Elastase-Like, Esteroprotease Activity in Human and Rabbit Alveolar Macrophage Granules[1] (35426)

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(Introduced by Z. Ovary)

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One of us previously reported that human peripheral blood leukocytes contain an elastase-like esteroprotease which differs in several respects from pancreatic elastase (1), but which nevertheless hydrolyzes synthetic substrates of the latter enzyme (2, 3). Fractions of human leukocyte lysosomes containing the elastase-like esteroprotease were shown to attack several components of connective tissues (at neutral pH) including arterial elastic fibers (1, 4), vascular basement membrane (4, 5), and cartilage-matrix proteinpolysaccharide (6). The effects could be demonstrated in a variety of tissues and organs; for example, extracts of the granules caused lung hemorrhages following endotracheal instillation in mice and also decreased the staining of arterial elastic fibers after incubation with frozen sections of human lung (7). It has been suggested (8, 9) that elastolytic enzymes may play a role in the pathogenesis of elastic tissue destruction in lung (and other organs) associated with $alpha_1$ antitrypsin and antielastase deficiency states. In this connection, further study of the leukocyte elastase-like protease appears warranted. However, in addition to circulating leukocytes, which may be broken down in lung during pulmonary infections or in the course of the physiologic turnover of these cells (10), the alveolar macrophages could constitute an additional source of tissue-damaging elastase-like proteolytic activity in this organ. To our knowledge, neither blood monocytes nor tissue macrophages have been examined to date for enzyme activity of the foregoing type. In the present experiments, therefore, human and rabbit alveolar macrophages (both whole cells and granule fractions) were tested for the presence of an esteroprotease with activity against elastase substrates.

Materials and Methods. Collection of cells and cell fractions. Human alveolar cells were collected by washing out lungs obtained within 12 hr postmortem. Death was usually due to trauma or narcotoxicosis, in one case to staphylococcal meningitis, but at all times only grossly normal-appearing lungs were used. Perfusion was carried out via the major airways exposed by section at the hilum, contamination with blood being minimized by prior ligation of associated pulmonary vessels. Granulomatous alveolar cells were similarly collected from rabbit lungs, 3 weeks after intravenous vaccination of the animals with 0.1 mg of lyophilized, heat-killed, attenuated tubercle bacilli (Vaccine BCG, Institut Pasteur, Paris) suspended in an emulsion of sterile mineral oil and Falba (Pfaltz and Bauer, Inc., New York, N.Y.) according to methods described by Myrvik *et al.* (11). Lungs of both species were irrigated with ice-cold, mammalian Ringer–Locke solution containing 0.1% dextrose and buffered with 0.015% sodium bicarbonate. The pH was adjusted to 7.1–7.4 immediately before use by gassing with CO_2. Lung washings were filtered through several layers of gauze, stored at 0°, and samples were removed for: total cell counts, differential counts using Wright's stain, observation of cell-types by phase-contrast microscopy, and dye-exclusion tests with trypan blue (viable cell count).

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Cells in the bulk of the filtrate were then sedimented, washed, homogenized, and their granules were recovered and extracted by the same techniques used to process human peripheral blood leukocytes (1), except that sedimentation of macrophage granules was carried out at a higher force (17,000*g* for 30 min). In addition to granule lysates, crude extracts of whole cells were also prepared by repeated freeze–thawing of washed cell suspensions.

Assay procedures. Protein was determined by the method of Lowry *et al.* (12) using crystalline bovine serum albumin as reference standard. Acid phosphatase was measured with *p*-nitrophenyl phosphate as substrate (Sigma Chem. Co., St. Louis, Mo.) according to Nigam and co-workers' method (13), and beta-glucuronidase was assayed on phenolphthalein glucuronic acid (Sigma Chem. Co.) by Fishman's method (14). Tertiary butyloxycarbonyl (*t*-BOC)-*l*-alanine *p*-nitrophenol and *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester were purchased from Cyclo Chemical Corp. (Los Angeles, Calif.), and served as substrates for elastase-like esterases in macrophage granules and whole cell extracts. Alanine-*p*-nitrophenyl esterase activity was determined by measuring absorbance of liberated *p*-nitrophenol as has been described for leukocyte granules (2), while acetyltrialanine methyl esterase activity was measured in the pH Stat by methods which have also been previously described (3). Elastolysis by granules and by whole cell extracts was monitored by Sachar's method (15) using orcein-impregnated, bovine ligamentum nuchae elastin as substrate (Worthington Biochemical Corp., Freehold, N.J.). This assay was modified according to previously described procedures for measuring elastolysis by leukocyte granules (1, 4). Enzymes used as standards in the foregoing assays were: wheat germ acid phosphatase (EC 3.1.3.2), beef liver beta-glucuronidase (EC 3.2.1.31), and electrophoretically-purified pancreatopeptidase E (elastase, EC 3.4.4.7). Enzymes were purchased from Worthington Biochemical Corp.

Results. Recovery of alveolar macrophages. Using the washout procedures described above, the yield of lung cells (total of all types) recovered per lung averaged 20×10^8 (for human lungs) and 24×10^7 (for lungs of BCG-vaccinated rabbits). Of these cells, macrophages constituted 90–98% in man and 65% in the rabbit. In human lung washouts, scattered columnar epithelial cells represented the principal contaminant, while lymphocytes (25%) and eosinophiles (10%) were the main contaminants in cell preparations from BCG-vaccinated rabbits. Human alveolar macrophages contained two distinct types of inclusions: some cells appeared filled with prominent, lipid-laden vacuoles under phase microscopy while others contained small carbon-like particles ("dust cells"). Large numbers of the second type of macrophage were generally present in the human lung preparations causing the cell pellets to have a mud-colored appearance and the granule pellets derived from these cells to appear black. Granule extracts, however, were clear following sedimentation of debris by high-speed centrifugation. Viability of human macrophages collected by the foregoing procedures was generally low, averaging 34% according to dye-exclusion tests. In the case of rabbit cells, where lungs were available immediately after death, viability was considerably improved (90%).

Acid-hydrolase activity of macrophage granules. Acid-phosphatase and beta-

TABLE I. Acid-Hydrolases Recovered from Alveolar Macrophage Granules.

Cell source	Acid phosphatase[a]	β-Glucuronidase[a]
Rabbit lung[b]		
Normal	2.63	0.083
BCG vaccinated	4.70	0.101
Rabbit lung[c]	2.53	0.585
Human lung[d]	6.47	0.291
	5.13	0.469

[a] Enzyme activities (μm substrate hydrolyzed/mg of protein/hr) (37°); acetate buffer, pH 5.0 (acid phosphatase), pH 4.5 (β-glucuronidase).

[b] Published values (16).

[c] BCG-vaccinated animals, present experiments, pool of 14 lungs.

[d] Postmortem organs, present experiments, results of 2 separate preparations.

TABLE II. Relative Hydrolysis[a] of Elastase Substrates by Various Cell Fractions.

	Cell fraction				
	Human			Rabbit[c]	
Substrate[b]	PMNg	AMg	AMwc	AMg	AMwc
NBA	100	5	4	14	5
AAAOMe	20	0.2	—	0.1	—
EO	5	0.7	0.6	—	0.08

[a] Relative hydrolysis expressed as the *reciprocal* of the no. of mg protein in the fraction required to produce substrate breakdown equal to that given by 5 μg of purified pancreatic elastase. Average values of 3 determinations. PMN, polymorphonuclear leukocytes; AM, alveolar macrophages; g, granule extract; wc, whole cell extract.

[b] NBA, tertiary butyloxycarbonyl-*l*-alanine *p*-nitrophenyl ester; AAAOMe, *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester; EO, orcein-impregnated beef ligamentum nuchae elastin.

[c] BCG-vaccinated animals.

glucuronidase activity of alveolar macrophage granules from human lungs and lungs of BCG-vaccinated rabbits were compared with levels of activity found in rabbit alveolar macrophages by others (16). The results are given in Table I. In general, the acid-hydrolase activities recovered in the present experiments (from both human and rabbit preparations) were as high or higher than those previously reported for rabbit alveolar macrophages.

Elastase-like activity of macrophage granules and whole cell extracts: Table II shows the results of assays for elastase-like esteroprotease activity performed on the macrophage fractions prepared from human and rabbit lung. For purposes of comparison, results obtained in previous work with human peripheral blood leukocytes (1–3) are included in Table II. Human alveolar macrophage preparations consistently showed activity against two elastase substrates: alanine-*p*-nitrophenyl ester and orcein-dyed elastin. Clearly, however, the sp act of macrophage fractions on these substrates was considerably weaker than that found previously in granules from human blood leukocytes. Thus, the sp act of macrophage granule preparations on the alanine *p*-nitrophenyl ester and the dye-impregnated elastin was 5 and 14%, respectively, of that shown by leukocyte granules. When a highly specific synthetic substrate for elastase was used, namely *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester (17), specific rates of hydrolysis by human alveolar macrophage preparations were even lower. Instead of the anticipated 5–14% of leukocyte granule values, only 1% of the latter was observed. It should be remembered, however, that macrophage lysosomes prepared by simple differential centrifugation are heavily contaminated with other cell particulates (especially mitochondria), unlike lysosomal granules prepared from polymorphonuclear leukocytes. A corresponding reduction of specific hydrolase activity of certain enzymes might therefore be expected. For example, comparison of specific activities of acid phosphatase and beta-glucuronidase measured in granules of rabbit leukocytes and alveolar macrophages by other investigators (16, 18) shows the alveolar macrophage granule fraction to have only 30 to 50% of the acid-phosphatase activity of leukocyte granule fraction and only 16% of the latter's beta-glucuronidase activity. At least part of this difference may be based on the lysosome-dilution effect indicated above. Applying these same considerations to the data shown in Table II, it would appear that elastolytic activity of human alveolar macrophage granules (about 14% of that found in PMN granules of man) is not unexpectedly low. On the other hand, levels of esterolytic activity in the macrophage granules against both synthetic substrates, are lower than can be accounted for by the above considerations. Rabbit alveolar macrophage granules appeared to be two or three times more active than their counterparts from human lung when alanine *p*-nitrophenol was the substrate, but again hydrolysis of the more specific trialanine methyl ester was very weak and more significant, little breakdown of dyed-elastin could be detected when relatively large amounts of rabbit macrophage protein were tested. This finding was consistent with our previous observation of minimal elastinolytic activity in rabbit polymorphonuclear leukocyte granules (7), despite the report of some alanine-*p*-nitrophenyl esterase

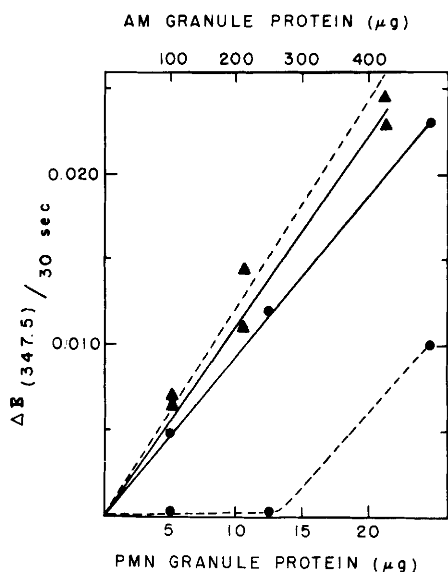

FIG. 1. Hydrolysis of *t*-BOC-*l*-alanine *p*-nitrophenol by granule extracts of human polymorphonuclear leukocytes (PMN) and alveolar macrophages (AM): (ordinate), increase in absorbance (at 347.5 nm) of free *p*-nitrophenol/30-sec incubation; (abscissas), µg of granule protein added; (●), PMN granules; (▲), AM granules; (—), granule extracts tested alone; (- - -), Granule extracts tested in the presence of 300 µg of human PMN cytosol proteins.

activity in leukocyte granules of this species (19).

Discussion. The collection of human alveolar macrophages from postmortem lungs resulted in low viability of most cells. Part of the reduction in elastase-like esteroprotease activity recovered from these cells, as compared with the viable blood leukocytes previously studied, might have been due to the moribund condition of many of the macrophages. However, the excellent recovery of acid-hydrolase activity in our preparations (Table I) would argue against the foregoing interpretation, although persistence of acid hydrolases in dying cells may not reflect stability of other enzymes.

The possibility exists that the elastase-like enzyme activity found in alveolar macrophages is actually derived from circulating leukocytes which have been destroyed in the lung (10). One additional experiment which was performed in the present study appears to rule out this possibility. The human leukocyte alanine-*p*-nitrophenyl esterase is inhibited by a factor present in the cytosol fraction of leukocyte homogenates (20). When this factor was tested against human alveolar macrophage alanine-*p*-nitrophenyl esterase, comparable inhibition was not detected. These results are given in Fig. 1.

Our results suggest that weak elastase-like esteroproteolytic activity is present in human alveolar macrophage granules. One reasonably specific synthetic elastase substrate (*t*-BOC-*l*-alanine *p*-nitrophenol) was hydrolyzed by granule extracts, and some elastin-degrading activity was consistently detectable. Hydrolysis of these substrates could not be accounted for by trypsin or chymotrypsin-like activity, since neither the *p*-nitroanalide ester of benzoyl-*dl*-arginine nor of glutaryl-*l*-phenylalanine was broken down by granule extracts (7). The acid-cathepsins of macrophage granules presumably would not be operative in the pH range employed.

The potential role of this type of enzyme activity in the mediation of lung damage by alveolar macrophages remains to be explored. Weak activity of this type could serve merely to supplement the degradative enzyme(s) present in leukocytes. Alternatively, the alveolar macrophage enzyme, although seemingly present in low concentration, could play a more critical role if (as the preliminary data suggest) it is less susceptible to inhibition by endogenous antiproteases than is the leukocyte esteroprotease. Additional inhibition experiments with the macrophage esterase and serum antielastase or alpha$_1$ trypsin inhibitor should be undertaken to explore this possibility. It is tempting to speculate that leukocyte elastinolytic protease participates in the early-onset type of emphysema associated with alpha$_1$ trypsin-inhibitor deficiency, while the macrophage enzyme is involved in late-onset obstructive lung disease less frequently associated with protease-inhibitor deficiencies than with chronic exposure to irritants.

Summary. Human alveolar macrophages were collected postmortem and their granules were analyzed for acid phosphatase, beta-glucuronidase and elastase-like esteroprotease. Alveolar macrophages from BCG-vaccinated rabbits were also studied. The al-

veolar macrophage granule preparations showed satisfactory levels of acid hydrolases but were nevertheless low in elastase-like esteroprotease activity when compared to human polymorphonuclear leukocyte granules. Part of the reduction in enzyme may be accounted for by differences in lysosomal content of the granule preparations from these two cell types. Human alveolar macrophage granules showed elastinolytic activity commensurate with their esterolytic activity against *t*-BOC-*l*-alanine *p*-nitrophenol. The macrophage alanine-*p*-nitrophenyl esterase was not affected by an endogenous inhibitor of the leukocyte esterase. The possible role of these enzymes in the pathogenesis of emphysema remains to be explored.

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