1 μmol of *p*-nitrophenol from NBA, 1 μmol of β-naphthylamine from GGPNA, and TCA soluble peptides equivalent to 1 μmol of leucyl-leucine from casein, histone, and elastin.

‡ Elastase and trypsin from porcine, chymotrypsin from bovine pancreas.

Laboratories (Holyport, Berks, U. K.); [^{3}H]thymidine, 5 Ci/mmol, from Radiochemical Centre (Amersham, U. K.); histone, elastin, and *N*-tert-butoxycarbonyl-L-alanine *p*-nitrophenyl ester (NBA), from Sigma Chemical Company (St. Louis, Mo.); soybean trypsin inhibitor, from Worthington Biochemical Corp. (Freehold, N. J.).

Results

The specific activities of purified elastase and cathepsin G from azurophil granules of human PMN are given in Table I, together with the values obtained with pancreatic proteinases. In addition to casein, elastin, and histone, the synthetic substrates NBA and GGPNA which are specific for elastase (17) and chymotrypsin (11), respectively, were used. The data underline the chymotrypsin-like properties of cathepsin G and the similar substrate specificity of PMN and pancreas elastase. Electrophoretic analysis in polyacrylamide gels (Fig. 1) reveals for both enzymes a characteristic pattern of bands which is already visible in the crude granule extract and which remains unchanged in the course of purification. All protein bands which are detectable on electropherograms of the purified enzyme preparations show hydrolytic activity on AS-D acetate which is a general substrate of PMN proteinases (1).

Giemsa-stained cytocentrifuge smears of lymphocyte cultures which were exposed to the neutral PMN proteinases show great numbers of blast cells[4] and frequent mitotic figures. By light microscopy, these blast cells appear identical to LPS-transformed lymphocytes.

The lymphocyte-stimulating effect of PMN proteinases was then explored by measuring the extent of [^{3}H]thymidine incorporation after the addition of increasing amounts of enzyme preparations to the cultures. Fig. 2 shows the dose-response curves obtained with mouse spleen cells. Maximum stimulation was obtained with 20–40 μg of enzyme protein per milliliter of culture. A similar dose-response curve has been obtained previously with trypsin (5). The stimulatory effects of PMN elastase, cathepsin G, trypsin, and PHA on both human and mouse lymphocytes are shown in Table II. Mouse spleen cells seem to respond

[4] See Table V.

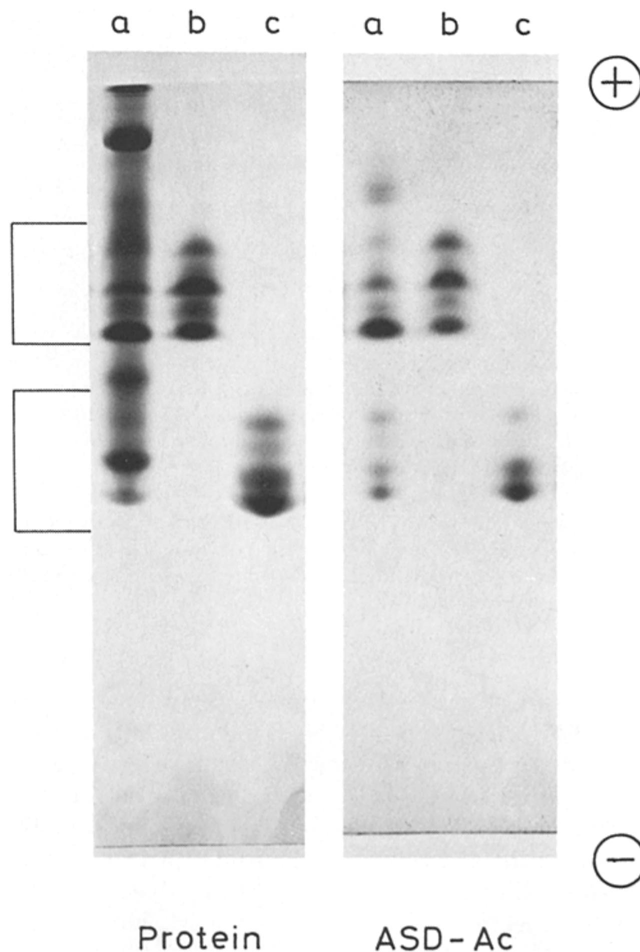

FIG. 1. Polyacrylamide gel electrophoresis of purified neutral proteinases. One part of the gel was stained for protein with Coomassie Brillant Blue, the other part for esterolytic activity of proteinases with AS-D acetate as substrate (1). The electropherograms (*b*) and (*c*) represent purified PMN elastase, and cathepsin G, respectively, while (*a*) is a total granule extract run as a reference. Electrophoresis was carried out as described previously (1). The gel was 20% acrylamide, pH 4.3. The samples contained 0.2 M acetate buffer pH 4.5 and 10% glycerol.

better to proteinase stimulation than do human peripheral blood lymphocytes. This difference, which is characteristic for B-cell mitogens (18), is more pronounced with trypsin than with the PMN proteinases. As compared to the latter, trypsin appears to be a stronger lymphocyte stimulator in both species tested. However, these differences in stimulatory potency do not correlate with the hydrolytic activities shown in Table I.

Table III indicates that the proteinases must be enzymatically active in order to display their stimulatory effect on lymphocytes. Two proteinase inhibitors, Aprotinin and soybean trypsin inhibitor, were found to block the stimulatory action of cathepsin G, trypsin, and chymotrypsin, but these inhibitors had little

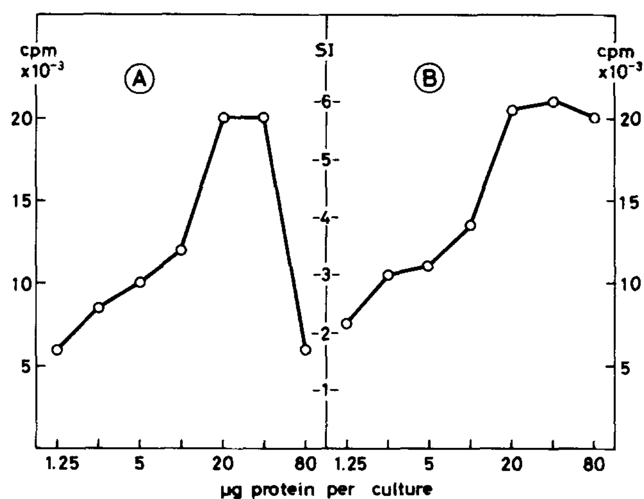

FIG. 2. Increasing amounts of PMN elastase (A), or cathepsin G (B) were added to 1 ml cultures containing 5×10^6 spleen cells. Tritiated thymidine (1 μCi) was added 16 h before completion of the experiments, and its incorporation was measured by scintillation counting.

TABLE II
*Stimulation of [^{3}H]Thymidine Incorporation by Neutral Proteinases**

Stimulant	Mouse spleen cells		Human peripheral blood lymphocytes	
	cpm	SI‡	cpm	SI‡
None	3,454	–	610	–
PMN elastase (20 μg)	19,978	5.8	2,427	4.0
Cathepsin G (10 μg)	20,492	5.9	2,710	4.4
Trypsin (2.5 μg)	58,773	17.0	1,997	3.3
PHA (5 μl)	150,372	43.2	41,947	68.8

* 5×10^6 Balb/c spleen cells, or 0.5×10^6 human peipheral blood lymphocytes were cultured for 72 h with or without stimulant in 1 ml of serum-free medium. Tritiated thymidine was added 16 h before completion of the experiments.

‡ Stimulation index (SI) is the ratio between the average counts per minute values of stimulated and control cultures.

if any effect on the stimulation induced by either PMN or pancreas elastase. More potent proteinase inhibitors, such as phenylmethylsulfonyl fluoride and chloromethyl ketones, could not be tested because of their cytotoxicity. Mixtures of enzymes and inhibitors corresponding to those used in the lymphocyte cultures were also tested for casein hydrolysis. Inhibition was found to be dependent on the time of preincubation of enzyme and inhibitor. After a preincubation which was sufficiently long to completely block the action of trypsin, both cathepsin G and chymotrypsin were strongly inhibited, while the elastases were only slightly affected. This is in accord with the inhibition of effects seen in culture.

As shown in Table IV, PMN elastase and cathepsin G stimulate spleen cells

TABLE III
*Effects of Inhibitors on Lymphocyte Stimulation by Neutral Proteinases**

Proteinases	None		Inhibitors: Aprotinin (100 IU)		Inhibitors: Soybean trypsin inhibitor (0.4 mg)	
	cpm	SI	cpm	SI	cpm	SI
None	2,134	–	1,783	–	2,086	–
PMN elastase (20 μg)	12,255	5.7	8,840	5.0	8,713	4.2
Pancreatic elastase (5 μg)	7,879	3.7	6,756	3.7	6,059	2.9
Cathepsin G (20 μg)	9,253	4.3	2,499	1.4	3,027	1.4
Trypsin (5 μg)	38,070	17.8	1,661	0.9	2,824	1.3
Chymotrypsin (2.5 μg)	15,840	7.4	2,367	1.3	2,742	1.3

* Proteinases and inhibitors were present in the serum-free cultures of 5×10^6 Balb/c spleen cells for 72 h. Tritiated thymidine was added 16 h before completion of the experiments.

TABLE IV
*Stimulation of nu/nu Spleen Cells by Neutral Proteinases**

Stimulant	[^{3}H]Thymidine incorporation: cpm	SI
None	3,400	–
PMN elastase (22 μg)	13,246	3.9
Cathepsin G (13 μg)	16,691	4.9
Trypsin (5 μg)	36,393	10.7
PHA (5 μl)	1,638	0.5

* Spleen cells from homozygous nude mice (5×10^6 cells in 1 ml) were cultured for 72 h in serum-free medium with and without stimulants. Tritiated thymidine was added 16 h before completion of the experiments.

from homozygous nude mice as effectively as those from normal mice. This indicates that the proteinases are B-cell stimulants and that the presence of functional T cells is not required for their stimulatory action. T lymphocytes themselves do not appear to respond to proteinases. Thymic cells from both normal and hydrocortisone-treated mice, which were strongly stimulated by PHA, failed to show any increase in [^{3}H]thymidine incorporation when exposed to various amounts of PMN elastase, cathepsin G, and trypsin. Further evidence for the B-cell specificity of the proteinase action is provided by our immunofluorescence studies. As shown in Table V, surface immunoglobulins are found on about 80% of the blast cells in 48-h cultures. At 96 h, high numbers of cells appear to be engaged in antibody production, as suggested by the presence of intracellular immunoglobulins.

Discussion

The results presented demonstrate that the two main neutral proteinases of human PMN stimulate human and mouse lymphocytes. These conclusions are in accord with the results of Tchorzewski et al. (19) who observed lymphocyte

TABLE V
*Occurrence of Surface and Intracellular Immunoglobulins in Cultured Mouse Spleen Cells**

Stimulant	48-h cultures		96-h cultures
	Transformed cells (% of total cells)	Cells with surface immunoglobulins‡ (% of transformed cells)	Cells with intracellular immunoglobulins§ (% of viable cells)
None	2.1±1.1	51.0±7.4	5.2±2.8
PMN elastase (22 μg)	17.6±6.2	77.5±13.3	28.3±7.7
Cathepsin G (13 μg)	19.3±5.5	75.9±11.4	22.2±5.7
Trypsin (5 μg)	21.5±5.5	80.5±9.5	25.7±8.5
PWM (5 μl)	12.3±1.9	46.1±6.4	10.5±7.8
LPS (40 μg)	21.0±4.8	78.6±11.5	34.3±7.9

* Values are mean ±SD from three to six different experiments.
‡ The cells were washed and stained at 4°C with a fluorescein-conjugated antibody to mouse immunoglobulin.
§ The cells were washed, and smears prepared in a cytocentrifuge. The smears were fixed with methanol and stained with the above antibody. Numbers of viable cells were similar in all samples tested.

stimulation by PMN extracts, and with the work of Havemann and Schmidt[5] demonstrating that purified leukocyte extracts potentiate the PHA-induced stimulation of lymphocytes.

As shown by the experiments with mouse cells, both PMN elastase and cathepsin G appear to be B-cell mitogens. They induce the transformation of spleen cells from both normal and congenitally athymic mice into antibody-synthesizing cells, and they seem not to require the help of T cells for this action. With respect to these properties, PMN proteinases closely resemble trypsin (5). More extensive studies have shown that trypsin, like the classic B-cell mitogen LPS (20), potentiates in vitro antibody production, and substitutes for T-helper cells (21) in the Mishell-Dutton system (22). It is, therefore, reasonable to assume that PMN proteinases may have similar adjuvant effects when released at the sites of chronic inflammation.

The mechanism of the lymphocyte-stimulating action of neutral proteinases is not known. Our data, however, strongly suggest that the stimulatory effect is dependent on the proteolytic activity of the enzymes. Preliminary experiments which were carried out with enzyme preparations at various stages of purification showed that the stimulatory effect increased with the degree of enzyme purity. Furthermore, proteinase inhibitors were found to lower the stimulatory action in accordance with their inhibitory potency toward the proteolytic activity of the respective enzymes. A strict correlation between stimulatory effect and proteolytic activity cannot be established since the substrate involved in the

[5] Schmidt, W., U. Bogdahn, and K. Havemann. 1975. Mechanism of release of granulocytic neutral proteases and their effect on lymphocyte activation and migration inhibition. Reported at the 6th Workshop on Leukocyte Cultures. Basle, Switzerland.

lymphocyte activation process is not known. As serum is not required for stimulation, the substrate involved is very likely to be a component of the lymphocyte membrane. Lymphocytes are stimulated by an impressive variety of neutral proteinases. These enzymes cannot all be expected to hydrolyze the same kind of peptide bonds. It appears, therefore, that stimulation may be initiated by any proteolytic cleavage of a given surface substrate or, alternatively, be induced by a nonspecific degradation of exposed membrane proteins. Presumably, any neutral proteinase which is released at a site of inflammation could modulate lymphocyte function. Neutral proteinases are not only released from PMN during phagocytosis but are also secreted by activated macrophages (23–25) and possibly by lymphocytes themselves as lymphokines (26).

Our data suggest that PMN proteinases, as well as similar enzymes from other inflammatory cells, may have more complex effects than tissue destruction (3) and complement activation (27, 28). Brinolase, a proteolytic enzyme preparation, was found to restore tuberculin reactivity in anergic cancer patients (29). This, together with the in vitro effects on lymphocytes described here, would seem to support the view that proteinases may act as immunostimulants. Such an hypothesis, however, requires adequate support from animal experiments.

Summary

Two neutral proteinases from human polymorphonuclear leukocytes (PMN), an elastase and the chymotrypsin-like cathepsin G, were purified, and their actions on lymphocytes in culture were studied.

Both PMN proteinases stimulate lymphocytes from human peripheral blood and from mouse spleen in vitro, but do not affect thymic cells from either normal or hydrocortisone-treated mice. In stimulated mouse spleen cell cultures, most of the developing blast cells bear surface immunoglobulins, and subsequently appear to engage in antibody synthesis. In their stimulatory action, the two PMN proteinases thus resemble the classic B-cell mitogen LPS and neutral pancreatic proteinases such as trypsin, chymotrypsin, and elastase. The effects of proteinase inhibitors indicate that lymphocyte stimulation is dependent on the proteolytic activity of the enzymes.

This work suggests that PMN proteinases, which are released at sites of inflammation, may modulate the function of lymphocytes.

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