ELASTASE SECRETION BY STIMULATED MACROPHAGES

Characterization and Regulation*

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Elastin is a fibrous protein which is widely distributed in elastic tissues such as lung, arteries, and skin. The protein contains numerous lysine-derived cross-links, is hydrophobic and insoluble, and resists proteolysis by many mammalian proteinases (1–4). Two mammalian serine proteinases have been identified which are able to hydrolyze and solubilize elastin, a porcine pancreatic enzyme investigated in considerable detail (5, 6) and an elastinolytic proteinase obtained from human spleen,[1, 2] granules of polymorphonuclear leukocytes (PMN),[3] and platelets (7–10). The role of these enzymes in elastin degradation in the body has not, however, been defined.

In previous work we have shown that activated macrophages are able to modify their extracellular environment by secreting specific neutral proteinases such as plasminogen activator(s) (11) and collagenase (12). Since macrophages are present in large numbers in pulmonary emphysema and other chronic diseases associated with destruction of elastic tissue (7), we have studied the role of macrophages in the degradation of elastin.

In this report we show that cultivated mouse macrophages, obtained after intraperitoneal stimulation with thioglycollate broth, secrete a distinctive proteinase which lyses insoluble elastin fibrils. We have characterized the enzyme and studied some of the factors which regulate elastase secretion in vitro. Unstimulated macrophages secrete little enzyme, but can be induced to secrete higher levels for prolonged periods of time by phagocytosis of indigestible latex particles.

Materials and Methods

Reagents. Reagents were obtained from the following sources: Dithiothreitol, soybean trypsin inhibitor (Kunitz), lima bean trypsin inhibitor, Tos-PheCH$_2$Cl (L-1-tosylamido-2-phenylethyl-

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[1] Starkey, P. M., and A. J. Barrett. Neutral proteinases of human spleen. Purification and properties of elastase and cathepsin G. Manuscript submitted for publication.

[2] Starkey, P. M., and A. J. Barrett. Lysosome elastase. Catalytic properties. Manuscript submitted for publication.

[3] *Abbreviations used in this paper:* AT-FBS, acid treated-fetal bovine serum; CM, conditioned medium; Dip-F, di-isopropyl phosphorofluoridate; LH, lactalbumin hydrolysate; PMN, polymorphonuclear leukocytes; SDS, sodium dodecyl sulfate.

chloromethyl ketone), lysozyme (from egg white), elastin (from bovine ligamentum nuchae), agarose, hog pancreatic elastase (type III, chromatographically purified, 58 U/mg, where 1 U = 1 mg elastin hydrolyzed/20 min at pH 8.8, 37°C), elastin-orcein, cycloheximide, Sigma (London) Chemical Co., Ltd., Kingston-on-Thames, Surrey, England; Tos-LysCH_2Cl,Ac-$(Ala)_3$-4-nitroanilide, Bachem Feichemikalin, Liestal, Switzerland; 4-nitrophenyl-4'-guanidinobenzoate, Nutritional Biochemical Corp., Cleveland, Ohio; Chelex 100, Bio-Rad Laboratories Ltd., Richmond, Calif.; dextran sulfate 500, Pharmacia Fine Chemicals Ltd., London, England. Dulbecco's modification of Eagle's medium, Neumann and Tytell serumless medium, lactalbumin hydrolysate (LH), Gibco-Biocult Ltd., Paisley, Renfrews., Scotland; latex particles, 1.1 μm in diameter, Micro-Bio Labs., London, England.

Purified human spleen (granulocyte or lysosomal) elastase was the gift of Dr. P. M. Starkey, Strangeways Research Laboratory, Cambridge, England. Ac-$(Ala)_2$-Pro-AlaCH_2Cl and Ac-$(Ala)_3$-AlaCH_2Cl were gifts of Doctors J. C. Powers and P. M. Tuhy, School of Chemistry, Georgia Institute of Technology, Atlanta, Ga. The esterase substrates, benzyloxycarbonyl-L-alanine-2-naphthol ester (Z-Ala-2-ONap) and *N*-benzoyl-L-phenylalanine-2-naphthol ester (Bz-Phe-2-ONap) were gifts of Doctors C. G. Knight and A. J. Barrett, Strangeways Research Laboratory. Chicken ovoinhibitor and turkey ovomucoid were gifts of Dr. A. J. Barrett, Strangeways Research Laboratory. Azocasein, prepared by the method of Charney and Tomarelli (14) was the gift of Dr. P. M. Starkey, Strangeways Research Laboratory.

Cell Culture. Unstimulated and thioglycollate-stimulated macrophages were harvested from the peritoneal cavity of mice and cultured in Dulbecco's medium supplemented with 15% AT-FBS as described previously (11–13). Conditioned media (CM) were prepared by washing the monolayers three times and culturing the cells for 1–4 days in serum-free Dulbecco's medium supplemented with 0.2% LH. CM was centrifuged (500 *g*, 20 min) and stored at −20°C. Cell lysates were collected by scraping the monolayers into 0.2% Triton X-100. For phagocytosis experiments the cells were cultured for 24 h, then exposed to 1.1 μm diameter latex particles as before (12). In some experiments media were dialyzed and concentrated by freeze drying (12).

Other Sources. L-cell fibroblasts, 3T3 cells, and mouse melanoma cells were obtained and cultured as before (12). Rabbit synovial fibroblast cultures were prepared as described previously (15). Alveolar macrophages were obtained from the lungs of rabbits injected with heat-killed *Mycobacterium tuberculosis*, var. BCG (16), and lysates of washed cells prepared in 0.1% Triton. Rabbit heterophil leukocytes were obtained from the peritoneal cavity of New Zealand white rabbits 4 h after injection of saline solution containing shellfish glycogen and a granule fraction prepared by the method of Cohn and Hirsch (17).

Assays of Elastase Activity. Radial diffusion in agarose gels containing elastin was used for the standard determination of elastinolytic activity, employing a modification of the method of Schill and Schumacher (18, 19). Elastin was finely pulverized (mesh size <100) in a vibratory grinding mill (A. Gallenkamp & Co. Ltd., London, England). Agarose was dissolved at 10 mg/ml in 50 mM Tris-HCl buffer, pH 7.6, containing 200 mM NaCl and the mixture cooled to 65°C. Elastin (usually 0.8 mg/ml) was added to the agarose and suspended by 10 sec of treatment with an Ultraturrax homogenizer (Jacob & Kunkel, K. G., Ikawerk, Stauferi Breisgau, W. Germany). Sodium dodecyl sulfate (SDS:elastin ratio 1:4, by weight) was added to the mixture and sodium azide (0.05%) included to prevent bacterial growth. Aliquots (11 ml) were poured onto 8 × 8 cm plates on a leveling table and the plates kept overnight to allow the SDS to bind to the elastin (20, 21). Plates were stored in a moist chamber at 4°C before use.

For assay of elastase activity 5-mm (20 μl) diameter wells were cut in the gel. Samples were placed in the wells and allowed to diffuse into the gel on a leveling table, then incubated at 40°C in a moist chamber for up to 2 wk. Zones of lysis were examined with darkground illumination and measured in two perpendicular dimensions after 16, 24, 48, and 72 h, although on occasion they were developed for longer times. Reactions were stopped with 7% (vol/vol) acetic acid, in which the plates were stored. Plates could also be dried and stained for protein. All samples were assayed in duplicate or triplicate and compared with standards of porcine pancreatic elastase and a standard sample of macrophage CM. Trypsin and chymotrypsin (1 mg/ml) gave no visible lysis even after 7 days of incubation. 1 U of elastase activity was defined as the amount of enzyme giving a zone of lysis of 7.0 mm in 24 h under standard experimental conditions.

For assays with inhibitors the enzyme samples were mixed with reagents before loading the wells. In some cases the inhibitor was incorporated in the gel. On occasion assays for elastase activity were made using a suspension of elastin-orcein as substrate (22).

Assays with N-Acetyl-L-Alanyl-L-Alanyl-L-Alanine-p-Nitroanilide as Substrate. The assays were made essentially as described for other 4-nitroanilide substrates (23). Reaction mixtures (1 ml) containing enzyme, 0.02% BRIJ-35, 100 mM Tris-HCl buffer, pH 7.8, and 20 μl of Ac-$(Ala)_3$-4-nitroanilide (dissolved at 5 μg/ml in dimethyl sulfoxide) were incubated at 40°C. Positive controls of porcine pancreatic elastase (0.01–10 μg per tube) and buffer blanks were included in each assay. The assays were stopped with 1 ml of 5 M sodium formate buffer, pH 3, and the OD_{410} determined.

Esterase assays with Z-Ala-2-ONap and Bz-Phe-2-ONap, substrates used for human spleen elastase and cathepsin G respectively, were made as described by Starkey (10, footnotes 1, 2). Porcine pancreatic elastase (10 μg/tube) was used as standard.

Assays with Azocasein Proteolytic activity was assayed in reaction mixtures (0.5 ml) containing 625 μg azocasein in 100 mM Tris-HCl buffer, pH 7.6, with 5 mM $CaCl_2$. After 15 h at 40°C the reactions were stopped by the addition of 3% (wt/vol) trichloroacetic acid and the OD_{366} of the filtrates measured. The azocasein preparation used had an $OD^{1\%}_{366}$ of 37.6.

Collagenase assays were made using ^{14}C-glycine-labeled collagen as described previously (12, 15). Protein determinations were made by the method of Lowry et al. (24) using egg lysozyme standard. All results are shown as ± SEM and tests of statistical significance were made with Students' *t* test.

Results

Conditions for Assay of Macrophage Elastase. The elastinolytic activity of macrophages was measured from the zones of lysis produced by incubating CM in wells of elastin-agarose plates. Schill and Schumacher (18, 19) had observed that this assay allowed comparison of samples of widely differing enzyme concentrations. For the range of enzymic activities normally encountered with macrophage CM, wells 5 mm in diameter gave zones of lysis more accurately measurable than wells of 2 mm diameter. The 5-mm wells could be refilled so that up to 80 μl of enzyme solution was assayed. The edge of the zone of lysis was sharp even with low levels of the macrophage enzyme suggesting that the macrophage enzyme binds firmly to elastin.

In order to obtain an accurate measurement of the enzymic activity present in CM, optimal assay conditions were established. Conditioned medium (10 ml) was produced by culturing 1.2×10^7 thioglycollate-stimulated macrophages for 72 h in Dulbecco's medium supplemented with 0.2% LH. The unprocessed CM was placed in 20-μl wells in the elastin-agarose plates.

EFFECT OF SDS. Elastin binds hydrophobic ligands such as SDS strongly and both porcine pancreatic elastase (20, 21) and human spleen elastase (10) are more active against elastin-ligand complexes. Accordingly, the effect of SDS on elastinolysis by macrophage CM was first examined. Agarose plates containing 1 mg elastin/ml were prepared with varying concentrations of SDS. As shown in Table I the incorporation of SDS into the gel enhanced elastinolytic activity considerably. Enzymic activity was maximal at an SDS:elastin ratio of 1:4, whereas increased concentrations of SDS inhibited lysis. At the optimal ratio the activity of macrophage elastase was at least 20 times that in the absence of SDS. Pancreatic elastase was affected much less by the addition of SDS to the elastin (not shown).

EFFECT OF SALT. The interaction between pancreatic elastase and elastin is electrostatic and high salt concentrations effectively depress enzyme activity (20). Since CM contained physiological salt concentrations and other preparations had varying salt concentrations, the effect of salt on elastinolysis by macrophage CM was examined. Elastin-SDS-agarose plates were prepared in 50 mM Tris-HCl buffer, pH 7.6, containing no added salt, 200 mM NaCl, or 1 M

TABLE I

Effect of SDS on Elastinolysis by Macrophage Conditioned Medium

SDS:elastin ratio	Elastinolysis (maximum)
wt/wt	%
0	2
0.1	38
0.25	100
0.5	3
1.0	0

Agarose plates containing 0.1% elastin in 50 mM Tris-HCl, pH 7.6, with 200 mM NaCl and 5 mM $CaCl_2$ were prepared and zones of lysis measured after 24 h incubation at 40°C; 100% equals 8.2 mm diameter.

NaCl. There was little difference between the lysis of elastin by macrophage enzyme in the presence of 0 or 200 mM NaCl but 1 M NaCl reduced the activity to about 10% of the maximal level. 200 mM NaCl was routinely included in all assays.

RELATIONSHIP OF ELASTINOLYSIS TO ENZYME CONCENTRATION. The relationship between the area of the zone of lysis and the amount of macrophage elastase was linear except at very high or low enzyme concentration, as shown in Fig. 1. Over a wider range, 5.5–9 mm zone of lysis, the relationship of log enzyme concentration to area (or μg elastin hydrolyzed) gave a straight line of high correlation coefficient. It was noted that pancreatic elastase standard curves had a different slope from that for macrophage elastase. Since the hydrolysis of elastin by pancreatic elastase is known to involve a complex reaction (5, 6), these empirically derived relationships (linearity of concentration with area of elastin solubilized) were used to prepare standard curves and it should be emphasized that a similar relationship may not be valid for purified macrophage elastase. In practice, CM samples were usually assayed at two concentrations and standards of varying concentration were also included. Deviation from linearity of the standard curves could be reduced by loading samples of the same volume and by filling the wells of the elastin agarose plates with 50 mM Tris-HCl buffer, pH 7.6, containing 200 mM NaCl and 5 mM $CaCl_2$, 60 min before adding the samples.

ELASTINOLYSIS AS A FUNCTION OF TIME. One of the advantages of the visible elastin-agarose assay is that samples are allowed to react until accurate measurements are possible. At 40°C the area of the zone of lysis increased linearly for 80–100 h and then increased somewhat more slowly for up to 24 days, with a reasonably linear relationship between area of zone of lysis and log time. Readings were taken three times at 16, 20, 24, 40, 48, or 72 h and compared with standard curves for each time. The ratio of activities of different samples therefore remained constant with assay time. Semiquantitative comparisons between samples could be made at much longer times of incubation. These findings also suggest that the elastase is stable for long periods of time in the presence of substrate.

REPRODUCIBILITY. Samples of macrophage elastase were placed in 25 wells in

two elastin-SDS-agarose plates of the same batch. A high degree of reproducibility was found (±2.6% for diameter of the zone of lysis and ± 4.7% for elastase units). The variability between plates of different batches was somewhat higher and standards of pancreatic elastase were used to compare results.

UNITS OF MACROPHAGE ELASTASE ACTIVITY. The unit of macrophage elastase was defined as the amount of enzyme producing a zone of lysis of 7.0 mm diameter (from a well 5 mm in diameter) in 24 h at 40°C and pH 7.6, on agarose plates containing 800 μg elastin/200 μg SDS per ml, and measured in unstained plates; this represented the hydrolysis and solubilization of approx. 1 μg of elastin/h. Under these conditions approx. 0.02 μg of purified pancreatic elastase was equivalent to 1 U of macrophage elastase activity.

SENSITIVITY. The assays with elastin-SDS-agarose plates were more sensitive than assays with elastin-orcein. An experiment was made in which 20 μl and 100 μl of macrophage CM were incubated in a final vol of 1 ml with 2 mg elastin-orcein in 50 mM Tris-HCl buffer at 40°C for 24 h. Virtually no activity could be detected in this reaction whereas 20 μl of medium gave a zone of lysis of 6.5 mm diameter in 24 h at 40°C in the plate assay.

PROCESSING AND STABILITY OF THE MACROPHAGE ELASTASE. In a previous study (12) macrophage CM was dialyzed and lyophilized to concentrate the collagenase released in culture. Although the macrophage elastase was routinely measured in unconcentrated CM, it was often convenient to examine samples for both enzymes. Conditioned media were analyzed for elastase directly, after dialysis for 2 days in 8/32 Visking tubing at 4°C against three changes (100 vol) of 10 mM Tris-HCl buffer pH 7.6, containing 1 mM $CaCl_2$, and after freeze-drying and reconstitution in water at 10% of the original volume. Widely varying losses of enzymic activity (25–80%, usually about 50%) were found after dialysis, but there was no further loss after freeze-drying. However, the loss with dialysis was

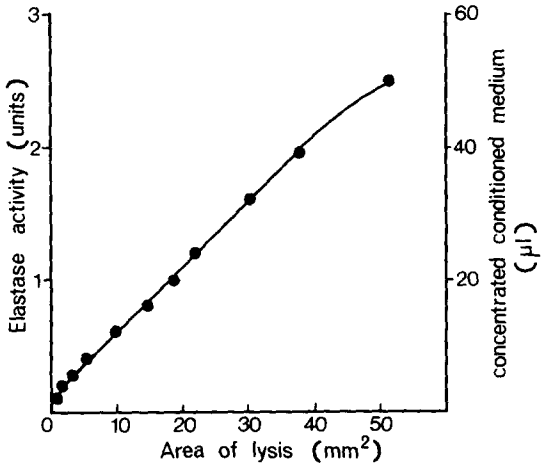

FIG. 1. Relationship between hydrolysis of elastin and concentration of macrophage elastase. CM from thioglycollate-stimulated macrophages was concentrated by lyophilization as described in the Materials and Methods section and reconstituted at 5% of the original volume. Aliquots were diluted as indicated and 40 or 50 μl were placed in 5-mm diameter wells in agarose-elastin-SDS plates. Readings were made after 24 h at 40°C.

constant within each experiment and the relative ratios of elastase to collagenase activity of processed and unprocessed samples differed by less than 5%.

Enzymic activity in unprocessed CM was stable at −20°C for at least 3 mo and unaffected by several cycles of freezing and thawing. After dialysis and freeze-drying the reconstituted elastase was often lost after repeated freezing and thawing, perhaps by sticking to the glass or small precipitates of protein usually present in the concentrates.

Secretion of Macrophage Elastase

DISTRIBUTION OF ELASTASE IN CELL LYSATES AND CONDITIONED MEDIUM. Both the plasminogen activator and collagenase have been found to be macrophage secretion products for which only a small amount of active enzyme is stored within the cells (11, 12), and the elastase also had this distribution. Although elastase activity was detected readily in CM, little activity was found directly in cell lysates (Table II). The presence of inhibitors within cell lysates was tested in

TABLE II

*Distribution of Elastase in Stimulated Macrophage Cultures**

Source of enzyme	Elastase activity
	U/culture
Cell lysate	55
Conditioned medium	810

* 4×10^7 thioglycollate-stimulated macrophages were cultivated in Dulbecco's medium containing 15% AT-FBS. After 48 h the cells were washed and placed for 72 h in Dulbecco's medium with 0.05% LH. Conditioned medium (30 ml) and cell lysates were processed as described in text and reconstituted in 1.5 ml and 0.3 ml respectively. The recovery of elastase activity in the CM was 78% after processing.

mixing experiments with active preparations of CM and no inhibition was detected. These experiments suggested that elastase was another secretion product of thioglycollate-stimulated macrophages.

TIME-COURSE OF SECRETION OF ELASTASE FROM STIMULATED MACROPHAGES. The ability of macrophages to continue to produce and release elastase in culture was next examined. Stimulated macrophages were maintained in the presence of AT-FBS for 48 h, washed, and then placed in serum-free medium containing 0.2% LH. Conditioned medium was collected and replaced with fresh serum-free medium every 2 days for six consecutive cycles. As shown in Fig. 2 the macrophages continued to release elastase into the medium at a remarkably constant rate for as long as 12 days in culture.

REQUIREMENT FOR PROTEIN SYNTHESIS. The effect of cycloheximide on the extracellular accumulation of elastase activity was next studied. As shown in Table III cycloheximide, even at 0.5 μg/ml, abolished the appearance of elastase in the medium. There was no increase in the cellular level of elastase under these conditions and hence protein synthesis is required for the extracellular appearance of elastase.

ELASTASE SECRETION IN RELATION TO CELL NUMBER. The relationship between

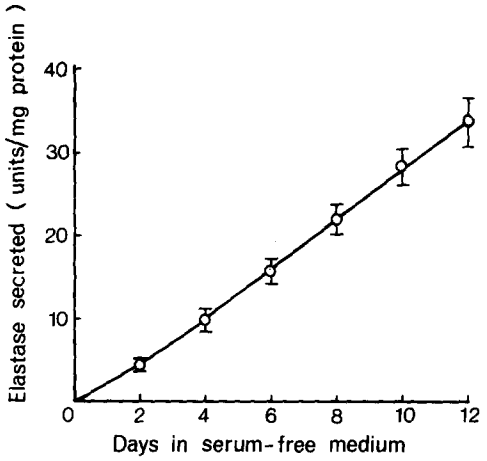

FIG. 2. Time-course of elastase secretion from thioglycollate-stimulated macrophages. The figure shows cumulative secretion of elastase by 7×10^6 macrophages (0.71 ± 0.12 mg cell protein). The bars show the mean and SEM of triplicate cultures.

TABLE III

Effect of Cycloheximide on Elastase Secretion from Stimulated Macrophages

Treatment	Concn.	Time	Elastase	
			U/flask	% Control
	μg/ml	*h*		
None	—	11	11.4	100
	—	22	19.8	100
Cycloheximide	2	11	0	0
		22	0	0
	0.5	11	0	0
		22	1.1	5.5

Thioglycollate-stimulated macrophages were plated in 50-ml flasks (approx. 7×10^6 macrophages), and cultured for 48 h in Dulbecco's medium + 15% AT-FBS. The cultures were then washed three times with medium and 2.5 ml of Neumann and Tytell medium added, with or without cycloheximide, to duplicate cultures. An aliquot (0.3 ml) of medium was taken from each flask at 11 h and the rest of the medium harvested at 22 h.

elastase secretion, the number of thioglycollate-stimulated cells plated and cell protein recovered is shown in Fig. 3. Elastase secretion was directly proportional to cell number and protein up to an optimum plating density of 5×10^6 cells/flask. This experiment shows that cell density does not influence elastase secretion.

Regulation of Macrophage Elastase

CELL STIMULATION. Both collagenase and plasminogen activator secretion are regulated by macrophage activation (11, 12, 25); elastase secretion is also controlled by this mechanism. After thioglycollate stimulation the elastase

secretion was increased as much as 30-fold over the secretion from unstimulated cultures whereas the levels of cathepsin D, a lysosomal hydrolase, were similar for both the stimulated and unstimulated cultures (12). Cell lysates prepared before and after cultivation contained little elastase activity in the case of both the stimulated and unstimulated macrophages as was also found for macrophage collagenase (12). The difference in the levels of elastase secreted from thioglycollate-stimulated and unstimulated macrophages is illustrated in the photograph of the zones of lysis in an elastin plate produced by CM concentrated by lyophilizing (Fig. 4 *a*).

EFFECT OF PHAGOCYTOSIS ON THE SECRETION OF ELASTASE FROM UNSTIMULATED MACROPHAGES. Since phagocytosis of latex particles by macrophages is able to stimulate the production and secretion of plasminogen activator (25) and

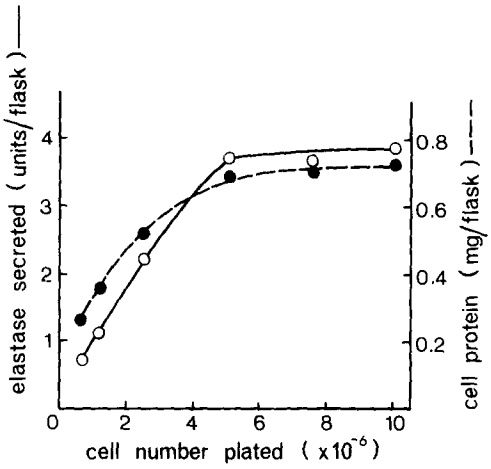

FIG. 3 Elastase secretion in relation to macrophage number. Thioglycollate-stimulated cells (0.6 – 10 × 10^6) were plated in 50-ml flasks in the presence of AT-FBS. The cultures were washed and refed at 2 and 24 h and placed in Dulbecco's and 0.2% LH from 48 to 144 h.

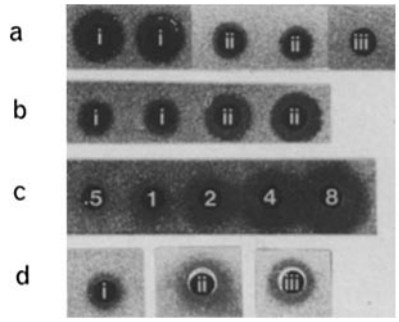

FIG. 4. Elastinolysis by elastases. (*a*) Conditioned medium from 1 × 10^7 macrophages (*i*) thioglycollate-stimulated, (*ii*) unstimulated, (*iii*) Dulbecco's medium only, no cells. (*b*) Conditioned medium from 1.2 × 10^7 macrophages (*i*) unstimulated, (*ii*) unstimulated + latex. (*c*) Pancreatic elastase (0.5 – 8 μg/ml). (*d*) (*i*) rabbit fibroblast conditioned medium, (*ii*) rabbit heterophil leukocyte granule lysates, (*iii*) human lysosomal elastase (20 μg/ml). 24 h incubation.

collagenase (12), the effect of latex uptake on elastase secretion by unstimulated macrophages was next examined. Although ingestion of latex increased the elastase secretion at least threefold (Table IV, and Fig. 4 *b*), this was still only 25% of the secretory rate of thioglycollate-stimulated macrophages. The increased rate of secretion of elastase and collagenase was sustained for at least 13 days after phagocytosis and these secretory rates were maintained equally well in Dulbecco's medium containing 0.2% LH or in Neumann-Tytell medium (Table V). These data suggest that the control of release of elastase is strongly co-ordinated with the release of collagenase (12) and plasminogen activator (25).

TABLE IV

*Effect of Phagocytosis on the Secretion of Elastase from Unstimulated Macrophages**

Macrophages	Elastase secretion
	U/72 h/mg cell protein
Unstimulated	8.8 ± 1.7 (6)‡
Unstimulated + latex	29.4 ± 2.7 (6)
Thioglycollate-stimulated	115.5 ± 3.6 (3)

* Average of three experiments in which $1 - 2 \times 10^7$ cells were plated initially (0.3 – 0.7 mg cell protein finally; phagocytosis increased the cell protein by 10 to 82%). The unstimulated macrophages were fed latex particles as described in the Materials and Methods section. Conditioned media were prepared by exposing the monolayers for 72 h to Dulbecco's medium containing 0.2% LH. The difference between the elastase secretion from the unstimulated macrophages and the macrophages fed latex was statistically significant ($P < 0.001$).

‡ Number of samples.

TABLE V

Time-course of Secretion of Elastase and Collagenase from Unstimulated Macrophages after Phagocytosis of Latex

Collection period (days after phagocytosis)	Elastase secreted	Collagenase secreted
	U/flask	*U/flask*
1–4	39	0.038
4–7	54	0.040
7–10	50	0.029
10–13	44	0.026

Unstimulated macrophages (1.6×10^7) were cultivated in Dulbecco's medium in 15% AT-FBS, in duplicate flasks. After 24 h the cells were fed latex and placed in the same medium for a further period of 24 h. The cells were then washed and placed in either Dulbecco's medium + 0.2% LH or in Neumann-Tytell serumless medium. Conditioned media were collected for four 3-day cycles and assayed for elastase and, after concentration, for collagenase. Final cell protein was 1.2 mg/flask. 0.7×10^7 thioglycollate stimulated macrophages (0.71 mg cell protein) secreted 62 U elastase/72 h in a parallel experiment. Values shown are the average of two flasks.

STUDIES WITH OTHER CELLS. Both collagenase (12, 26) and plasminogen activators (11) are also synthesized and secreted by cells other than macrophages. Accordingly, elastase secretion by a number of cell types was examined. As shown in Table VI both stimulated, and to a lesser extent unstimulated, mouse macrophages secreted elastase, but only a very low level of elastase activity could be detected in the CM of rabbit fibroblasts and none in CM of melanoma and other established mouse cell lines. A trace of activity was found in rabbit alveolar macrophage lysates and rabbit heterophil leukocyte granules.

TABLE VI
*Elastases from Various Cell Types**

Cell type	Elastase activity		
	Cell lysate	Medium	
		U/24 h/mg cell protein	U/24 h/10^7 cells
	U/10^7 cells		
Mouse macrophages			
Unstimulated	<1	3	1.8
Thioglycollate-stimulated	10	61	68
Mouse established cell lines			
L-cell fibroblasts (1R)	0	0	0
3T3 cells	0	0	0
Melanoma cells (PG 19)	0	0	0
Rabbit synovial fibroblasts‡			
(*a*)	0	< 1.5§	< 12§
(*b*)	ND	0	0
BCG-induced rabbit alveolar macrophage lysates	< 10§	ND	ND
Rabbit heterophil leukocyte granules‖	< 10§	ND	ND

* Mouse macrophages, established mouse cell lines and rabbit fibroblasts were cultured in serum-free Dulbecco's medium +LH for 72 h. Media were concentrated before assay. Alveolar macrophage lysates and leukocyte granule lysates were prepared in 0.1% Triton X-100.

‡ (*a*) Secreting ~ 2 U collagenase/10^6 cells/24 h, (*b*) secreting < 0.1 U collagenase/10^6 cells/24 h.

§ Weak diffuse zones of lysis on elastin plates.

‖ Enzyme not EDTA sensitive and is inhibited by soybean trypsin inhibitor. ND, not determined.

The granulocyte enzyme was inhibited by soybean trypsin inhibitor (5 mg/ml) and unaffected by EDTA (20 mM), and thus is distinct from the macrophage and fibroblast (unpublished observation) enzymes.

Characterization of the Macrophage Elastase

EFFECT OF INHIBITORS. The elastase present in the culture medium from thioglycollate-stimulated macrophages was characterized with the aid of potential inhibitors. As shown in Table VII, the enzyme is a serine proteinase because of inhibition by Dip-F and phenylmethane sulfonylfluoride. It was not inhibited effectively by the chloromethyl ketones derived from tosyl-lysine or tosyl-phenylalanine, nor by the two tetrapeptide alanyl chloromethyl ketones which

TABLE VII

*The Effect of Potential Inhibitors on Macrophage Elastase**

Compound	Concn. in preincubation mixture	Time of preincubation	Activity
		h	%
None	—		100
Dip-F	40 mM	0.1	0
	4 mM	0.1	19
	1 mM	1.0	39
	1 mM	20	7
Phenylmethane sulfonylfluoride	10 mM	3	34
	1 mM	3	76
Ac-$(Ala)_3$-$AlaCH_2Cl$	2.0 mM	1.0	61
	0.5 mM	1.0	69
Ac-$(Ala)_2$-Pro-$AlaCH_2Cl$	2.0 mM	1.0	40
	0.5 mM	1.0	93
	0.2	0.1	111
Tos-$LysCH_2Cl$	2 mM	0.1	81
	0.2	0.1	102
Tos-$PheCH_2Cl$	2 mM	0.1	78
	0.2 mM	0.1	96
Isopropanol	20% (vol/vol)	0.1	100
4-nitrophenyl-4′-guanidinobenzoate	0.2 mM	0.1	62
NaCl	1.1 M	0.1	29
EDTA, calcium salt	40 mM	0.1	8.5
EDTA, trisodium salt	50 mM	0.1	0
	20 mM	0.1	23
Chelex-calcium salt	—	0.1	103
1,10-phenathroline	2 mM	0.1	7
4-chloromercuribenzoate	1 mM	0.1	68
Dithiothreitol	20 mM	0.1	8.5
	1 mM	0.1	83
Normal human serum	5% (vol/vol)	0.1	0
Soybean trypsin inhibitor	8 mg/ml	0.1	88
	2 mg/ml	0.1	129
Lima bean trypsin inhibitor	2 mg/ml	0.1	102
Chicken ovoinhibitor	2 mg/ml	0.1	19
Turkey ovomucoid	2 mg/ml	0.1	87
Dextran sulfate 500	200 μg/ml	0.1	100

* 100 μl of enzyme was mixed with 25 μl of inhibitor to give concentrations listed above, and the mixture preincubated for indicated times. 25 μl aliquots were placed in 5.0 mm diameter wells in the elastin plates. Zones of lysis were measured after 20 h incubation at 40°C and 100% activity was equivalent to a zone of lysis of 8.2 mm diameter.

were designed as active site reagents for pancreatic elastase (27) and which also inhibit human spleen elastase[2] (10). The active site titrant for trypsin, 4-nitrophenyl-4′-guanidinobenzoate gave partial inhibition at relatively high concentrations. Chelating agents inhibited the elastase. However, it is likely that the divalent cations act by stabilizing the enzyme or enzyme-substrate complex since pretreatment of the enzyme with chelating resin (Chelex) had no effect on enzyme activity. Thiol reagents such as dithiothreitol inhibited the enzyme either by reducing essential disulfide bridges, or by acting as chelating agents. Although serum abolished the enzyme activity completely, trypsin inhibitors from soybean and lima bean, and turkey ovomucoid (28) were without effect. In fact, soybean trypsin inhibitor (1 mg/ml mixed with the elastase) often enhanced the elastase activity by up to 40%, presumably by inhibiting another proteinase which was destroying the elastase. Chicken ovoinhibitor, an inhibitor of human spleen elastase (10, footnote 2) and other serine proteinases (29), was an effective inhibitor. Since soybean trypsin inhibitor does inhibit human granulocyte elastase, an experiment was made to test its failure to inhibit mouse macrophage elastase. Elastin-SDS-agarose plates were prepared containing in the gel 1 mg of soybean trypsin inhibitor/ml. No inhibition of macrophage elastase activity was seen in this experiment. Dextran sulfate, a stimulator of collagenase (20) and plasminogen-activator activities (S. Gordon, unpublished observation) had no effect on the elastase.

BINDING TO ELASTIN. The tightness of binding of macrophage elastase to elastin was studied in an experiment in which elastin was used to adsorb the enzyme from solution. Tubes containing 50 μl CM from thioglycollate-stimulated macrophages were incubated with 0.5 ml of 50 mM Tris-HCl buffer alone, buffer containing 4 mg elastin, buffer containing 4 mg elastin:1 mg SDS complex, or supernatant fluid from the elastin-SDS complex (to check for the presence of free SDS) for 30 min at 25°C. The elastin was removed by centrifugation and the supernates tested for enzymic activity. The activities found were 100%, 85%,, 0%, and 95% respectively showing that the enzyme bound more firmly to elastin-SDS than to free elastin. The strong binding of the elastase to elastin indicated that it was a cationic protein like other elastases.

ACTIVITY OF MACROPHAGE ELASTASE ON CHROMOGENIC-ESTER SUBSTRATES. CM containing elastase activity was tested at pH 7.8 for the ability to hydrolyze Ac-$(Ala)_3$-4-nitroanilide, a substrate less susceptible to hydrolysis by nonspecific esterases than the usual elastoesterase substrates. This substrate is similar to the Ac-$(Ala)_3$-OMe substrate of Gertler and Hofmann (30), but more stable. No activity was found even after 60 h at 40°C. Comparable levels of elastinolytic activity of pancreatic elastase readily hydrolyzed this substrate. There was also no detectable activity against Z-Ala-2-ONap or Bz-Phe-2-ONap (10 min, 50°C) by 100 μl concentrated culture medium containing 250 U of elastase whereas high activities were seen with equivalent elastinolytic preparations of pancreatic or spleen elastase.

ACTIVITY AGAINST AZOCASEIN. In addition to their elastinolytic activities both pancreatic and granulocyte elastases have a broad spectrum of proteolytic activity (6, 7, 10). The proteolytic activities of conditioned medium from thioglycollate-stimulated macrophages and pancreatic elastase were compared

against elastin and azocasein. The macrophage CM had elastinolytic activity equivalent to 2.0 μg of pancreatic elastase/ml but the trace of azocasein-degrading activity observed represented less than 0.1 μg of pancreatic elastase. Macrophage CM does contain an azocasein-degrading proteinase which becomes measurable after dialyzing and concentrating conditioned medium, but this is probably a different proteinase.[4] Hence it seems likely that the macrophage elastase has a narrower range of specificity than the other two elastases.

Discussion

Our evidence suggests that the elastase secreted by mouse macrophages differs from the better known mammalian elastases (Table VIII). In comparison with the porcine pancreatic and human lysosomal enzymes which have rather broad proteolytic and elastinolytic activity (7, 10), the macrophage elastase is more restricted in its substrate specificity. Unlike these enzymes the macrophage

TABLE VIII

Properties of Mammalian Elastases in Three Species

	Macrophage (mouse)	Pancreas (pig)	Lysosomal (human)
Substrate			
Ac-$(Ala)_3$-4-nitroanilide	−	+	+
Chloroacetate naphthol AS-D	−	+	+
Elastin	+	+	+
Azocasein	±	+	+
Inhibitor			
Dip-F	+	+	+
Chicken ovoinhibitor	+	+	+
EDTA	+	±	−
Ac-$(Ala)_3$-$AlaCH_2Cl$	−	+	+
Turkey ovomucoid	−	+	+
Soybean trypsin inhibitor	−	−	+

elastase does not attack synthetic substrates such as Ac-$(Ala)_3$-4-nitroanilide, or the less specific elastoesterase substrates in a way which releases the chromogenic end groups. It is also relatively resistant to the active site-directed alanyl chloromethyl ketones designed for pancreatic elastase (27) and is unaffected by turkey ovomucoid, inhibitor of both the other elastases (10, 28, 29). Although all three types of elastase are serine proteinases sensitive to Dip-F and chicken ovoinhibitor (10, 28), the macrophage and pancreatic enzymes differ from the granulocyte elastase in their sensitivity to chelating agents and resistance to soybean trypsin inhibitor. With respect to its limited protein substrate specificity and metal ion requirements, the macrophage elastase resembles the microbial elastases isolated from *Flavobacterium elastolyticum* (32) and *Pseudomonas aeruginosa* (33), respectively, the latter being a common contaminant of water supplies and thus, possibly, elastin plates.

The differences between the mammalian elastases are consistent with a

[4] Werb, Z., and S. Gordon. Manuscript in preparation.

distinct catalytic site for the macrophage enzyme as already seems likely for the pancreatic and granulocyte enzymes. However, species differences may contribute significantly to the distinctive properties since human and porcine pancreatic elastases also differ (34). Our preliminary studies suggest that human monocytes in culture also secrete an elastinolytic enzyme which differs from both human pancreatic and lysosomal elastases and which resembles the mouse macrophage enzyme in its inhibition by EDTA and resistance to soybean trypsin inhibitor.[5] Janoff and his co-workers detected a small amount of elastin-degrading activity in human alveolar macrophage lysates which hydrolyzed the synthetic Ac-$(Ala)_3$-OMe esterase substrate less readily than comparable pancreatic and granulocyte elastases (35), and human blood monocytes do not stain with naphthol AS-D chloroacetate, an elastoesterase substrate which stains neutrophil granules (31). Further study using purified macrophage elastase is clearly needed to establish its unique physical and catalytic properties.

The macrophage elastase can be differentiated from other neutral proteinases present in the conditioned medium from thioglycollate-stimulated macrophages. The plasminogen activator, also a serine proteinase, resists chelating agents, is sensitive to 4-nitrophenyl-4′-guanidinobenzoate, and its fibrinolysis is strictly dependent on plasminogen (11). The collagenase, a metalloproteinase, is insensitive to Dip-F (12). Macrophages also secrete neutral proteinase(s) active against azocasein, denatured fibrinogen and gelatin which display(s) a different inhibitor profile.[4]

The regulation of all these different neutral proteinases, however, seems to be closely co-ordinated in the macrophage. Induction of their secretion is associated with cell stimulation, for example, by thioglycollate treatment in vivo, and secretion is enhanced by phagocytosis. They are all secreted in parallel over prolonged periods in culture (11, 12, 25). As a class, these products can be readily differentiated from other secretory products, such as lysozyme and intracellular acid hydrolases, which are regulated independently (13).

It is probable that macrophage elastase plays a central role in tissue injury associated with chronic pulmonary disease such as emphysema. Leukocyte neutral proteinases have been implicated in the pathogenesis of this disease since it was shown that hereditary deficiency of α_1-trypsin inhibitor results in premature emphysema (37, 38) and that aerosols of leukocyte lysates and proteinases induce lesions resembling emphysema in dogs (39) and rodents (40). Although the role of the pulmonary alveolar macrophage in emphysema has not been defined, they are active during primary response to dusts and air pollutants and are present in large numbers in the superimposed recurrent bronchiolitis (36). Our studies clearly indicate that the prolonged secretion of elastase, and other neutral proteinases, by viable macrophages could contribute a great deal to the breakdown of the elastic tissue and to injury to blood vessels. The uptake of nondegradable irritant materials from the atmosphere could result in high levels of elastase secretion by the alveolar macrophages. It is already known that alveolar macrophages are more numerous in smokers and that these cells utilize more glucose and contain more prominent Golgi vesicles, endoplasmic reticulum, and residual bodies than macrophages obtained from nonsmokers (41).

[5]Barrett, A. J., Z. Werb, and S. Gordon. Unpublished work.

It should be possible to demonstrate enhanced secretion of elastase by macrophages from such human subjects directly in culture. Cultivated mouse macrophages, however, provide a well characterized model system for further studies on regulation of elastase secretion and its role in disease.

Summary

Thioglycollate-stimulated mouse peritoneal macrophages secrete a proteinase which degrades insoluble elastin. There is little elastase activity in cell lysates but the bulk of the enzyme accumulates extracellularly during culture in serum-free medium. The secretion of elastase is sustained for over 12 days in culture and continued secretion of elastase requires protein synthesis. Unstimulated macrophages secrete very little elastase activity but can be triggered to secrete higher levels of this enzyme by phagocytosis and intracellular storage of latex particles.

The macrophage elastase is a distinctive proteinase differing from the elastases of pancreas and granulocytes and is distinct from the other secreted proteinases of macrophages, namely, collagenase and plasminogen activator. The macrophage elastase is a serine proteinase and is inhibited by di-isopropyl phosphorofluoridate, ovoinhibitor, EDTA, dithiothreitol, and serum. Its activity is little affected by soybean trypsin inhibitor, turkey ovomucoid and chloromethyl ketones derived from tosyl lysine, tosyl phenylalanine, and acetyltetra alanine. Hydrolysis by macrophage elastase of chromogenic ester substrates for pancreatic elastase could not be detected.

Elastase secretion by stimulated macrophages exceeds that by primary and established fibroblast cell strains. It is likely that elastase secretion by macrophages plays a major role in the pathogenesis of chronic destructive pulmonary diseases such as emphysema.

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