Inhibition of the Elastase-Like Esterase in Human Leukocyte Granules by Human Leukocyte Cell Sap[1] (35425)

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Human leukocyte granules contain marked proteolytic activity at neutral pH (1). Despite this, the granule extract shows little or no affinity for synthetic substrates of trypsin or chymotrypsin (1), but does show considerable affinity for alanine esters (2, 3). The latter are considered to be specific substrates for elastases (4, 5), and the extracts degrade elastin itself (6, 7). Thus, an elastase-like esteroprotease appears to be present in human leukocyte granules. This elastase-like enzyme (7, 8) and a specific collagenase of human granulocytes (9) have recently been suggested as possible mediators of connective tissue damage in man. Inhibitors of the leukocyte enzymes might therefore prove useful in the management of acute inflammatory conditions.

An inhibitor has recently been described (10) in the postgranule supernatant fraction of rabbit leukocyte homogenates which suppresses one of the neutral proteases found in rabbit leukocyte lysosomes. We therefore tested human leukocyte cytosol fractions for inhibitory activity against the elastase-like esterase found in the granules of these cells. Detection of esterase-inhibition and preliminary characterization of the responsible agent are the subjects of the present report.

Materials and Methods. Enzymes and substrates. Enzymes used were pancreatic elastase (EC 3.4.4.7), trypsin (EC 3.4.4.4), chymotrypsin (EC 3.4.4.5) (Worthington Biochemical Corp. Freehold, N.J.), and pronase (Calbiochem, Los Angeles, Calif.). Substrates used were tertiary butyloxycarbonyl (*t*-BOC)-*l*-alanine *p*-nitrophenol (Cyclo Chemical Corp., Los Angeles, Calif.), benzoyl-*dl*-arginine-*p*-nitroanalide (BAPA), and glutaryl-*l*-phenylalanine-*p*-nitroanalide (GPA NA) (Mann Research Labs., New York, N.Y.). Yeast RNA was purchased from Worthington Biochemical Corp.

Leukocyte fractions. Granule extracts from human peripheral blood leukocytes were prepared as before (6). Crude supernatant fractions from the same cells (referred to hereafter as cytosol) were prepared as follows. Following sedimentation of granules at 17,000*g* for 30 min, the leukocyte homogenates (in 0.34 *M* sucrose) were diluted to a final sucrose concentration of 0.28–0.30 *M* and then centrifuged at 100,000*g* for 90 min. The water-clear, high-speed supernatants were next dialyzed for 24 hr against two changes of isotonic saline buffered with 0.01 *M* sodium phosphate to pH 7.0, and the dialyzed material was stored at —60° until used. Heparin was never added at any point in the preparation of the leukocyte fractions.

Assays. Protein was measured, according to Lowry *et al.* (11), using crystallized bovine serum albumin as reference standard (Pentex Inc., Kankakee, Ill.). DNA was determined by Burton's method (12) with calf thymus DNA (Calbiochem) as reference. Total nucleic acids were estimated from absorbance values at 260 and 280 nm using a nomograph derived from Warburg and Christian (13). Determination of hexuronic acid was carried out according to Dische (14) with glucuronyl lactone as reference. Immunodiffusion experiments were performed using Immuno-Plates-Pattern D (Hyland Div. of Travenol Labs. Inc., Los Angeles, Calif.). Standardized human serum was obtained from the Behring

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TABLE I. Inhibition of Esterase Activity by Leukocyte Cytosol.

Cytosol protein (μg)	Inhibition (%) of: Human leukocyte granule protein[a] (25 μg)	Pancreatic elastase[a] (10 μg)	Trypsin[b] (5 μg)	Chymotrypsin[c] (12 μg)
A. Human cytosol				
50	11	0	0	0
100	25	0	0	0
200	53	0	0	0
300	64			
400	70	0		
500	72			
600	72	0		
1000			0	
B. Rabbit cytosol	Human granules[a] (25 μg)			
230	60	—	—	—
460	71	—	—	—

[a] Esterase activity measured with *t*-BOC-*l*-alanine *p*-nitrophenol. Incubation volume, 3.0 ml.
[b] Esterase activity measured with BAPA. Incubation volume, 5.0 ml.
[c] Esterase activity measured with GPANA. Incubation volume, 5.0 ml.

Diagnostics subsidiary of Amer. Hoechst Corp., Woodbury, N.Y., and rabbit antisera to alpha$_2$ macroglobulin and alpha$_1$ globulin fractions of human serum were purchased from Certified Blood Donor Service, Inc., Woodbury, N.Y. Elastase-like esterase activity was measured with *t*-BOC-*l*-alanine *p*-nitrophenol as substrate (2). Hydrolysis of BAPA by trypsin was measured by the method of Erlanger *et al.* (15) and that of GPANA by chymotrypsin according to the same authors (16).

Results and Discussion. Inhibition of esterase activity by cytosol. Table IA shows that human leukocyte cytosol added to incubation mixtures containing enzymes and synthetic ester substrates caused inhibition of esterolysis by leukocyte granule extracts but not by any of three other enzyme preparations. Amounts of cytosol added were calculated in terms of micrograms of cytosol protein, although there was no evidence at this point in our experiments that the inhibition was necessarily due to a protein component. As shown in Table I, the inhibition of the granule elastase-like esterase was incomplete even at the highest concentrations of cytosol tested. This is similar to the incomplete inhibitions of other enzymes observed with many naturally-occurring protease inhibitors (17).

The failure of human cytosol to inhibit esterolysis by pancreatic elastase was not unexpected, in view of previously demonstrated differences between leukocyte and pancreatic enzymes with respect to inhibition by other substances (2, 6, 7). However, the absence of antitrypsin activity in human leukocyte cytosol (over the range of protein tested) is a significant observation in that it suggests that leukocyte cytosol inhibitor is different from most other known protease inhibitors in mammalian tissues, which do inhibit trypsin (17). The lack of trypsin inhibition by human cytosol is clear from the experiment in which 1 mg of cytosol protein (corresponding to 20%, v:v, of cytosol in the incubation mixture) failed to suppress esterolysis by 5 μg of the crystalline enzyme. In the same assay, human pregnancy urine (20% final concentration, v:v) caused 100% inhibition of this amount of trypsin, showing that a different agent (urinary trypsin inhibitor) (18) was active under the conditions of the test.

TABLE II. Effect of Various Treatments of Cytosol on Its Esterase-Inhibiting Activity.

Treatment	Esterase inhibition[a] (%)	Loss of inhibition (%)
None	57, 63, 66	0
60 min at 37°	50	19
20 min at 56°	25	60
20 min at 80°	25	60
TCA[b]	13	79
Pronase[c]	30	50

[a] Percentage inhibition of esterolysis by human leukocyte granules with *t*-BOC-*l*-alanine *p*-nitrophenol as substrate. Incubation volume, 3.0 ml; 25 μg of granule protein; volume of cytosol added always corresponded to 300 μg of protein before treatment.

[b] Trichloroacetic acid added to give a final concentration of 6.6% (w:v), precipitate discarded. Excess TCA eliminated by dialysis. TCA-supernatant of cytosol tested as above.

[c] Pronase treatment (10 μg of enzyme × 3-hr incubation) followed by dialysis resulted in a 65% loss of cytosol proteins in the same experiment.

Evidence that inhibition of leukocyte granule esterase by human cytosol was not due to contamination with serum trypsin inhibitors was also obtained from double diffusion experiments involving cytosol and rabbit antisera against human alpha$_1$ trypsin inhibitor and alpha$_2$ macroglobulin. Antialpha$_1$ trypsin-inhibitor antiserum gave a single precipitin line when tested against standardized human serum diluted 1:64, 1:16, 1:4, and undiluted. Antialpha$_2$ macroglobulin antiserum gave two precipitin lines against undiluted human serum and serum diluted 1:4. Neither antiserum gave detectable precipitin reactions with cytosol (1:1, 1:2, or 1:4). Previous data had shown that elastin digestion by 200 μg of leukocyte granule protein could be inhibited 25% by a 0.2% (w:v) concentration of human serum proteins (6). Identical tests of human cytosol proteins showed that the same inhibition could be achieved with a concentration of 0.03% (w:v). Thus, cytosol appeared to be about 7-fold more effective than serum (on a protein wt basis) in the suppression of leukocyte granule elastase-like enzyme. Had the inhibition by cytosol been due to the presence of serum inhibitors in the human cytosol fraction, one might have expected detection of these agents by the specific antisera.

Some properties of the cytosol inhibitor. Inhibitory activity of cytosol was routinely measured after a 24 hr dialysis against 100 vol (2 changes) of cold phosphate–saline (see Methods). Marked inhibition was present after this treatment; and although no comparison was made to undialyzed cytosol, it seems reasonable to suggest that the responsible agent is nondialyzable. Activity was mostly lost (see Table II) following addition of trichloroacetic acid (TCA) to a final concentration of 6.6% (w:v) and separation of the resultant precipitate. Excess TCA was eliminated by dialysis before testing. Heating at 56° for 20 min destroyed most of the inhibition, but some activity persisted* even after heating to 80° (Table II). Treatment of cytosol with the broad-spectrum bacterial protease, pronase (10 μg/ml of cytosol, pH 7.0, 37° × 3 hr) followed by elimination of dialyzable peptides resulted in essentially parallel depletion of cytosol protein content and inhibitory activity (Table II). Pronase, by itself, had no effect in the assay. This observation suggested that most inhibition was due to a protein or to a protein-containing complex. Cytosol DNA (possibly released from nuclei during homogenization) was too low to be detected by our assay (see Methods). Other nondialyzable nucleic acids were also low in amount (40 μg/ml of cytosol, calculated from E_{260}/E_{280} ratios). Twenty μg of yeast RNA corresponding to the total nucleic acid content of 0.5 ml of cytosol (which normally inhibited 70% of the esterase) proved inactive when tested against the granule enzyme, although 10 times that amount (200 μg) was inhibitory. Hexuronic acid content of the cytosol was also low (7.7 μg/ml of cytosol) and heparin failed to give significant esterase inhibition when added to the assay in amounts below 100 μg. Finally, inhi-

* Note added in proof: Thermal-inactivation tests using purified cytosol inhibitor (Janoff and Blondin, in press) show the agent to be heat-stable. Partial loss of activity seen above after heating crude cytosol could result from inhibitor-trapping in aggregates of heat-labile cytosol proteins.

bition of human leukocyte granules appeared not to be species-specific, since a cytosol fraction prepared from rabbit peritoneal exudate polymorphonuclear leukocytes also showed antiesterase activity (see Table IB).

In conclusion, our results suggest that a proteinaceous agent in leukocyte cytosol fractions inhibits the hydrolytic activity of human leukocyte granules on an alanine ester reportedly specific for elastase-like enzymes. Further work will be required to determine if the hydrolysis of other alanine esters (5) and proteolysis and elastolysis by human granule enzyme(s) are equally affected by the cytosol fractions. Attempts at purification of the human cytosol inhibitor are in progress.

Summary. Human leukocyte cytosol inhibits esterolysis of a synthetic elastase substrate by human leukocyte granule extract. Hydrolysis of the same substrate by pancreatic elastase is not affected, nor is esterolysis of BAPA by trypsin or GPANA by chymotrypsin. Cytosol gives no cross reaction with antisera to human alpha$_1$ trypsin inhibitor or alpha$_2$ macroglobulin, even though cytosol protein is more potent than serum protein in suppressing leukocyte granule hydrolytic activity. The cytosol inhibitor appears to be nondialyzable, thermolabile,* and TCA-precipitable. Treatment of cytosol with pronase causes loss of inhibition which is approximately equivalent to the degree of protein digestion in the cytosol. No evidence was obtained for DNA, RNA, or hexuronic acid-containing substances being responsible for inhibition by cytosol.

* See footnote above.

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