ELASTASE SECRETION BY PERITONEAL EXUDATIVE AND ALVEOLAR MACROPHAGES*

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There is reason to believe that elastolytic enzymes play a role in the pathogenesis of emphysema (1, 2). Since alveolar macrophages represent a potential source of elastase in the lung, and their numbers are greatly increased in cigarette smokers (3, 4), we have sought secretion of elastase by alveolar macrophages and compared it with that of peritoneal macrophages (5). Although inhibitor studies and pH optima suggest that the enzymes produced by macrophages from either source are similar, we observed some differences in the secretory properties of the two types of cell.

Materials and Methods

Harvesting Macrophages. Peritoneal exudative macrophages from male DBA/2 mice 9-15 wk of age (The Jackson Laboratory, Bar Harbor, Maine) were harvested by a modification of the method of Stewart et al. (6) on the 4th day after intraperitoneal injection of 1.5 ml thioglycollate medium (Difco Laboratories, Detroit, Mich.).

Alveolar macrophages were harvested by the method of Brain and Frank (7).

Preparation of Conditioned Medium (CM). Freshly harvested macrophages were centrifuged for 10 min at 500 g and washed twice with Dulbecco modified Eagle's minimal essential medium (DMEM). The cells were cultured in DMEM supplemented with 10% acid-treated (AT) fetal calf serum and 5% AT horse serum (5, 6) in 4 chamber Lab-Tek slides (Miles Laboratories Inc., Elkhart, Ind.). At the end of 4 days the cultures were washed four times, the medium replaced with DMEM supplemented with 0.2% lactalbumin hydrolysate (DMEM-LH) (5), and incubation continued for 1-4 days. Elastase was measured in medium and in cell lysates prepared in 1.0% Triton X 100 in 0.9% NaCl.

Preparation of Particles for Phagocytosis. Latex beads, (Dow Diagnostics, The Dow Chemical Co., Indianapolis, Ind.) 1.1 μm in diameter were washed twice in sterile saline and resuspended in DMEM-LH at a concentration to provide 30-50 particles/cell. The suspension was subjected to three 20-s cycles of 2,500 AMP sonication to sterilize and disperse the beads. Carbonyl iron particles 0.5-5.0 μm, were sterilized in a 110°C drying oven and suspended in DMEM-LH at a stock concentration of 1 mg/ml. Cultures receiving a phagocytic challenge were maintained for 4 days in DMEM supplemented with 15% AT serum, and washed four times with DMEM-LH before adding the particles.

* Supported by U. S. Public Health Service research grants RO1 HL-19746, PO1 HL-16118, and training grants ES-00128.

† Recipient of Research Career Development Award KO4 CA-70730.

Abbreviations used in this paper: AM, alveolar macrophage; AT, acid treated; CM, conditioned medium; DMEM, Dulbecco modified Eagle's minimal essential medium; LH, lactalbumin hydrolysates; PiMM, normal serum; PiZ, alpha anti-protease-deficient serum; PM, peritoneal macrophage.

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FIG. 1. Secretion of elastase in prolonged cultures. The DMEM-LH from macrophage cultures was harvested, assayed, and replaced with fresh DMEM-LH at 48-h intervals for five consecutive cycles. Assays were conducted on 75-μl samples of CM. The total area of lysis is the accumulated activity calculated from the addition of the lytic zones assayed after each cycle. The vertical bars represent the SEM.

Elastase Assay. For elastase assays, aliquots of medium or cell lysates were added to wells 5 mm in diameter, cut in agar plates containing suspended elastin particles (<400 mesh) and sodium dodecyl sulfate, and buffered to the desired pH with Tris maleate (5). The plates were incubated at 40°C in a humidified chamber for up to 10 days. Elastase activity was quantitated from the area of the zone of lysis surrounding the wells. For assays employing inhibitors, the reagents were mixed and incubated for 6 min before loading the well.

Enzyme Inhibitors. Lavage fluids tested for elastase inhibitory activity were prepared by reducing the cell-free lavage supernates to 1% of their original volume by membrane filtration. Human serum, of phenotypes PiZ and PiMM and purified α-1-anti-protease were gifts of Dr. John Pierce, Washington University School of Medicine, St. Louis, Mo. Synthetic inhibitors, Ac-(Ala)3-Pro-Ala CH2Cl, Ac-(Ala)3Ala CH2Cl, Ac(Ala)3-Pro-Val-CH2Cl, were gifts of J. C. Powers, School of Chemistry, Georgia Institute of Technology, Atlanta, Ga.

Results

Secretion of Elastase by Alveolar Macrophages. Alveolar macrophages (AM) cultured in DMEM-LH secrete elastase at a constant rate for at least 10 days (Fig. 1).

In contrast to the active secretion of elastase by AM, it has been reported that unstimulated peritoneal macrophages secrete low levels of elastase, but that phagocytosis of indigestible particles significantly increases their secretion (5). Therefore, we next examined effects of phagocytosis on elastase secretion by AM, resident, and exudative peritoneal macrophages.

Fig. 2 shows the results of an experiment comparing the effect of adding 0.1 mg/ml of carbonyl iron particles on 1 × 10⁶ adherent macrophages of each type. Resident peritoneal macrophages secreted barely detectable levels of elastase, but increased their release of elastase after phagocytosis. Peritoneal exudative macrophages produced twice as much elastase as phagocytosing resident cells. The exudative cells, like the resident cells, could be further stimulated by the
ingestion of nonmetabolizable particles. In contrast, the amount of elastase secreted by AM was not increased by phagocytosis but was similar to that of the maximally stimulated exudative peritoneal cell. Neither the substitution of latex beads for iron particles nor variations in the concentration of carbonyl iron from 0.01 to 1 mg/ml altered these results.

Addition of cycloheximide to the cultures during the period of incubation in serum-free medium completely eliminated secretion at concentrations of cycloheximide above 0.25 μg/ml. With concentrations of 0.125 and 0.25 μg/ml a trace of activity was detected only when the assay was extended to 72 h. Removal of the cycloheximide after 24 h restored elastase secretion. Cycloheximide added to the medium at the end of the period of culture did not affect the assay. Thus, protein synthesis is required during elastase secretion.

**Properties of the CM.** Alveolar and peritoneal macrophage CM were compared to purified elastases from porcine pancreas and human leukocytes by their inhibition profiles. Enzyme concentrations were adjusted to yield zones of similar size at pH 7.6 in the absence of inhibitors. Alveolar and peritoneal macrophage activities were similar to one another, but differed from the other two enzymes (Table 1). This was shown by the small molecular weight site-specific synthetic cloromethyl ketone inhibitors (8) and by soybean trypsin inhibitor, which were effective inhibitors of the leukocyte and porcine pancreatic elastase, but had no effect on either of the macrophage preparations. Both alveolar lavage fluid and normal (Pi-MM) and α₁-anti-protease-deficient (PiZ) sera completely inhibited all four preparations but purified α₁-anti-protease only partially inhibited macrophage elastase (Table 1, Fig. 3).

The pH optima for alveolar and peritoneal macrophage enzymes were similar, between 7.6 and 7.8, while those of purified porcine pancreatic and human leukocytes were 8.6 and 8.2, respectively (Fig. 4).
TABLE I

| Inhibitor                           | Concentration | AM  | PM  | Porcine pancreatic | Human leukocyte |
|-------------------------------------|---------------|-----|-----|-------------------|----------------|
| Ac(Ala)₂AlaCH₂Cl                    | 2 mM          | -   | -   | +                 | +              |
| Ac(Ala)₂ProAlaCH₂Cl                 | 2 mM          | -   | -   | +                 | +              |
| AcAlaProVal-AlaCH₂Cl                | 2 mM          | -   | -   | +                 | +              |
| Serum (PiZ or PiMM)                 | 5% vol/       | ++  | ++  | ++                | ++             |
| Soybean trypsin inhibitor           | 8 mg/ml       | -   | -   | +                 | +              |
| Phenylmethane sulfonylfluoride      | 10 mM         | ++  | ++  | ++                | ++             |
| Alpha-1-anti-protease               | 60 µg/ml      | +   | +   | +                 | +              |
| Lavage supernate                    | 20% vol/vol   | ++  | ++  | ++                | ++             |
| 4-Cloromercuribenzoate              | 1 mM          | -   | -   | -                 | +              |

40 µl of conditioned media was mixed with 10 µl of inhibitor to yield the final inhibitor concentrations indicated above. These were preincubated for 6 min before charging the 5-mm diameter well of the elastin agar gel assay. Each well contained 25 µl, and was charged three consecutive times with fresh conditioned medium inhibitor mixtures, for a final vol of 75 µl. The zones of elastolysis were measured after 24 h incubation.

++++, complete inhibition; +, partial inhibition; and −, no inhibition.

Discussion

The elastolytic enzymes secreted by alveolar and peritoneal macrophages appear to be different from either human leukocytes or porcine pancreatic elastase. Although this may reflect species differences, human leukocyte and human pancreatic elastase are antigenically different, indicating that a given species can have more than one elastase (direct communication with P. Hubner, Washington University School of Medicine, St. Louis, Mo.). These enzymes are similar in their sensitivities to phenyl methane sulfonyl fluoride and are therefore all serine proteinases (9), but differing pH and inhibition profiles indicate that they are separate enzymes (Table I). The three mammalian elastases...
are sensitive to PiZ and to PiMM serum as well as to the inhibitory activity of pulmonary lavage fluid (Table I). The macrophage enzyme, however, seems to be only partially inhibited by purified alpha-1-anti-protease (Fig. 3).

One of the limitations of the assay used in this study is that measurement of zone size alone does not give valid quantitative data on inhibition. The lytic zone surrounding the well containing CM and alpha-1-anti-protease in Fig. 3 is of a larger diameter than with CM alone, but the lysis is incomplete. The zone of lysis appears cloudy because only the smaller elastin particles were completely hydrolyzed. The diameter of the lytic zone surrounding a well is limited not only by dilution of the enzyme as it diffuses radially from the well, but also by binding of the enzyme to the suspended substrate. An enzyme-inhibitor complex which does not bind to substrate would be expected to diffuse more readily than the active enzyme despite its higher molecular weight. If the inhibition is reversible, it is possible to obtain a wide zone of incomplete lysis from relatively little active enzyme as a consequence. Alpha-1-anti-protease is slowly hydrolyzed by the enzymes it inhibits releasing active enzyme (10). The inference from the appearance of the large zone obtained by mixing CM with alpha-1-anti-protease, then, is that the enzyme inhibitor complex is unable to bind to the elastin substrate, and that active enzyme is being liberated from inhibitor enzyme complexes during the assay.

The alpha-1-anti-protease-deficient (PiZ) serum was effective in inhibiting the macrophage CM, despite giving a final concentration of alpha-1-anti-protease of only 10 μg/ml, one-fifth that of the PiMM serum and substantially less than the 60 μg/ml of purified alpha-1-anti-protease which failed to provide complete inhibition. This indicates that although alpha-1-anti-protease alone is a poor inhibitor, other serum inhibitors are effective against macrophage elastase, such as alpha-1-macroglobulin, the levels of which are normal in PiZ serum.
Since lung tissue damage does not always occur in mice, despite the high levels of elastase secretion by alveolar macrophages, we examined the inhibitory properties of concentrated lavage fluids as an approach to sampling the alveolar lining fluid. The lavage fluid effectively inhibited pancreatic, leukocyte, and macrophage elastase. Although lavage fluids contain α1-anti-protease, they also contain a heat labile high molecular weight elastase inhibitor which is not a serum protein (11), and which may account for the inhibition of macrophage elastase.

Hydrolytic enzymes in macrophages fit into at least three groups. Lysosomal hydrolases are stored intracellularly, and normally function intracellularly, although appreciable extracellular release occurs with phagocytosis or activation of the cell. Some hydrolases, exemplified by lysozyme are secreted continuously at a rate independent of phagocytosis by the cells (12). The third group which includes collagenase, elastase, plasminogen activator, and neutral proteases which digest azacasein and gelatin, are also secreted. The rate of secretion varies with the level of activation (5, 13, 14). Resident peritoneal macrophages secrete low levels, which increase with phagocytosis; activated macrophages in peritoneal exudates secrete still higher concentrations. Our observations indicate that alveolar macrophages differ from peritoneal macrophages in that they secrete high levels of elastase without additional stimulation. Unlike thioglycollate medium-induced peritoneal exudative macrophages, which further augmented their rate of elastase secretion with a phagocytic load, AM did not. The basis for this difference in secretory regulation between alveolar and peritoneal exudative macrophages is unclear. The limit of the secretory capacity of the AM had not been reached, because they can be induced to increase their secretion by cytochalasin B or colchicine (R. White and C. Kuhn, unpublished observations).

Elastolytic enzymes have been implicated in the pathogenesis of emphysema (1, 2) and macrophages are attractive as a source of these enzymes. Macrophages are present in the early lesions of emphysema (15). In young cigarette smokers who died accidently, the only abnormality attributable to smoking was large collections of macrophages in respiratory bronchioles, sites which are prone to the development of emphysematous lesions (16). The macrophages from smokers are more highly activated than those of nonsmokers, having greater glucose uptake (17) and greater esterolytic activity (4). Secretion of elastase by such activated macrophages could be a mechanism in the eventual development of emphysema.

Summary

Mouse alveolar macrophages (AM) cultured in the absence of serum secrete an elastolytic enzyme. The elastase from AM resembles the previously described elastase from peritoneal macrophages (PM) in pH optimum and inhibition profile. The macrophage enzymes do not appear to be stored, and with periodic changes in the culture medium, accumulate extracellularly for up to 10 days.

Resident PM produce barely detectable levels of extracellular elastase unless given a phagocytic load. Thioglycollate-stimulated peritoneal exudative macrophages (PEM), however, secrete easily detectable levels of elastase, which can be further increased with a phagocytic load. Without any additional stimula-
tion, AM secrete an elastolytic activity comparable to that of the PEM receiving a phagocytic load, but unlike PM they do not increase elastase secretion after phagocytosis.

Received for publication 7 March 1977.

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