Effects of Elastase, Collagenase, and Papain on Structure and Function of Rat Lungs In Vitro

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ABSTRACT Present concepts of the roles of collagen and elastin in lung elastic behavior and maintenance of lung structure have been largely inferred from anatomical observations or from studies of isolated fibers in vitro. Based on the intimate association of elastin and collagen it has been postulated that elastin contributes little to elastic behavior and that collagen is the major determinant of lung structure. Using clostridial collagenase, pancreatic elastase, and papain we have selectively degraded these fibers and studied the resulting changes in elastic behavior and structure of rat lungs in vitro.

Pressure-volume curves were recorded during continuous slow air inflation and deflation (10.5 ml/min) before and after the intratracheal instillation of 0.5 ml of control or enzyme solution. Surface tension–lowering activity of lavaged material was studied. All lungs were fixed inflated at 25 cm H_2O pressure and whole lung sections were stained for elastin, collagen, and reticulin.

Collagenase produced a marked susceptibility to pleural rupture but did not alter elastic behavior or lung structure. Elastase and papain produced segments of lung with increased compliance; this change was not due to alteration in surface forces but was associated with decreased tissue elastic recoil. Histologically, altered tissue recoil correlated well with evidence of damaged elastin fibers. In contrast to previous concepts these results suggest that elastin is the major connective tissue determinant of lung structure and elastic behavior.

INTRODUCTION

Although the etiology of human emphysema remains obscure, proteolysis has been proposed as a possible mechanism, based on the observation that individuals genetically deficient in α_1-antitrypsin are particularly prone to develop emphysema (1). Circulating leukocyte or alveolar macrophage lysosomes may provide the necessary proteolytic enzymes. Recent studies have shown that these cells contain elastolytic (2), collagenolytic (3), and other proteolytic enzymes active at physiologic pH (4). The effect on the lung of such enzymes has received little attention.

The proteolytic enzyme papain produces emphysema in experimental animals when injected intratracheally or delivered as an aerosol (5–11). The histological features of this lesion include dilatation of air spaces, particularly alveolar ducts, and alterations in elastin and reticulin fibers (11). Since papain has broad proteolytic activity, including elastolytic activity (12), it seems likely that the observed histological changes are due to enzymatic attack on lung proteins.

The relative contributions of elastin, collagen, and reticulin to lung structure and elastic behavior have been largely inferred from anatomical observations and studies of isolated fibers in vitro. From such studies it has been suggested that collagen is the major determinant of lung structure (13) and that elastin plays little role in lung elastic behavior (14). In contrast, the present study indicates that enzymatic attack of elastin is associated with marked architectural changes and altered elastic behavior of the lung.

METHODS

White male Sprague-Dawley rats weighing 200–250 g were anesthetized with intraperitoneal thiamylal, the chest opened widely, and the animals promptly exanguinated by severing the subclavian vessels. The trachea was cannulated with a polyethylene catheter (length 1.0 cm, I.D. 0.16 cm), and the heart, lungs, and trachea resected *en bloc*. About one-third of the specimens were discarded because of pleural air leaks demonstrated by inflation under water. The volume of the heart, lungs, and residual air was measured by water displacement before and after each series of manipulations.

Pressure-volume curves were performed with the preparation suspended in a 1.5 liter plethysmograph and the air-

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TABLE I

Compliance and Ventilated Volume before and after Intratracheal Instillation of Control or Enzyme Solutions*

Agent	Compliance			Ventilated volume		
	Before	After	Per cent change	Before	After	Per cent change
	ml/cm H_2O		%	*ml*		%
Saline	0.60 ±0.03‡	0.36 ±0.08	−40	8.94 ±0.31	5.94 ±0.19	−34
0.05 M Tris	0.65 ±0.06	0.42 ±0.05	−35	9.25 ±0.81	6.75 ±0.53	−31
0.2 M Tris	0.61 ±0.08	0.38 ±0.06	−38	7.94 ±0.70	5.44 ±0.64	−31
Collagenase (0.5 mg/ml)	0.55 ±0.05	0.33 ±0.04	−40	8.19 ±0.39	5.88 ±0.14	−28
Papain (10.0 mg/ml)	0.53 ±0.04	1.34 ±0.11	+149	7.45 ±0.48	5.40 ±0.41	−31
Elastase (4.5 mg/ml)	0.70 ±0.09	1.91 ±0.20	+173	10.13 ±0.63	6.75 ±0.84	−36

* Ventilated volume refers to the volume of air recovered during deflation.

‡ Mean ±SE.

way connected to a Harvard No. 600-000 infusion pump. The pump inflated and deflated the lungs with air at a rate of 10.5 ml/min. Inflation was continued until the upper plateau of the pressure volume was clearly established, as determined by direct observation of the tracing. Transpulmonary pressure was measured by a Statham PM5±.7 differential transducer connected to the airway and the plethysmograph. Pressure changes in the plethysmograph were measured with a Statham PM5±.3 transducer. The plethysmograph was calibrated with known volume injections. Signals were recorded with a Hewlett-Packard 1100 series recorder, stored on magnetic tape, and displayed on a Hewlett-Packard 1240A oscilloscope.

Initial pressure-volume curves were continuously recorded until reproducible plots were obtained. The preparation was removed from the plethysmograph, its water displacement measured, and 0.5 ml of one of the following solutions was instilled: 0.15 M saline; 0.2 M Tris buffer [tris(hydroxymethyl) aminomethane] pH 8.8; 0.001 M calcium chloride in 0.05 M Tris buffer, pH 7.6; papain[1] 10 mg/ml in 0.15 M saline; pancreatic elastase[2] 4.5 mg/ml in 0.2 M Tris buffer; clostridial collagenase[3] 0.5 mg/ml in 0.001 M calcium chloride in 0.05 M Tris buffer. Distribution of the solutions was made more uniform by injecting slowly while turning the lungs from side to side. Deposition of the solution in the lung periphery was achieved by immediately inflating the lungs with air until there was no visible atelectasis. The preparations were incubated for 30 min at room temperature (25°C), and the pressure-volume curves were repeated.

Continuous flow curves were used because most lungs developed small leaks during static curves which rendered the results unreliable. Such leaks occasionally occurred in control lungs but regularly appeared after the instillation of enzymes. Leaks were considered significant when the injected volumes of air could not be recovered or accounted for by an increase in residual air; such lungs were discarded. Since the inflation limb of the pressure-volume curve was more variable due to airways' opening pressure and nonuniform inflation, all data reported were obtained from the deflation limb. Compliance (C_L) was calculated from the steepest slope as determined by visual inspection of the tracings.

Surface tension activity of washings from control and enzyme-treated lungs was assessed. Repeated tracheal lavage with 2.0 ml volumes of saline was performed until at least 5 ml were recovered. Washings from two preparations incubated with each solution were pooled, and aliquots applied directly to the trough of a modified Wilhelmy balance.[4] After aging, tracings of surface tension and relative areas were displayed on an x-y plotter,[5] and tracings were analyzed by the technique of Clements, Hustead, Johnson, and Gribetz (15).

For histological study, the lungs were inflated and fixed with 10% neutral buffered Formalin at a pressure of 25 cm H_2O. Mid-coronal blocks of both lungs were cut, imbedded in paraffin, and sections of 6, 10, and 40 μ were prepared. Sections were stained with hematoxylin and eosin, aldehyde fuchsin stain for elastic tissue, Weigert's reticulin stain, and Gomori's trichrome stain for collagen. The mean linear intercept, L_M (the distance between alveolar surfaces) (16), was measured on aldehyde fuchsin–stained sections. In control lungs and lungs treated with collagenase, a 3 mm^2 grid was applied to the slide and single fields in alternate squares counted. In papain- and elastase-treated lungs, areas with normal and abnormal elastic tissue were counted separately; random selection of these areas was not possible because of the nonuniform distribution of enzyme.

The in vitro elastolytic activity of the enzyme preparations was assayed with the orcein-elastin technique described by Sachar, Winter, Sicher, and Frankel (17). Duplicate tubes were prepared containing increasing amounts of orcein-elastin (Worthington Biochemical Corp.) over a range of 2.5–20.0 mg, and 2 mg of elastase in 2 ml of 0.2 M Tris, pH 8.8. After 24 hr incubation at 37°C and centrifugation, aliquots were read in a Beckman spectrophotometer at 590 mμ. A standard curve, assuming 100% solubilization of elastin, of milligrams of elastin solubilized was plotted against optical density. Elastase, collagenase, and papain were

[1] Papain, Difco, Difco Laboratories, Detroit, Mich.

[2] Elastase, 2× crystallized, Worthington Biochemical Corp., Freehold, N. J.

[3] Clostridial collagenase, type III, Fraction "A", Sigma Chemical Co., St. Louis, Mo.

[4] Cahn RG Electrobalance, Cahn Instrument Company, Paramount, Calif.

[5] Model 560 x-y recorder, Honeywell, Inc., Test Instrument Division, Denver, Colo.

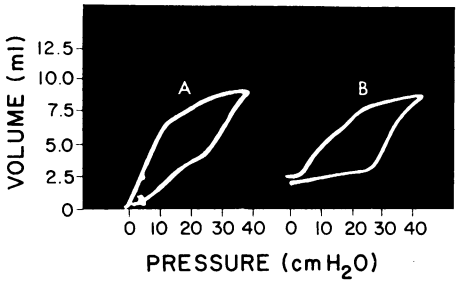

FIGURE 1 Pressure-volume tracing obtained from a control preparation before (A) and after (B) the instillation of 0.5 ml saline. A similar reduction in compliance was seen after the instillation of Tris buffers and collagenase.

studied in the same solutions as instilled into the lungs using 20 mg of orcein-elastin substrate. Incubation at 37°C was stopped after 20 min and the amount of elastin solubilized determined by reference to the standard curve.

RESULTS

After intratracheal instillation of 0.5 ml saline, or Tris buffers, the compliance and ventilated air volumes decreased proportionately (Table I, Fig. 1). Instillation of collagenase was followed by similar decreases. In contrast, papain and elastase produced an increase in compliance although the ventilated volumes decreased similarly to control lungs (Fig. 2). In all groups the residual air volume was increased and total lung capacity (the lung volume at a transpulmonary pressure of 25 cm H_2O) was slightly reduced after the instillation of liquid; no significant differences were found between control and enzyme groups. The mean percentage of the total lung capacity which demonstrated increased compliance after papain or elastase was 27% (range 23–34%). Greater concentrations than 10 mg/ml of papain or 4.5 mg/ml of elastase were attempted but regularly caused the preparations to leak. Although collagenase at a concentration of 0.5 mg/ml had a dissimilar effect on compliance, at greater concentrations it also produced air leaks.

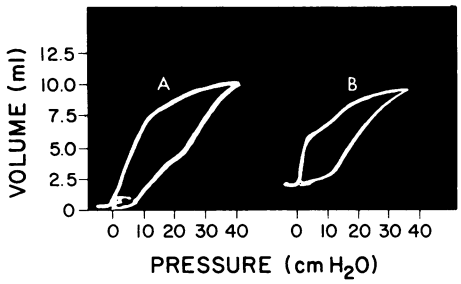

FIGURE 2 Pressure-volume tracings obtained from a preparation before (A) and after (B) the instillation of elastase, 4.5 mg/ml. Similar segments with increased compliance were seen after the instillation of papain, 10.0 mg/ml.

TABLE II

Surface Tension Activity of Washings from Lungs after Incubation with Control or Enzyme Solutions

Solution	Surface tension (γ)		Stability index
	Maximum	Minimum*	($\bar{S}$)‡
	dynes/cm		
Control§	39	17	0.78
Saline	41.5	16.5	0.86
0.05 M Tris	39	17.5	0.77
0.2 M Tris	41.5	17	0.87
Collagenase (0.5 mg/ml)	48	17	0.95
Papain (10.0 mg/ml)	49	19	0.88
Elastase (4.5 mg/ml)	51	19.5	0.88

* Minimum values for surface tension recorded after an 85% decrease in area.

‡ $\bar{S} = 2(\gamma \text{ max} - \gamma \text{ min})/(\gamma \text{ max} + \gamma \text{ min})$.

§ Two controls were not incubated with any solution before lavage.

The increase in compliance which occurred after elastase and papain could have been due to increased surfactant activity in these lungs. However, the surface tension–lowering activity of material recovered by lavage was similar in all groups (Table II).

Papain and elastase demonstrated significant elastolytic activity in the orcein-elastin assay system (Table III). Collagenase did not have demonstrable elastolytic activity.

Histologically, lungs which were instilled with saline or Tris buffer appeared normal. Lungs instilled with elastase or papain demonstrated a marked distortion of architecture. Enlargement of centrilobular air spaces, principally alveolar ducts, occurred focally throughout the lungs and were separated by areas with normal structure (Fig. 3). In abnormal areas, and only in such areas, elastin fibers in the lung parenchyma either failed to stain or stained with a coarsely granular appearance. The distance between alveolar surfaces (L_M) of the elastase- and papain-treated lungs was significantly increased in areas with abnormal elastin compared to

TABLE III

*Comparison of Elastolytic Activity**

Enzyme	Elastin solubilized	Activity compared to elastase 4.5 mg/ml
	mg	%
Collagenase (0.5 mg/ml)	0	0
Papain (10.0 mg/ml)	1.17	19.5
Elastase (4.5 mg/ml)	6.0	100

* Orcein-elastin assay with 20 min incubation (15).

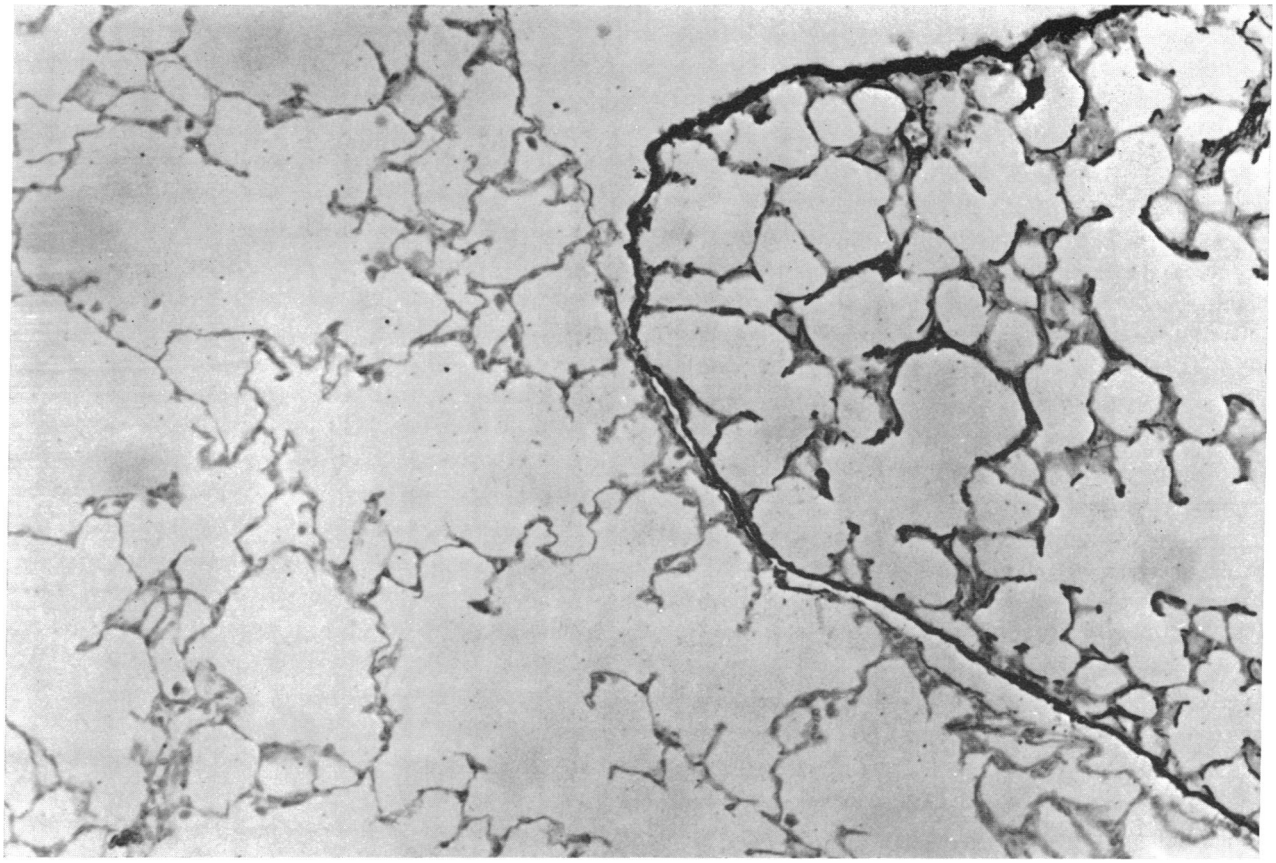

FIGURE 3 Low power photomicrograph of a rat lung instilled with elastase, 4.5 mg/ml. Normal elastic tissue is present in the pleura and parenchyma of the lobe seen on the right but remnants of elastic tissue remain only in the pleura of the lobe on the left. Dilatation of alveolar ducts and alveoli corresponds to the absence of staining elastic tissue. (Aldehyde fuchsin stain, × 285)

normal areas of the same lungs or compared to control lungs (Table IV). Normal areas of elastase- and papain-treated lungs, collagenase-treated lungs, and controls had similar values for L_M ($P > 0.05$).

Alveolar reticulin fibers were diminished after papain and elastase in the most markedly abnormal areas, and occasional alveolar walls were devoid of all silver-staining fibers. Collagenase also produced distinct fragmentation of reticulin; this finding was not associated with altered lung architecture. None of the enzymes caused demonstrable alterations in larger collagen fibers.

DISCUSSION

A decrease in compliance of in vitro lungs after the intrabronchial instillation of small volumes of saline in the absence of measurable changes in surface tension forces has been observed previously by others (18). While the small quantities of control solutions instilled in the present study may have altered *in situ* surface forces not measured in lavaged material, most of the decrease in compliance was explained by the observed smaller ventilated volumes due to the exclusion of some lung units by fluid retained in small airways.

TABLE IV
Mean Linear Intercept (L_M)

Group	L_M	
	mm	
Control	0.075 ±0.003*	
Collagenase (0.5 mg/ml)	0.068 ±0.002	
Papain (10.0 mg/ml)		
Normal areas	0.069 ±0.002	($P < 0.001$)
Abnormal areas	0.094 ±0.003	
Elastase (4.5 mg/ml)		
Normal areas	0.065 ±0.003	($P < 0.001$)
Abnormal areas‡	0.107 ±0.004	

* Mean ±SEM.
‡ "Abnormal areas" refer to areas in which the elastin failed to stain or had a coarsely granular appearance with aldehyde fuchsin.

At least three mechanisms could explain the increased compliance observed in segments of the lungs treated with elastase or papain: (*a*) reduced surface tension, (*b*) reduced tissue elastic recoil, or (*c*) altered geometry of the alveolar spaces. Since the surfactant activity of material recovered during lung lavage was similar in all groups, it seems unlikely that elastase or papain produced an increase in surfactant activity.

An estimate of tissue elastic recoil can be obtained from the measurement of alveolar dimensions after liquid-filled fixation at a constant pressure. Surface forces are minimized in liquid-filled lungs, so the size of air spaces in the specimens fixed for histological study is directly related to tissue elastic properties. While the L_M of control and collagenase-treated lungs were quite uniform and agreed well with published data on alveolar size in the rat (19), the markedly enlarged air spaces observed after treatment with elastase or papain indicate that tissue elastic recoil was reduced in these areas.

An increase in alveolar diameter could have reduced transpulmonary pressure at a given lung volume by virtue of the Laplace relationship without a change in either surface or tissue forces. The observed increase in lung compliance could have occurred if papain and elastase caused either focal destruction and loss of lung parenchyma or a loss of tissue elastic recoil without actual tissue loss, leaving in either case enlarged air spaces surrounded by normal tissue. Our data cannot differentiate between the effects of tissue loss and a change in elastic properties. However, since tissue elastic recoil has been reported to account for only 33% of total lung recoil in the rat (20), we can conclude that the increase in compliance which we observed after papain and elastase cannot be explained by a simple loss of tissue recoil alone; changes in alveolar geometry must have contributed to the altered pressure-volume relationships at low lung volumes.

Several lines of evidence support the hypothesis that the increased compliance and enlarged air spaces were related and due to enzymatic degradation of elastin: (*a*) the two effects always occurred together; (*b*) both changes were seen only with enzymes which had elastolytic activity by separate assay; (*c*) both changes occurred only in lungs with altered elastin; and (*d*) alveolar spaces were enlarged only in areas with histologically abnormal elastin. While papain, elastase, and collagenase all produced changes in reticulin, no enzyme produced histologic alteration in collagen bundles at the concentrations utilized. The nonspecific proteolytic activity of both papain and elastase (21) dictates that these observations be interpreted with caution. It is possible that elastase and papain attack an elastic element in the lung which behaves like elastin in addition to attacking elastin per se.

Setnikar proposed a model for lung tissue elastic behavior composed of two elements (22). One element, being basically inelastic, limits distensibility at high lung volumes, but, because of the anatomical arrangement of its fibers, it contributes little to elastic recoil at low lung volumes. The other element has nearly linear stress-strain characteristics similar to elastin. In our system, if papain and elastase selectively destroyed the element with linear stress-strain characteristics in some areas of the lung, the observed steep segment of the pressure-volume curve would reflect only the other, collagen-like element. Hogg, Nepszy, Macklem, and Thurlbeck have used a similar analysis to explain the elastic behavior of emphysematous human lungs examined in vitro (23).

Elastase, but not collagenase, increases the compliance of in vitro tracheal segments at low transmural pressures (24); this observation is in agreement with our findings in whole lung preparations. Collagenase increases distensibility of the trachea at high pressures (24). The fragility of collagenase-treated lungs prevented the investigation of changes occurring at high transpulmonary pressures in the present study.

Consideration of the anatomical association of collagen and elastin in the lung has led to the view that collagen plays the major role in maintaining the structural integrity of the lung (13). In contrast, the present study suggests that major alterations in lung structure and elastic behavior are associated with alterations in elastin.

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