Lung Injury Induced by Leukocytic Proteases

Aaron Janoff, PhD, Richard White, PhD, Harvey Carp, BS,
Sarah Harel, PhD, Rosemarie Dearing, BS, and Dorothy Lee, BS

Human polymorphonuclear neutrophilic leukocytes (PMNs) contain large amounts of neutral proteases that can degrade elastin, collagen, proteoglycan, and basement membrane. The instillation of one of the purified enzymes (elastase) into dog lungs *in vivo* causes degradation of elastic fibers and other alveolar septal components and results in anatomic changes similar to those of human pulmonary emphysema. Cigarette smoking is a major risk factor associated with pulmonary emphysema in man. One mechanism for this association may be interference with the regulation of PMN elastase activity by alveolar antiproteases. This possibility is supported by the observation that the oxidizing activity of tobacco smoke inactivates α_1-proteinase inhibitor *in vitro*. Macrophages also secrete an elastolytic protease, albeit at low levels. The short-term exposure of cultured mouse macrophages to cigarette smoke augments the rate of elastase secretion by these cells. Mouse macrophage elastase is not inhibited by α_1-proteinase inhibitor or α_2-macroglobulin. This unusual property of macrophage elastase may facilitate its attack upon elastin over prolonged intervals despite very low levels of macrophage elastase production. A unified hypothesis of lung injury in pulmonary emphysema is presented, involving both PMN and macrophage elastases and the actions of cigarette smoke. (Am J Pathol 97:111-136, 1979)

Neutrophil Elastase

Proteases of Human Polymorphonuclear Neutrophilic Leukocytes (PMNs) and Their Potential Substrates in the Lung

The protease content of the cytoplasmic granules of human PMNs has recently been reviewed by Baggiolini and co-workers.[1] The azurophil granules of these cells contain elastase,[2] cathepsin G (a chymotrypsin-like protease),[3] and a third enzyme, which (like the aforementioned two) is also a serine-proteinase.[1] In addition, the azurophil granules contain cathepsins D and B. The specific granules of PMNs contain two metalloproteinases, of which one is the specific collagenase originally described by Lazarus.[4] Thus, all four families of proteolytic enzymes (carboxyl, thiol, serine, and metallo) are represented in the two classes of granules of these cells.

From the Department of Pathology, State University of New York at Stony Brook, Stony Brook, New York.

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Address reprint requests to Dr. Aaron Janoff, Department of Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794.

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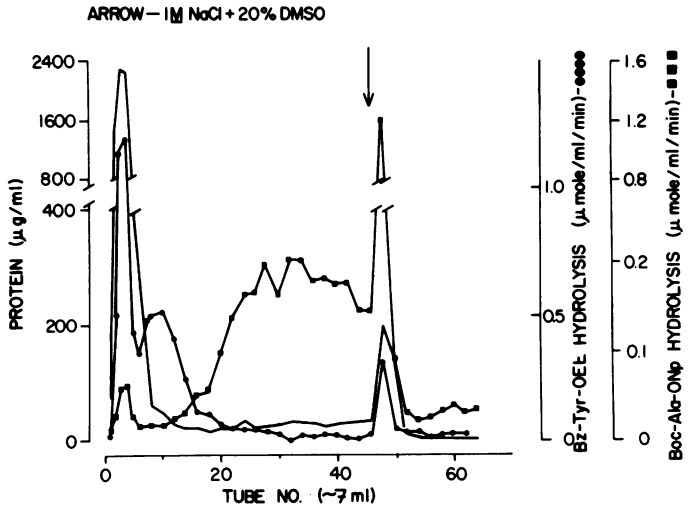
One of the neutral serine proteases of human PMNs, elastase is capable by itself of degrading several different structural components of lung connective tissue. The potential substrates of this important enzyme include elastin,[5,6] proteoglycan,[7-9] basement membrane,[10] and collagen.[9] In addition, pulmonary surfactant apoproteins are digested by PMN elastase,[11] as are intermediates of the kinin, complement, and clotting systems and immunoglobulins.[12] Thus, the liberation of elastase from PMNs during lung inflammation can cause injury to a wide spectrum of structurally and functionally important molecules. Since the granule-stored enzymes, including elastase, are all released extracellularly during phagocytosis of immune complexes by PMNs,[13-15] these enzymes can be important mediators of lung injury during immunopathologic reactions affecting that organ.

Purification of PMN Elastase

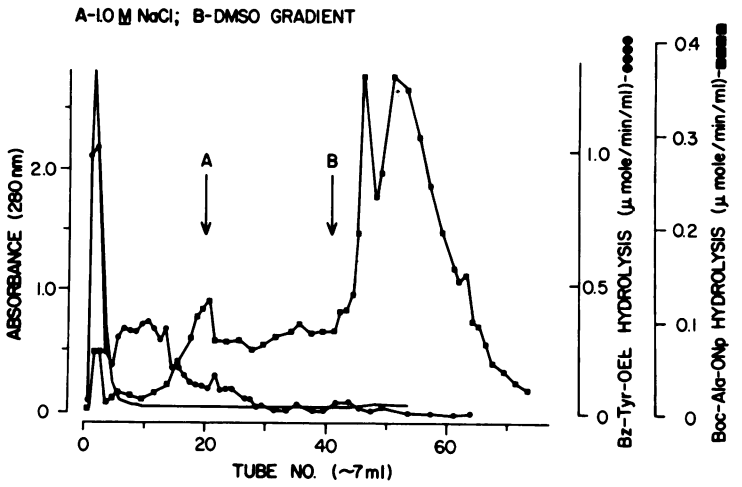
To facilitate studies of tissue injury by PMN elastase, purification of the enzyme became an early goal of investigators in this field. Human PMN elastase was successfully purified by a number of different laboratories.[3,17-22] One method [20] for the isolation of the enzyme is the following.



TEXT-FIGURE 1—Affinity chromatograph of low-salt (0.10 M NaCl) granular extract on PhBuN-Affi-Gel column. The PhBuN-Affi-Gel column (10 cm × 1.2 cm) was equilibrated and run in the cold with pH 7.5, 0.01 M $NaPO_4$ buffer containing 0.5 M NaCl. The sample was applied to the column in the same buffer. Buffer change (*arrow*) was to phosphate buffer containing 1.0 M NaCl and 20% Me_2SO. Flow rate, 20 ml/hr. Substrate: Bz-Tyr-OEt, ●——●; Boc-Ala-ONp, ■——■. Bz-Tyr-OEt = Benzoyl-tyrosine ethyl ester. Reprinted with permission.[20]

Leukocytes are harvested from normal human peripheral blood by dextran sedimentation and hypotonic lysis of erythrocytes. The leukocytes are then suspended in 0.34 M sucrose and disrupted by mechanical means (passage through fine wire-mesh screen under suction) or by the addition of heparin, and the granule fraction is finally collected by differential centrifugation. Granular proteins are extracted and crudely fractionated by freezing and thawing the granule pellet in buffers of varying salt concentrations. The elastase is nearly completely solubilized in 0.1 M NaCl, buffered with 0.01 M phosphate to pH 7.5. Affinity chromatography of this extract on a sepharose column containing covalently linked phenylbutylamine (PhBuN-Affi-Gel) yields 90–100% pure neutrophil elastase with 60–70% recovery of starting enzyme.

Text-figures 1 and 2 represent two typical elastase purification runs with 0.1 M salt extracts of leukocyte granules. In both cases, the bulk of granular proteins is eliminated in the run-through fractions. Either of two procedures is then followed in order to obtain purified elastase. In one procedure (Text-figure 1), column washing with 0.5 M NaCl (pH 7.5) is



TEXT-FIGURE 2—Affinity chromatograph of low-salt (0.10 M NaCl) granular extract on PhBuN-Affi-Gel column. The PhBuN-Affi-Gel column (10 cm × 1.2 cm) was equilibrated and run in the cold with pH 7.5, 0.01 M $NaPO_4$ buffer containing 0.5 M NaCl. The sample was applied to the column in the same buffer. First buffer change (*arrow A*): same $NaPO_4$ buffer containing 1.0 M NaCl. Second buffer change (*arrow B*): 0–20% Me_2SO gradient in pH 7.5, 0.01 M $NaPO_4$ containing 1.0 M Nacl. Flow rate, 20 ml/hr. Substrate hydrolysis: Boc-Ala-ONp, ■——■: Bz-Tyr-OEt, ●——●. Reprinted with permission.[20]

continued until elastase activity, monitored by hydrolysis of tert-butyloxycarbonyl-L-alanine-p-nitrophenyl ester (Boc-Ala-ONp), begins to appear in the column effluent. Washing with this buffer is then continued until elastase activity of effluent fractions begins, in turn, to decrease. At that time, the column wash is changed to 1.0 M NaCl + 0.01 M phosphate buffer, pH 7.5, + 20% dimethylsulfoxide (ME_2SO) (Text-figure 1, arrow). This wash elutes a sharp peak of additional elastase activity from the column, as shown in Text-figure 1.

Another procedure for recovery of purified elastase is shown in Text-figure 2. In this method, column washing with 0.5 M NaCl (pH 7.5) is stopped as soon as the bulk of chymotrypsin-like activity has passed through the column, and a 1.0 M NaCl (pH 7.5) wash without Me_2SO is begun (see arrow A in Text-figure 2). This wash is continued until hydrolysis of benzoyl-tyrosine-ethyl-ester(Bz-tyr-OEt) by effluent fractions (granulocyte chymotrypsin or cathepsin G) is no longer detectable, but before a significant amount of elastase activity has been eluted from the column. Thereafter, a Me_2SO gradient in 1.0 M NaCl (pH 7.5) is begun (arrow B in Text-figure 2). Under these conditions, the bulk of elastase elutes with that portion of the gradient between 5% and 12% Me_2SO and is obtained completely free of chymotrypsin-like activity. All traces of the latter have been eluted in the preceding 1.0 M NaCl wash.

The polyacrylamide disc gel electrophoretic pattern given by the purified elastase fraction is shown in Figure 1. A comparison of Gel 2A in this figure (purified elastase) and Gels 1A and 2 in Figure 2 shows the degree of isolation of the three well-characterized elastase isoenzymes achieved by the affinity chromatography procedures just described. The absence of detectable chymotrypsin-like protease (cathepsin G) in the purified elastase fraction is further substantiated by staining the gels with specific chromogenic ester substrates for the two proteases combined with oxidized p-rosaniline (compare 1B and 1C in Figure 2 with 2B and 2C in Figure 1). The faint bands in Gel 2C in Figure 1 in the elastase zone of the gel represent weak hydrolysis of the chymotrypsin substrate by elastase.

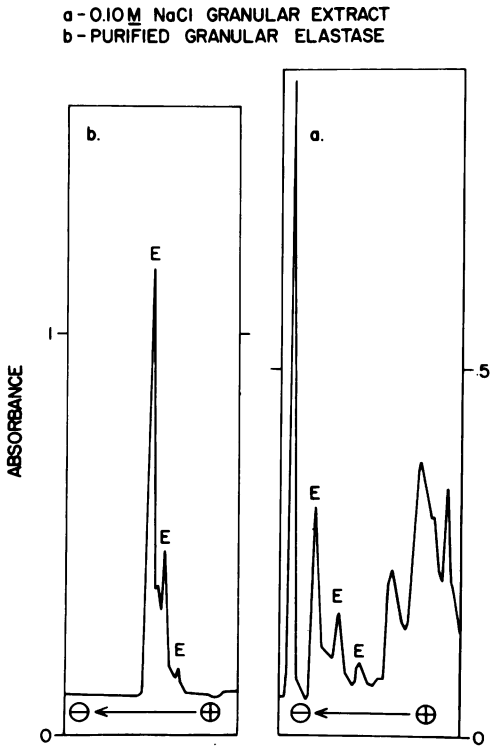
Text-figure 3 shows densitometric scans of gels 2A in Figure 1 and 1A in Figure 2 (panels b and a of Text-figure 3, respectively). The purified elastase constitutes 100% of the protein present in the gel pattern (Text-figure 3, b).

Induction of Experimental Emphysema by Endotracheal Instillation of Purified Human Neutrophil Elastase

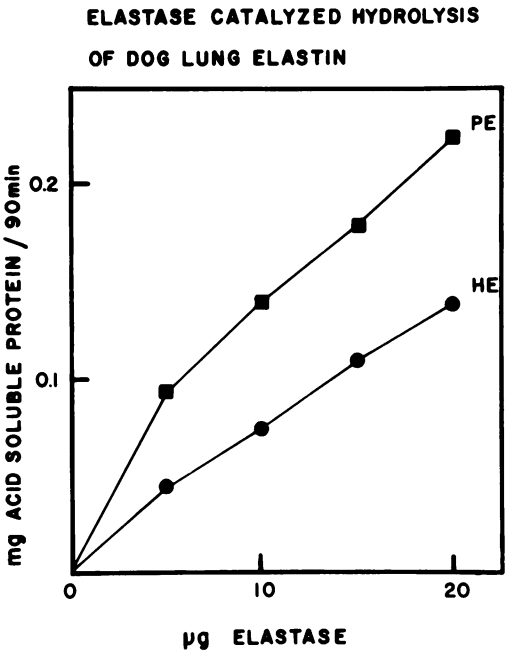
The solubilization of dog lung elastin *in vitro* by purified neutrophil elastase is shown in Text-figure 4. In this experiment, 10 mg dog lung elas-

tin was suspended in 1.0 ml of 0.01 M sodium phosphate buffer, pH 7.5, containing 0.25 M NaCl; and an appropriate amount of porcine pancreatic elastase or purified human neutrophil elastase was then added. The mixture was incubated with periodic shaking for 90 minutes at 37 C. The reaction was terminated by the addition of an equal volume of cold 10% trichloroacetic acid, and the acid-soluble peptides in the trichloroacetic acid supernatant were measured by the method of Lowry et al [23] by the use of a standard curve of bovine serum albumin. On an equal weight basis, the leukocyte enzyme was about 60% as active as the pancreatic enzyme on the lung substrate. On a molar weight basis, the neutrophil enzyme was calculated to have 75% of the activity of the pancreatic enzyme on this substrate.

Two different preparations of purified leukocyte elastase were administered to dogs via Swan–Ganz catheters placed into the lungs (under fluoroscopy) through an endotracheal tube. In one of these, the enzyme purity



TEXT-FIGURE 3—Scans of acrylamide disk gel electrophoretograms: *a*, low-salt (0.10 M NaCl) crude granular extract, 130 μg protein; *b*, elastase, 45 μg protein. Both stained with Buffalo black stain. Reprinted with permission.[20]



TEXT-FIGURE 4—Digestion of dog lung elastin by pancreatic (*PE*) and neutrophil (*HE*) elastases *in vitro*. Milligrams of acid-soluble peptides released are plotted on the ordinate versus micrograms of enzyme used on the abscissa. All values have been corrected for zero time and substrate minus enzyme controls. Reprinted with permission.[6]

was 90%, its concentration was 1 mg/ml, and a total of 1.33 mg was instilled. A separate lung site was treated with a 97% pure preparation at the same concentration (1 mg/ml), and a total of 2.52 mg of enzyme was instilled. Foci of alveolar destruction were observable at both sites 90 minutes after enzyme instillation. Morphometric analysis of the lesions was carried out, and the quantitative measurements of mean linear intercept were in agreement with the subjective impression gained from the histologic examination, namely, that PMN elastase had caused septal destruction (Table 1). The effects of the instilled enzyme on alveolar septal ultrastructure in the dog are shown in Figures 3 and 4.

Portions of dog lung that had been treated with human PMN elastase were also selected for enzyme localization studies using immunoelectronmicroscopy. Frozen sections 40 μ thick were cut and stained as floating sections with the following immunologic reagents. (Incubations were carried out at 37 C, to facilitate penetration of reagents.) Purified, monospecific rabbit anti-PMN elastase antibodies (see Figure 5) were diluted 1:20 in phosphate-buffered saline (PBS). Sheep antirabbit immunoglobulin antiserum (F[ab] fragments conjugated with horseradish peroxidase) was diluted 1:40. Peroxidase reaction product was developed by incubating the antiserum-treated tissue slices for 7 to 10 minutes with a

diaminobenzidine solution. Tissues were postfixed with glutaraldehyde and osmium tetroxide and dehydrated. After dehydration, sections were embedded in Epon and thin sections were cut by ultramicrotomy at 0.06 to 0.09 μ, using a Reichert OM-2 ultramicrotome. Sections were stained with uranyl acetate and lead citrate and examined using a JEM-7 transmission electron microscope operating at 80 kV. Controls were tissue sections exposed to PMN elastase, which were treated with nonimmune rabbit serum or absorbed antiserum as the primary reagent. Other controls were tissue sections exposed to the buffer vehicle alone and then treated with antielastase antibodies as the primary reagent. Control tissues were uniformly negative. Background activities of lung endogenous peroxidase and pseudoperoxidase (erythrocytes) were abolished by preincubation of tissues in absolute methanol for 20 minutes, followed by a rinse in 0.125% hydrogen peroxide in PBS for 20 minutes.

Figures 6 and 7 show the results of the immunoperoxidase staining to localize the instilled leukocyte enzyme in dog lung. Attachment of the elastase to lung elastic fibers can be seen in both figures.

Suppression of Endogenous Serum Elastase-Inhibitory Capacity by Cigarette Smoke—A Possible Mechanism for Augmentation of PMN-Elastase–Mediated Lung Injury in the Smoker

Persons who are genetically deficient in α_1-proteinase inhibitor (α_1Pi), a major serum inhibitor of elastase present in serum transudates such as alveolar lining fluid,[24] show increased susceptibility to emphysema.[25] However, α_1Pi deficiency accounts for only a small fraction of persons with emphysema, whereas cigarette smoking is recognized as the major risk factor in most emphysema patients.[26] Therefore, interference by cigarette smoke with local interaction between α_1Pi and elastase in the lung might help to explain the disease in smokers who are genetically normal for α_1Pi.

We recently approached this question experimentally. It was observed that freshly prepared, aqueous solutions of cigarette smoke could suppress

Table 1—Morphometric Analysis of *In Vivo* Experiment

Enzyme dose (mg)	Tissue zone*	Mean linear intercept value	
		Normal region	Diseased region
1.33	Central	0.072	0.143
	Peripheral	0.075	0.116
2.52	Central	0.073	0.185
	Peripheral	0.068	0.130

* Biopsies were done on tissue cores from a central zone adjacent to the tip of the instilling catheter and from a peripheral site more distant from the catheter tip.

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the elastase-inhibitory capacity of human serum. (Reaction mixtures were buffered at neutral pH.) Immunoelectrophoresis of mixtures of aqueous smoke solution, human serum, and pancreatic elastase showed decreased elastase–α_1-proteinase inhibitor complexes and increased free, active protease. Moreover, this action of cigarette smoke could be prevented by phenolic antioxidants known to trap free radicals, whereas model free-radical oxidants suppressed serum elastase-inhibitory capacity in a manner analogous to that of cigarette smoke.

The experiments outlined above have been described in greater detail elsewhere.[27] The major results of these experiments are summarized in Figures 8 and 9 and in Table 2 of the present report.

The fact that two phenolic antioxidants block smoke suppression of elastase inhibition by serum suggests that oxidation reactions in aqueous smoke solutions, perhaps proceeding through free radical formation, may be responsible for the serum suppression. This interpretation is consistent with evidence directly identifying free radical species in cigarette smoke, and with the wide range of free-radical–mediated activities such as peroxidation and alkylation displayed *in vivo* and *in vitro* by cigarette smoke.[28] The observations are also consonant with the report[29] that thymol blocks oxidation of cysteine sulfhydryl groups by aqueous solutions of cigarette smoke. The standard aqueous smoke solution used in our experiments was capable of oxidizing 3.4×10^{-4} moles of sulfhydryl.

α_1Pi contains sulfhydryl groups (cysteine residues) and thioethers (methionine residues). Both groups can be altered by oxidation or alkylation.[30] Indeed, pre vious work has shown that oxidation of methionyl thioether groups in chicken ovoinhibitor results in the loss of its elastase-inhibitory

Table 2—Summary of Radial Diffusion Elastinolysis Experiments

Reaction mixture	Area* of lysis	No. experiments
Elastase standard	100	14
Elastase, serum (control)	0	12
Elastase, serum (smoke)	49 ± 3	8
Elastase, serum (smoke), thymol	1 ± 1	5
Elastase, serum (smoke), hydroquinone	0	2
Elastase, smoke, thymol, or hydroquinone	100	5
Elastase, serum, N-chlorosuccinimide (model oxidant)	81 ± 4	3
Elastase, serum, hydrogen peroxide (model oxidant)	70 ± 10	3
Reaction mixtures containing model oxidants plus thymol	0	3

* Expressed as percentage of lysis given by the standard amount of pancreatic elastase tested alone (mean ± 1 SD).

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activity.[30] Cohen [31] and others [32] recently confirmed the loss of elastase-inhibitory activity of human α_1Pi following treatment with the oxidant chloramine-T. Cohen also showed that intravenous injection of chloramine-T results in transient depression of serum elastase inhibitory capacity *in vivo*.[31] Recent evidence suggests that a methionine residue may be present in the elastase-combining site of human α_1Pi.[33]

For all these reasons, we hypothesized that oxidation of methionine residues in α_1Pi by cigarette smoke was responsible for the observed loss of elastase-inhibitory capacity of smoke-treated serum. The view that methionine residues in α_1Pi may be an important target of smoke oxidants is further supported by the following type of experiment. Preincubation of the synthetic substrate butyloxycarbonyl-L-methionyl-p-nitrophenol with 10^{-3} M oxidizing agent (chloramine-T) for 5 minutes results in complete prevention of leukocyte elastase-catalyzed hydrolysis of the ester bond adjacent to methionine. Inhibition of hydrolysis is 98% ± 4% (four separate trials). In contrast, only 9% inhibition of hydrolysis occurs after identical pretreatment of butyloxycarbonyl-L-alanyl-p-nitrophenol with the oxidizing agent, indicating that loss of enzyme activity is dependent on the presence of a methionyl residue at the P_1 position in the substrate and is not due to effects of chloramine-T on elastase. Thus, oxidation of a susceptible methionine residue at the active site of α_1Pi could also prevent hydrolysis of the adjacent peptide bond by elastase and thus suppress formation of an inhibitor–enzyme complex. In this way, local suppression of elastase inhibitory activity of serum transudate in the alveolar lining fluid by inhaled oxidants (tobacco smoke, industrial waste gases, photochemically activated automobile emissions) may conceivably promote proteolytically mediated lung injury leading to the development of pulmonary emphysema.

Suppression of Serum Elastase Inhibitory Capacity by Reactive Oxygen Species Generated by Phagocytosing PMNs—A Possible Mechanism for Augmentation of PMN Elastase-Mediated Tissue Injury in Immune-Complex–Induced Lung Inflammation

Since leukocytes themselves produce and release several reactive oxygen species during phagocytosis [34] that also might be capable of inactivating α_1Pi, the local balance between protease and antiproteases may be disrupted during inflammation by the oxidative inactivation of α_1Pi in the microenvironment of PMNs. In this event, tissue components adjacent to PMNs at sites of inflammation would be even more susceptible to damage by proteases simultaneously released from these cells.

We tested this possibility in an *in vitro* system. Our results showed that human PMNs, phagocytosing opsonized antigen–antibody complexes,

produce dialyzable species of activated oxygen that are capable of partially suppressing the elastase-inhibiting capacity (EIC) of whole human serum or of purified human α_1-proteinase inhibitor. Serum EIC was partially protected by superoxide dismutase, catalase, or mannitol, suggesting that hydroxyl radical, formed by interaction of superoxide radical and hydrogen peroxide, might be responsible for this effect. Sodium azide also partly protected EIC, implicating myeloperoxidase-mediated reactions as well. An artificial superoxide radical-generating system, involving xanthine and xanthine oxidase, could be substituted for phagocytosing PMNs with similar resultant EIC suppression. Thus, oxidative inactivation of proteinase inhibitors in the microenvironment of PMNs accumulating at sites of inflammation may allow proteases released from these cells to damage adjacent connective tissue structures more readily. (See Figure 10). The experimental data that support this hypothesis have been reviewed in detail elsewhere.[35]

Macrophage Elastase

Macrophage Neutral Proteases

A considerable body of evidence has accumulated recently that shows that stimulated macrophages secrete a variety of neutral proteases in cell-culture systems. Protease secretion can be induced by a phagocytic challenge,[36] by nonspecific inflammatory stimuli such as intraperitoneal injection of thioglycollate,[36] or by lymphokines secreted from thymus-derived lymphocytes after exposure to specific antigens.[37–41] The macrophage-produced enzymes include a serine-dependent plasminogen activator with trypsin-like specificity [42] and a metallo-enzyme with specific collagenolytic activity,[43] which is secreted in proenzyme form and can be activated by trypsin or plasmin.[44] Macrophages also secrete an elastolytic protease that appears, in many of its properties, to be quite different from the PMN enzyme.[45,46]

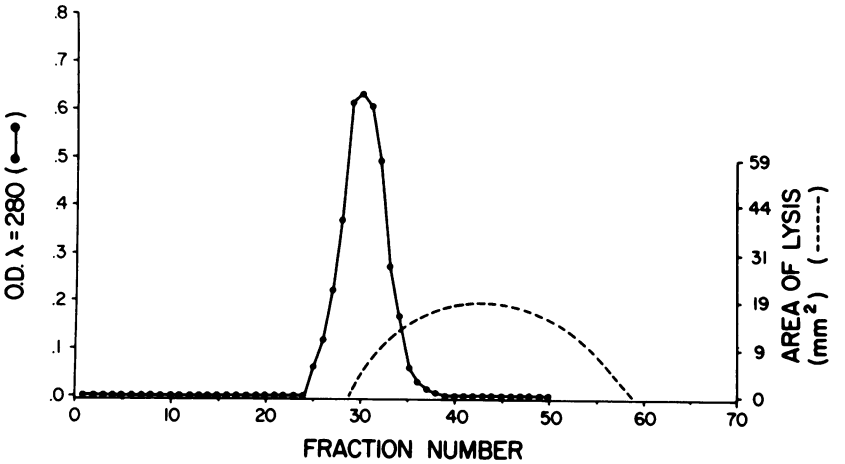
The discovery of elastase secretion by macrophages led to the suggestion that this enzyme might play a role in the degradation of lung elastin in pulmonary emphysema. Studies were therefore undertaken to isolate the enzyme and to explore its interaction with endogenous antiproteases.

Partial Purification of Mouse Peritoneal Exudative Macrophage Elastase

A scheme for purification of macrophage elastase based on multistep ion exchange and molecular-sieving chromatography has recently been reported by others.[47] One of the present authors has developed a simpler

method for isolation of this enzyme, using a single-step affinity chromatography procedure.[48] Serum-free conditioned medium of mouse peritoneal exudative macrophages is harvested at 2-day intervals over a 3-week culture period, and the pooled media are dialyzed extensively against 50 mM Tris buffer, pH 7.6, containing 200 mM NaCl, 5 mM $CaCl_2$ and 0.05% NaN_3. The dialyzed conditioned medium is then concentrated by ultrafiltration through a UM-2 Amicon Diaflo membrane. These procedures allow recovery of 80% of the elastase activity found in the original medium. The concentrated conditioned medium is applied to an affinity column containing sodium-dodecyl-sulfate(SDS)–treated, oxalic-acid–solubilized beef ligamentum nuchae elastin (α-elastin) covalently linked to sepharose beads. The optimal SDS:elastin ratio is 1:16 (wt:wt). The macrophage enzyme, although not permanently bound by the affinity column, is sufficiently retarded in its passage through the column to permit extensive purification. Using this one-step chromatographic procedure, we have obtained 90% purification of the enzyme with an average recovery of 80% of the activity present in the concentrated medium. Purification is monitored by SDS–polyacrylamide-gel electrophoresis. A more detailed description of the isolation and characterization of macrophage elastase has been presented elsewhere.[49]

Some results of our purification method are depicted in Text-figure 5



TEXT-FIGURE 5—Isolation of mouse peritoneal exudative macrophage elastase by affinity chromatography on SDS-treated, oxalic-acid–solubilized α-elastin, covalently linked to sepharose. Column dimensions: 1 cm × 25 cm. Flow rate, 4 ml/hr. Buffer: 0.05 M Trismaleate containing 0.2M NaCl + 0.005 M $CaCl_2$, pH 7.6. Fraction volume, 0.2 ml. Elastinolytic activity (----), measured as area of lysis in radial diffusion assays using SDS–elastin agarose plates, appears in the column effluent just after the major protein peak (●——●). Elastase is recovered nearly free of contaminating proteins in Fractions 40–50.

and Figure 11. It can be seen that a significant degree of purification can be achieved by using this convenient, single-step isolation procedure.

Studies on the Interaction of Mouse Peritoneal Exudative Macrophage Elastase with Endogenous Proteinase Inhibitors

Experiments were next carried out to test several endogenous serum proteinase inhibitors for activity against macrophage elastase. Some experiments were conducted with the use of crude conditioned medium from macrophage cultures, while others utilized the partly purified enzyme described in the preceding section. Inhibition assays employed SDS-treated ligament elastin as substrate. However, in experiments with α_2-macroglobulin, macrophage elastase was also tested for its ability to competitively block the binding of trypsin by the inhibitor.

Neither human nor mouse α_2-macroglobulin was found to complex with mouse macrophage elastase, as judged from failure of the enzyme to competitively block trypsin binding by α_2-macroglobulin. Elastolysis inhibition assays confirmed the inability of α_2-macroglobulin to inhibit mouse peritoneal exudate macrophage elastase. Similarly, α_1-proteinase inhibitor displayed no activity against this macrophage enzyme (see Table 3). Both of these antiproteases are well-established inhibitors of other elastases (eg, pancreatic elastase and PMN elastase). These results suggest unique behavior of macrophage elastase, namely, very high substrate specificity (a property not shared by the other two elastolytic enzymes). Since most

Table 3—Inhibition of Elastolytic Enzymes by Endogenous Antiproteases

Inhibitor	Elastolytic* enzyme tested		
	Porcine pancreatic elastase	Human PMN elastase	Murine macrophage† elastase
α_1-proteinase inhibitor (human)	+	+	−
α_2-macroglobulin (human)	+	+	−
α_2-macroglobulin (murine)	+	+	−
Whole serum (human, M phenotype)	+	+	+
Whole serum (human, Z phenotype)			+
Fractionated serum (human)‡			
α_1-proteinase inhibitor fraction	+	+	−
α_2-macroglobulin fraction	+	+	−
All other fractions			−

* The substrate, in all assays, was SDS-treated, bovine ligament elastin.
† Peritoneal exudative macrophages.
‡ Serum proteins were separated by preparative acrylamide-gel electrophoresis at pH 8.6.

endopeptidases hydrolyze a susceptible peptide bond in α_2-macroglobulin and are then complexed and inhibited by the antiprotease, the lack of interaction between α_2-macroglobulin and the macrophage elastase argues that this macrophage enzyme is not a broad-spectrum protease. Other elastolytic enzymes are broad-spectrum proteases.

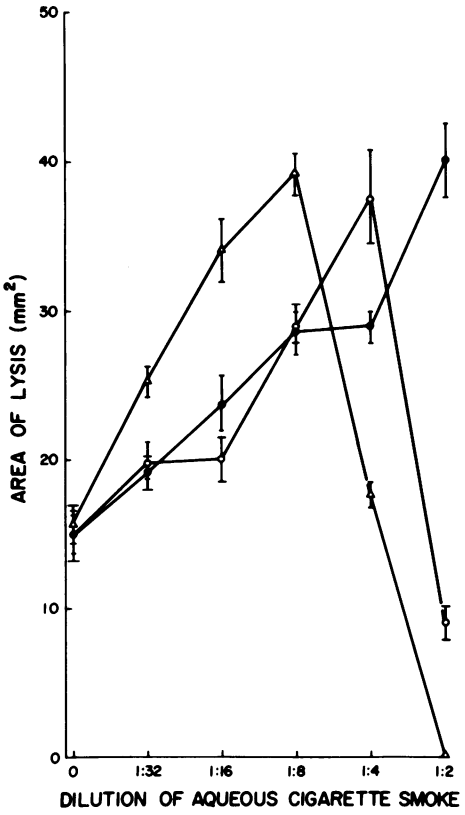
Despite the failure of α_1-proteinase inhibitor and α_2-macroglobulin to inactivate the macrophage enzyme, whole human serum does suppress this elastase (Table 3). Previous studies [46] showed that α_1-proteinase-inhibitor–deficient serum was as effective as normal serum in suppressing macrophage elastase, supporting the conclusion that α_1-proteinase inhibitor is not responsible for serum inhibition of macrophage elastase. The possibility was next explored that a serum component other than α-macroglobulin or α_1-proteinase inhibitor was responsible for serum suppression of macrophage elastase. Table 3 shows that no protein component that could be isolated by alkaline disc gel electrophoresis of serum inhibited the macrophage enzyme. Thus, the suppression of this elastase by whole serum may be due to nonspecific effects of serum proteins or other components (eg, binding to the elastin substrate), to the synergistic action of serum inhibitors (although no precedent for "synergistic inhibition" is known for other enzymes), or to a component of serum that is inactivated during the electrophoretic procedure. The foregoing results (summarized in Table 3), have been described elsewhere in greater detail, along with a fuller account of the methods used.[49]

The demonstration that macrophage elastase is less susceptible to endogenous proteinase inhibitors than is PMN elastase renders the macrophage enzyme a worthy candidate for further study in the context of lung injury. A role for this enzyme in emphysema should be considered, despite the generally low level of elastase secretion by macrophages. In this connection, recent findings suggest that there may be increased elastase secretion by the macrophages of smokers.[50] The following section describes some experiments designed to explore the enhancement effect of cigarette smoke.

Augmentation of Elastase Secretion by Mouse Peritoneal Exudative Macrophages Following Short-Term Exposure to Cigarette Smoke

Mouse peritoneal exudative macrophages cultured in the presence of aqueous cigarette smoke extracts show a sustained augmentation in elastase secretion that is dose- and time-dependent and can be inhibited by cycloheximide.

Text-Figure 6 shows the results of experiments designed to study the effect of different concentrations of smoke and different exposure times on



TEXT-FIGURE 6—The effect of various concentrations of cigarette smoke and durations of cigarette-smoke exposure on elastase secretion from mouse peritoneal exudative macrophages. Macrophages cultured in the absence of serum were pulsed for 5 minutes (●), 4 hours (○), or 24 hours (△) in the presence of aqueous extracts of cigarette smoke, diluted in Dulbecco's modified Eagle's medium containing lactalbumin hydrolysate as shown on the x axis. The cells were then cultured in the absence of smoke for the balance of 24 hours. The resultant conditioned medium was assayed using SDS–elastin–agar assay plates. Zones of lysis were measured after 72 hours' incubation of the plates at 37 C in a humidified environment. The vertical bars represent one standard deviation from the mean.

elastase activity recovered in the culture medium of mouse peritoneal exudative macrophages. As shown in the figure, low concentrations coupled with long exposure times, intermediate concentrations coupled with intermediate exposure times, and high concentrations coupled with short exposure times all provide stimulation of elastase production (approximate doubling of control values). Prolonged exposure of macrophages to a high concentration of aqueous smoke appears to inhibit enzyme production, which correlates closely with loss of cell viability (data not shown). In a separate experiment, it was determined that the presence of serum in the cultures during the interval of smoke exposure does not diminish enzyme response, supporting the view that enhanced elastase secretion by smoke-exposed macrophages might also take place under physiologic conditions.

When cells are exposed to a 1:2 dilution of smoke for 5 minutes, washed, and placed in medium containing cycloheximide (2.0 μg/ml) and the drug is maintained in the cultures for the duration of the 24-hour in-

cubation following smoke treatment, elastase activity in the culture medium falls below detectable levels. In the presence of this inhibitor of protein synthesis, neither control nor smoke-treated peritoneal exudative macrophages secrete measurable enzyme. Thus, the increased elastase activity of peritoneal exudative macrophages exposed to cigarette smoke involves an effect of smoke on enzyme production by the cells, which in turn requires active protein synthesis. (Moreover, the addition of aqueous smoke to the conditioned medium obtained from untreated control cultures does not increase the elastase activity of this medium, indicating that smoke does not directly enhance enzyme activity.)

If augmentation of macrophage elastase secretion by tobacco smoke also occurs in the lungs of cigarette smokers, and if the alveolar macrophage enzyme is also resistant to endogenous antiproteases, then this may be an important mechanism of lung injury in pulmonary emphysema (see Conclusions and Speculations). We are currently conducting experiments in our laboratory to determine whether chronic exposure of mice to cigarette smoke causes elevated elastase secretion by cultured alveolar macrophages harvested from such animals. Similar effects have been reported in human smokers.[50] If elevated secretion is observed, we will undertake further studies to learn the mechanism responsible for the enhancement. In addition to direct augmentation, other possibilities include a) recruitment of activated macrophages from the circulating monocyte pool and b) stimulation of macrophage secretion by lymphokines liberated from lymphocytes sensitized to tobacco antigens. Preliminary experiments with cultured mouse alveolar macrophages from normal animals have failed to show direct augmentation of elastase secretion after short-term exposure of these cells to aqueous smoke extract, although baseline elastase secretion by alveolar macrophages is higher than that of peritoneal cells.

Conclusions and Speculations

To conclude this brief review of our recent work on lung injury mediated by leukocytic proteases, we will propose a unified hypothesis of the role of elastase in the pathogenesis of pulmonary emphysema. According to this hypothesis, two different pathways can be envisioned for the production of lung injury in emphysema—one mediated by PMN elastase and the other by macrophage elastase. Which of the two is correct cannot be ascertained on the basis of present evidence.

In the first scheme, PMN elastase plays the central role. This enzyme is proposed as mediating the panacinar destruction of lung connective tissue

frequently seen in homozygous α_1-proteinase inhibitor deficiency states. The centrilobular destruction found in some individuals with normal inhibitor levels who smoke cigarettes is also attributed to the action of PMN elastase. The PMN enzyme is free to act because of suppression of α_1-proteinase inhibitor in the centrilobular respiratory lining fluid by inhaled cigarette smoke.

In the alternative scheme, macrophage elastase plays the central role. The panacinar disease of inhibitor-deficient individuals (minor population) is still mediated by the PMN enzyme, but the major population of emphysema subjects with centrilobular disease owe their lung destruction to macrophage elastase. Secretion of macrophage enzyme is enhanced by cigarette smoking in one or more of the ways proposed in the second part of this article. The lung damage produced by macrophage elastase is exacerbated by the enzyme's resistance to endogenous antiproteases.

The PMN scheme is attractive, in part, because of the large quantity of elastase present in these cells. A single neutrophil contains 4 to 5 pg of elastase,[51] a quantity at least four orders of magnitude greater than that secreted by an activated macrophage during 48 hours in culture. On the other hand, the macrophage scheme is attractive because of the apparent inability of α_1-proteinase inhibitor and α_2-macroglobulin to inhibit macrophage elastase and because alveolar macrophages are found in increased numbers in the centrilobular zones of the lungs of cigarette smokers.

If PMN elastase plays the key role in both forms of pulmonary emphysema in man, then synthetic inhibitors designed to inactivate this enzyme that are unaffected by oxidizing agents (eg, peptide chloromethyl ketones without methionyl residues) may afford widespread protection against the disease, both in smokers and in α_1-proteinase-inhibitor–deficient individuals. On the other hand, if macrophage elastase is the key determinant of injury in the smoker, then a new class of synthetic agents capable of inhibiting *this* enzyme will require development. Only future work will provide an answer to this very important question.

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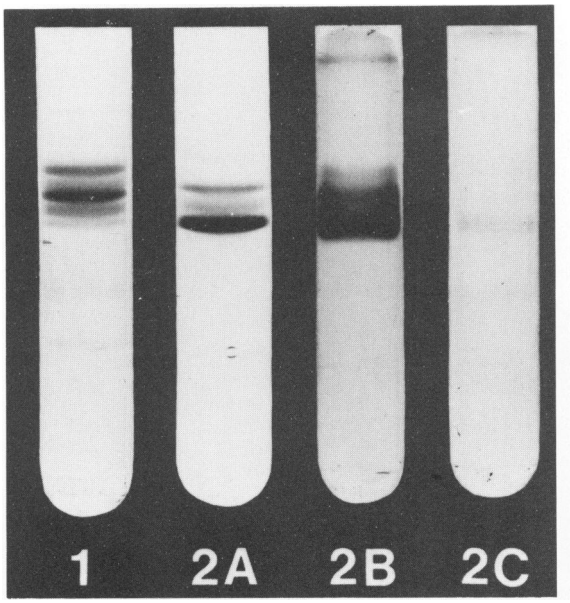
Parts of the discussions on granulocyte elastase and the role of elastase in emphysema have appeared in *Neutral Proteases of Human Polymorphonuclear Leukocytes*, edited by Klaus Havemann and Aaron Janoff (Baltimore and Munich, Urban and Schwarzenberg, 1978).

[*Illustrations follow*]



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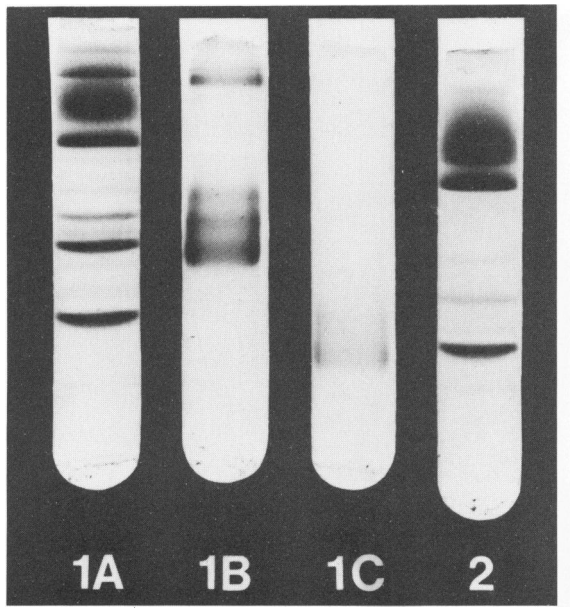

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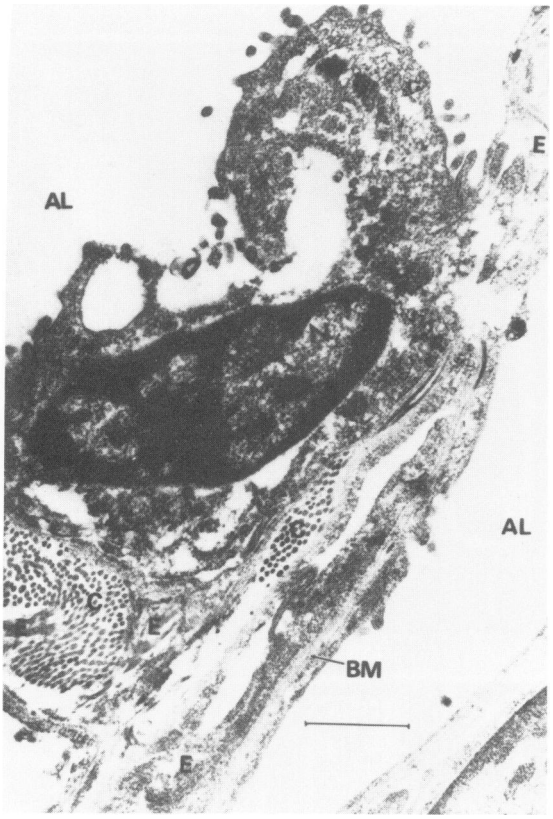
Figure 1—Acrylamide disk gel electrophoretograms. Purified elastase fractions. Early elastase (*1*); Elastase (*2*). Migration from top to bottom. Protein, 42.5 μg, Buffalo black stain (*1*); Protein, 45 μg, Buffalo black stain (*2A*); Protein, 12.9 μg, Ac-DL-Ala-1-ONap stain (*2B*); Protein, 43 μg, Ac-Phe-1-ONap stain (*2C*). (Ac-DL-Ala-1-ONap = acetyl-alanine-naphthyl ester, an elastase substrate; Ac-Phe-1-ONap = acetyl-phenylalanine-naphthyl ester, a chymotrypsin substrate.) Reprinted with permission.[20] **Figure 2**—Acrylamide disk gel electrophoretograms. Low-salt (0.10 M NaCl) crude granular extract (*1*) and "run-through" fraction from PhBuN-Affi-Gel chromatography (*2*). Migration from top to bottom. *1A* and *2*, 174 μg protein each, Buffalo black stain; *1B*, 116 μg protein, Ac-DL-Ala-I-ONap stain; *1C*, 116 μg protein, Ac-Phe-I-ONap stain. Reprinted with permission.[20]



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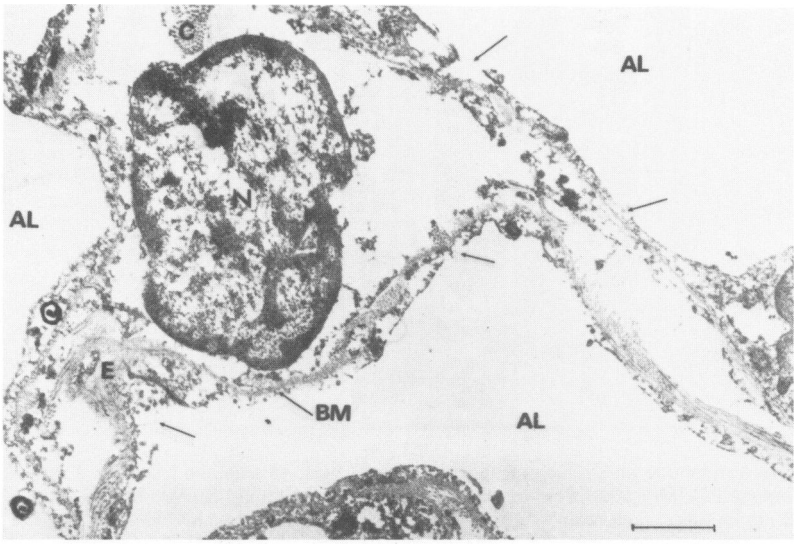

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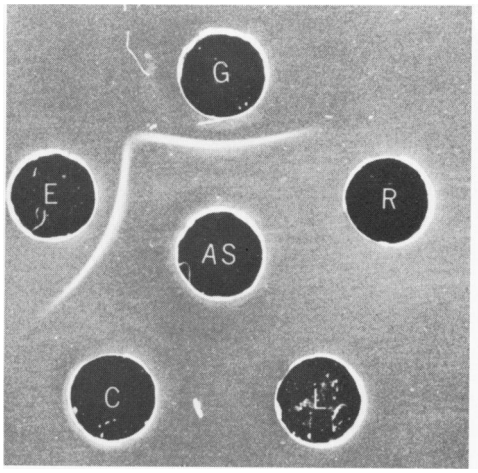
Figures 3 and 4—Production of emphysema by purified PMN elastase *in vivo*. Transmission electron micrographs of dog lung 90 minutes after buffer treatment (control) or treatment with PMN elastase. *In vivo* experiment. Uranyl acetate and lead citrate counterstain. A 1-μ marker is included for reference. **Figure 3**—Buffer-treated lung. Alveolar septum showing normal interstitial components including collagen bundles (*C*), elastin (*E*) and basement membrane (*BM*). *AL* = alveolar airspace. *N* = epithelial cell nucleus. Note intact septal structure. Reprinted with permission.[6] **Figure 4**—Enzyme-treated lung. Alveolar septum showing loss of interstitial components, except for residual collagen bundles (*C*), and a small amount of elastin (*E*). Numerous discontinuities in septal wall (*arrows*) are apparent. Note disrupted septal structure. See Figure 3 for definitions of other abbreviations. Reprinted with permission.[6]

Figure 5—Double immunodiffusion between rabbit antiserum to leukocyte elastase and different components of the granular extract. *AS* = anti-elastase antiserum, euglobulin-enriched fraction, 10 μl; *E* = purified leukocyte elastase, 2.8 μg; *G* = crude granular extract (0.1 M NaCl), 18.7 μg; *R* = run-through proteins of crude granular extract (PhBuN-Affi-Gel fractions), 17.4 μg; *L* = purified leukocyte lysozyme, 2.0 μg; *C* = purified leukocyte chymotrypsin-like enzyme, 3.9 μg. Reprinted with permission.[20] **Figures 6 and 7**—Localization of human PMN elastase in dog lung (from *in vivo* experiment). Uranyl acetate and lead citrate stain. A 1-μ marker is included for reference. **Figure 6**—Electron micrograph showing peroxidase reaction product distributed throughout lung connective tissue. Enzyme-treated lung. Rabbit antibody to neutrophil elastase as primary stain. Note microfibrillar structures (*small double arrows*) surrounding dense deposits of reaction product (*small single arrow*), which appears to be covering the amorphous component of elastic fibers. Collagen fibers (*C*) are relatively free of peroxidase reaction product. *N* = epithelial cell nucleus. Reprinted with permission.[6] **Figure 7**—Enzyme-treated lung. Antielastase antibody (rabbit) was used as primary stain. Two elastic fibers (*E*) are visible with attached peroxidase reaction product present at many locations (*single small arrows*), signifying adherent enzyme at these sites. The amorphous elastin component of the elastic fibers appears attenuated in several regions, exposing structures that may represent microfibrillar components of the elastic fiber (*double small arrows*). Intact collagen bundles (*C*) and a cell nucleus (*N*) are also visible. Reprinted with permission.[6]



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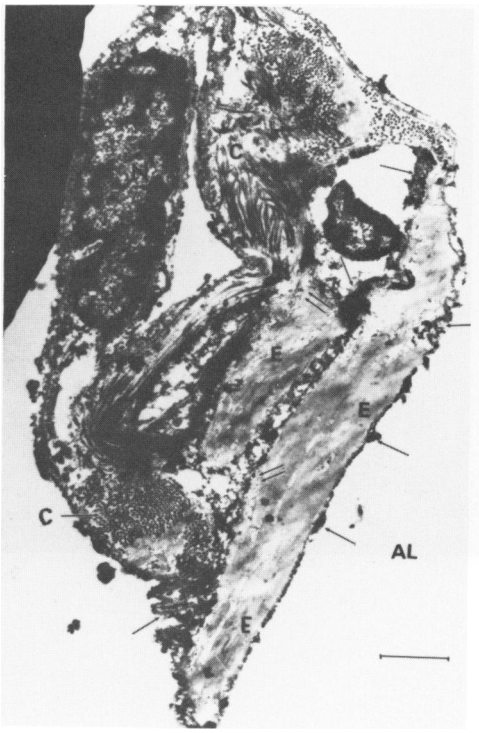

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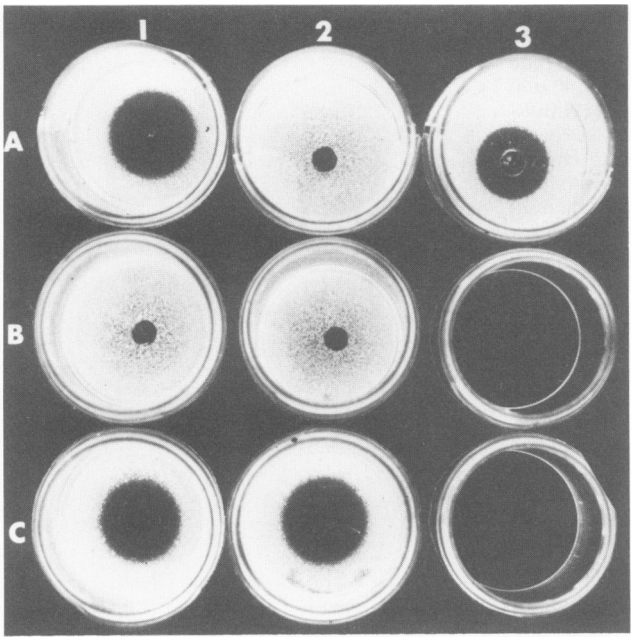

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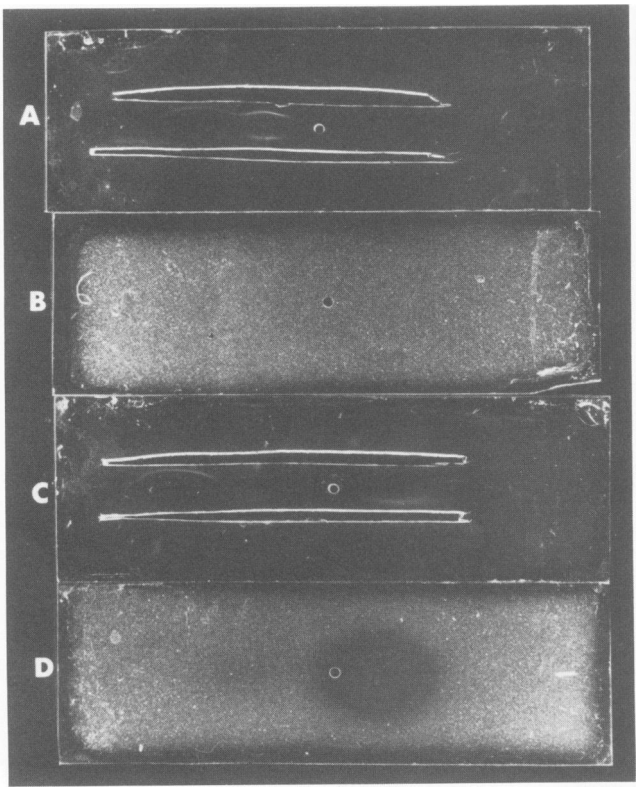

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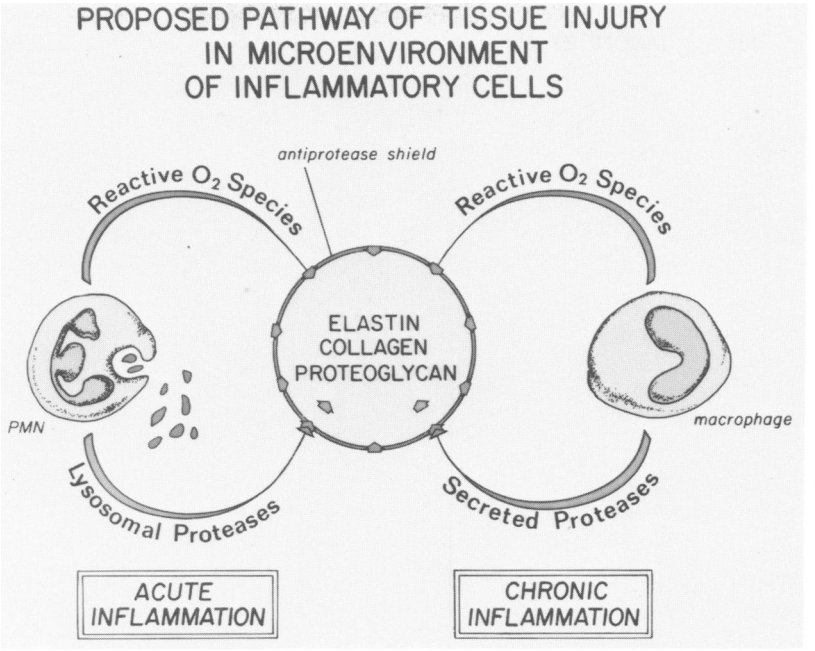
Figure 8—Analysis of elastase-inhibitory capacity of serum under different experimental conditions, by radial diffusion in elastin–agarose gels. *A1*, elastase alone in the presence of aqueous-soluble smoke components. *A2*, elastase incubated with untreated serum (control). *A3*, elastase incubated with serum in the presence of aqueous-soluble smoke components. *B1* and *B2*, conditions identical to *A3*, except that thymol (*B1*) or hydroquinone (*B2*) was incubated with aqueous smoke solution before the introduction of serum. Concentration of each phenolic antioxidant was 2.6×10^{-4} M. In wells *C1* and *C2*, conditions were identical to those in *A2*, except that N-chlorosuccinimide (*C1*) or hydrogen peroxide (*C2*) was incubated with the serum before the addition of elastase. Concentration of N-chlorosuccinimide was 6.6×10^{-4} M. Concentration of hydrogen peroxide was 8.8×10^{-2} M. *B3* and *C3* were unused wells. Reprinted with permission.[27]

Figure 9—Electrophoretic analysis of mixtures of serum and elastase with and without cigarette smoke. *A* and *C*, immunoelectrophoresis in agarose gels at a pH of 8.6. Upper trough, rabbit antiserum to human α_1Pi. Lower trough, rabbit antiserum to porcine elastase. *B* and *D*, electrophoresis in agarose gels at a pH of 8.6, containing finely divided ligament elastin. Anode to left, cathode to right. In *A* and *B*, untreated serum was incubated with elastase. Note presence of free α_1Pi migrating anodally, followed by complexes of α_1Pi–elastase. No free elastase was detectable immunologically (*A*) or enzymatically (*B*). In *C* and *D*, smoke-treated serum was incubated with elastase. Note increased free α_1Pi, decreased complexes of α_1Pi–elastase and cathodally migrating active uncomplexed elastase detected immunologically (*C*) and enzymatically (*D*). Note also that weak elastolytic activity was present in the region of the gel containing enzyme–inhibitor complexes (*D*). Thus, inhibition of elastase was incomplete, even in the case of complexes formed in the presence of smoke. Reprinted with permission.[27]



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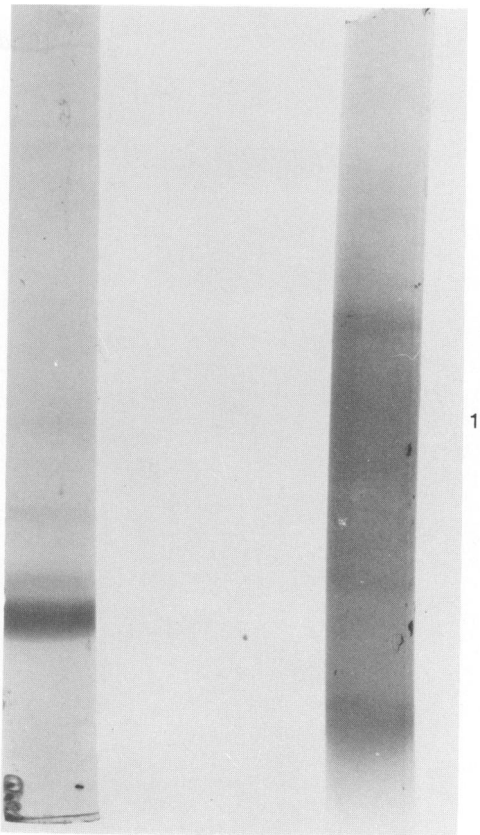

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Figure 10—A hypothetical scheme for exacerbation of protease-mediated tissue injury by reactive oxygen species liberated from PMNs. Phagocytosis of immune complexes by the cells leads to simultaneous release of granule-associated proteolytic enzymes and formation of activated species of oxygen, including superoxide radical, hydrogen peroxide, and hydroxyl radical. The reactive forms of oxygen inactivate antiproteases, such as α_1-proteinase inhibitor, in the microenvironment of the cells, thus allowing leukocytic proteases to more readily attack adjacent connective-tissue structures.

Figure 11—SDS-acrylamide gel electrophoretograms of the starting concentrated conditioned medium of mouse peritoneal exudative macrophage cultures (*right-hand gel*) and the reconcentrated, pooled elastinolytic activity recovered from the SDS-α-elastin-sepharose affinity column (*left-hand gel*). The purified material shows marked reduction in most protein components of the starting material, but shows simultaneous enrichment of one rapidly migrating protein species, which appears close to the anodal end of the gel (*anode at bottom*). Gels contained 10% acrylamide and 0.1% SDS. Gel pH = 8.8; current = 1 mA/gel; time of electrophoresis = 6 hours; Coomassie blue stain.

[*End of Article*]