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### Research Article

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The proteolytic activity in BAL fluid of ARDS patients was blocked by diisopropylphosphorofluoridate (0.1 mM), Trasylol, soybean trypsin inhibitor, and normal plasma, or plasma deficient in inhibition of the first component of complement. Alpha<sub>1</sub>-proteinase inhibitor ( $\alpha$ 1-Pi)-deficient plasma failed to inhibit the proteolytic activity and addition of  $\alpha$ 1-Pi to the deficient plasma reconstituted the inhibition.

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# Studies on the Pathogenesis of the Adult Respiratory Distress Syndrome

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**ABSTRACT** Bronchoalveolar lavage (BAL) fluid was obtained from 24 sequentially studied patients with adult respiratory distress syndrome (ARDS) for assessment of potential activating and mediating factors. Proteolytic activity of the fluids was observed by measuring cleavage of radiolabeled proteins of the contact (Hageman factor) and complement systems. Proteolytic activity was observed in 17 of 24 (71%) patients with ARDS, and BAL fluid of the 7 ARDS patients without demonstrable, active, enzyme exhibited inhibitory activity for the proteolytic activity. The enzymes cleaved Hageman factor, prekallikrein, plasminogen, high molecular weight kininogen, C4, C3, C5, and Factor B of the complement system. Cleavage of the contact system proteins produced fragments similar or identical in size to the fragments observed during activation of these molecules, although continued incubation invariably reduced the protein to small peptide fragments. None of 7 normal individuals, and 29 of 99 patients (29%) with other forms of pulmonary disease contained measurable enzymes.

The proteolytic activity in BAL fluid of ARDS patients was blocked by diisopropylphosphoridate (0.1 mM), Trasylol, soybean trypsin inhibitor, and normal plasma, or plasma deficient in inhibition of the first component of complement. Alpha<sub>1</sub>-proteinase inhibitor ( $\alpha$ 1-PI)-deficient plasma failed to inhibit the proteolytic activity and addition of  $\alpha$ 1-PI to the deficient plasma reconstituted the inhibition.

Much of the proteolytic activity of the BAL fluid from ARDS patients was identified as neutrophil elastase: the fluids cleaved elastin and synthetic peptide

substrate of neutrophil elastase, neutrophil elastase antigen was present in the BAL fluids as determined immunologically using antineutrophil elastase,  $\alpha$ 1-PI was the major inhibitor in plasma, and the enzyme was inhibited by diisopropylphosphoridate but not chelation. In addition, purified neutrophil elastase produced cleavage fragments of proteins of the contact system similar to those of the BAL fluids.

In each of the seven BAL fluids of ARDS patients that did not reveal active elastase,  $\alpha$ 1-PI was present in active form (as determined by <sup>125</sup>I-trypsin binding). In 9 of the 17 patients with active elastase in the BAL fluid,  $\alpha$ 1-PI antigen was present in the fluid, but was inactive (no binding of <sup>125</sup>I-trypsin). Immunoelectrophoretic analysis of elastase and  $\alpha$ 1-PI throughout proteins in these BAL fluids revealed the presence of both elastase and  $\alpha$ 1-PI that migrated with the same R<sub>f</sub>, suggesting the presence of an enzyme-inhibitor complex. Free, inactive  $\alpha$ 1-PI was also observed in these fluids.

The data reveal that in BAL fluids from all 24 patients with ARDS, leukocytic elastase and/or  $\alpha$ 1-PI exist. A complex of elastase and  $\alpha$ 1-PI was observed in BAL fluids, and in some cases where active enzyme and  $\alpha$ 1-PI coexisted, free, but inactive  $\alpha$ 1-PI was present.

## INTRODUCTION

The adult respiratory distress syndrome (ARDS)<sup>1</sup> can follow a variety of insults, including non-thoracic

<sup>1</sup> *Abbreviations used in this paper:* ARDS, adult respiratory distress syndrome;  $\alpha$ 1-PI,  $\alpha$ 1-proteinase inhibitor,  $\alpha$ 1-antitrypsin; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; DFP, diisopropylphosphoridate; HF, Hageman factor; HMWK, high molecular weight kininogen;  $\alpha$ <sub>2</sub>-M,  $\alpha$ <sub>2</sub>-macroglobulin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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causes as well as injury directly to the lung by inhalation (1). The former appear to injure the lung via a bloodborn mechanism and the latter through direct contact at the alveolar-capillary membrane. The pathophysiology of this process is poorly understood, although the syndrome itself may be characterized by a protein- and cell-rich pulmonary edema due to increased vascular permeability in the lung (2-4). The mediators of this increased permeability have been the subject of much study, and investigators have suggested causal relationships between proteases and this form of acute lung injury. Such has been suggested for complement, especially C5a, via neutrophil aggregation or direct microvascular injury (5). Heflin and Brigham (6) suggested the role of blood leukocytes in this increased pulmonary vascular permeability model using sheep. Increased consumption of fibronectin with subsequent microembolization has been suggested (7), and Bone et al. (8) demonstrated increased levels of fibrin degradation products in serum from patients with active ARDS. Leuterma et al. (9) have suggested that infusion of fibrin degradation fragment D in rabbits produces lung injury similar to that occurring in the ARDS. Hyers and Franks (10) and Haynes et al. (11) have demonstrated the presence of circulating atypical fibrin fragments, thus suggesting that enhanced fibrinolysis is associated with ARDS. All of these studies suggest that several agents may be responsible for increased vascular permeability observed in ARDS.

The activation of Hageman factor (HF) is capable of initiating coagulation through the activation of Factor XI and of Factor VII. Furthermore, the activated product, HF<sub>a</sub>, may proteolytically convert prekallikrein to kallikrein that subsequently can lead to plasmin generation and the release of the permeability factor, bradykinin, from high molecular weight kininogen (HMKW) (12). HF<sub>a</sub> has recently been found to increase cutaneous vascular permeability in quantities as low as 3 ng, i.e., 1/10,000th the amount of HF present in 1 ml of plasma.<sup>2</sup> In vitro studies of the system have demonstrated that negatively charged substances may activate the system. Vascular basement membrane (13) and collagen preparations (14, 16), bacterial lipopolysaccharides (17), and serine protease(s) released from cultured endothelial cells (18), are capable of cleaving and activating the system.

Thus through activation of complement, contact (HF), and clotting systems of plasma a number of effector agents are generated that could mediate many of the pathologic features of ARDS.

<sup>2</sup> Yamamoto, T., and C. G. Cochrane. 1981. Guinea pig Hageman factor as a vascular permeability enhancement factor. Manuscript in preparation.

We have undertaken studies of bronchoalveolar lavage (BAL) fluid from patients with ARDS in order to identify and characterize substances capable of activating components of the complement, contact, and clotting systems of plasma. We report herein the initial results of the definition of proteolytic activity in the BAL fluid. A portion of the data appeared in preliminary form (19).

## METHODS

### *Study population*

BAL fluid from 130 subjects was studied. Subjects ranged in age from 19 to 78 yr; 85 were male, 45 were female. Without knowledge of the biochemical analyses, patients were classified into diagnostic categories after review of the hospital record. A hierarchy of categories was used, and patients were placed into the highest appropriate category. These were ARDS, pneumonitis, carcinoma, chronic obstructive pulmonary disease (COPD), other, and normal. Categories were defined as follows:

**ARDS.** Onset over <7 d of panlobar alveolar infiltrates on the chest radiograph, with a pulmonary artery wedge pressure of <18 mm Hg, without evident pulmonary parenchymal infection (24 patients).

**Infectious pneumonitis.** Infiltrates on the chest radiograph and either isolation of an infectious organism (18 patients), or consistent lung biopsy results (1 patient), or a consistent clinical course (18 patients).

**Carcinoma.** Tissue diagnosis of carcinoma of the lung (24 patients) or metastatic to lung (3 patients).

**COPD.** History of asthma (four patients); chronic bronchitis (two patients); emphysema (two patients).

**Other.** Cutaneous burn (five patients), pulmonary fibrosis (five patients), lobar collapse (six patients), hemoptysis (four patients), alveolar proteinosis (two patients), bronchocentric granulomatosis (one patient), trauma (one patient), drug overdose (one patient), pulmonary embolus (one patient), rheumatoid arthritis (one patient).

**Normal.** Nonsmoking adults with normal pulmonary function tests, arterial blood gases, physical examinations and chest radiographs (seven volunteers).

### *BAL samples and preparation*

BAL fluid samples were obtained through a fiberoptic bronchoscope. Lavage of a basilar segment was performed using 20 ml of normal saline of which ~30-50% was recovered. These aliquots were then filtered through gauze, examined for cell count and differential, centrifuged immediately at 2,200 g for 10 min and the pellet was discarded. The supernate was then aliquoted and frozen in plastic vials at -70°C for later evaluation. The validity of the lavage procedure in sampling the area of inflammation was assessed three ways. First, the spectrum of proteins in the lavage samples of ARDS patients was compared with the spectrum of proteins in plasma in order to determine if the lavage fluid had sampled the zone of exudative proteins. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins in BAL fluid of all patients with ARDS were similar, if not identical, to the spectrum of proteins in plasma diluted to the same concentration as the BAL fluid. Second, the phospholipid content of the BAL fluid of the seven normal individuals was studied by Dr. Mikko Hal-

man and Dr. Louis Gluck of the University of California, San Diego, and was found to have the same lecithin/spinogomyelin ratio, phosphatidylglycerol, and desaturated lecithin content as normal alveolar fluid. And third, small volumes (0.5 ml) of saline containing colloidal carbon in 2% gelatin at 37°C were instilled into the bronchi at the carina of anesthetized rabbits, 4.5 cm from the pleural surface. Immediately after instillation, the lungs, showing carbon-black mottling on the pleural surface, were cooled in iced water to cause gelation of the fluid (20 s). Further movement of the carbon was thereby prevented. Histologic sections revealed that the carbon had filled the alveolar spaces throughout the lung sections. Instillation of carbon solution at the carina of breathing rabbits produced identical results. We were assured from these studies that even small amounts of lavage fluid reach alveolar spaces as the fluid is instilled.

### Chemicals

Diisopropylphosphorofluoridate (DFP) was obtained from Calbiochem-Behring Corp. American Hoechst Corp., San Diego, Calif.; [<sup>3</sup>H]DFP from Amersham Corp., Arlington Heights, Ill.; methyloxysuccinyl-L-alanyl-L-alanyl-prolyl-L-valyl-*p*-nitroanilide from Vega-Fox Biochemicals, Div., Newbery Energy Corp., Tucson, Ariz.; soybean trypsin inhibitor from Sigma Chemical Co., St. Louis, Mo.; Tryslyol from Mobay Chemical Co., N. Y.

### Proteins of the contact (HF) and complement systems

Human HF was purified from plasma by a previously reported method (20). HF prepared by this technique was tested for specific clotting activity, migration on polyacrylamide gels in the presence of SDS, and cleavage fragments generated in kaolin-activated human plasma. This 80,000 mol wt form of HF was demonstrated to be a single polypeptide chain on SDS-PAGE under nonreducing conditions, with dissociation into fragments of ~50,000 and 28,000 mol wt in the presence of reducing agents. Prekallikrein and HMWK were similarly purified from human plasma by published methods (21, 22) and were demonstrated to be homogeneous single proteins on SDS-PAGE in the presence and absence of reducing agents. Complement components were generously provided by Dr. H. J. Müller-Eberhard (Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif.). Each gave a single band when tested in Coomassie Blue-stained SDS-PAGE under nonreducing conditions. Upon reduction, the C4 band was dissociated into the typical three peptides and C3 dissociated into two fragments. Factor B and C5 did not dissociate upon reduction. Proteins were radiolabeled with <sup>125</sup>I by the chloramine T method (23). The radiolabeled proteins contained 1–3 μCi/μg. When mixed with whole plasma and exposed to glass, the <sup>125</sup>I-HF, prekallikrein, and HMWK underwent rapid, limited, proteolytic cleavage into characteristic heavy and light chains. Elastase was provided by Dr. Aaron Janoff, State University of New York at Stony Brook, or prepared in this laboratory by published methods (24, 25), and alpha1-antitrypsin (α1-PI) by Dr. James Travis, Department of Biochemistry, University of Georgia, Athens, Ga.

**Antisera.** Antisera to neutrophilic elastase were generously provided by Drs. Aaron Janoff, Department of Pathology, State University of New York, Stony Brook, and by James Travis, Department of Biochemistry, University of Georgia, Athens, Ga. Antiserum to elastase was prepared in

this laboratory in rabbits by biweekly multiple intradermal injections of 50 μg elastase (inactivated with DFP) in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Rabbits were bled 10 d after four biweekly injections. These antisera gave a single precipitin band in agarose against neutrophil extracts that coalesced with the precipitin bands formed with highly purified neutrophil elastase. Antisera to plasminogen, HF, and prekallikrein were obtained from goats by the same method. These latter antisera were mixed with kaolin (20 mg/ml serum) 20 min at 22°C, and then precipitated at 40% saturation with ammonium sulfate followed by dialysis and immunopurification on insolubilized antigen bound to Sepharose 4B. The antibodies were monospecific as determined by immunodiffusion assays and did not contain active components of the contact system or their inhibitors when tested with activated human component. Antisera to α1-PI was a gift of Dr. James Travis. A similar antibody was prepared in goats by us, using highly purified α1-PI for immunization. The antibody gave a single precipitin band against whole human plasma that reacted in identity with purified α1-PI.

**Concentrations of individual proteins.** The concentration of individual proteins in plasma or BAL fluid were obtained by quantitative double-diffusion immunologic assay. The antibody was incorporated at appropriate dilution (1:8–1:30) in 1% agarose and poured into leveled flat plastic plates. Wells, cut by template, were filled with 10 μl BAL fluid or varied quantities of purified proteins. The areas of precipitin bands forming after 2 d around the BAL fluid wells were compared with those of the standards.

**Elastase activity.** Elastase activity was determined by the elastin-agar plate method (26) and by cleavage of methyloxysuccinyl-L-Ala-L-Ala-Pro-L-Val-*p*-nitroanilide. Total serine protease activity of the BAL fluid was determined by the method of Wiggins.<sup>3</sup> Briefly, 10 μl BAL fluid was mixed with 10 μl [<sup>3</sup>H]DFP (Amersham Corp.) in 5 μl Tris (1 M, pH 7.2) at room temperature for 90 min. SDS and β-mercaptoethanol were added at 100°C as described below and, after 4 min at 100°C, the BAL fluid was analyzed by SDS-PAGE, followed by autoradiography. Under these conditions, the [<sup>3</sup>H]DFP was found to react with active HF or prekallikrein, but not with the zymogen proteins, offering evidence that binding of the [<sup>3</sup>H]DFP was specific for active serine proteases.

**Activity of α1-PI.** α1-PI activity was measured by incubating BAL fluid with <sup>125</sup>I-trypsin. Trypsin (TRL-trypsin, batch 3AQ Worthington Biochemical Corp. Freehold, N. J.) dissolved in 0.01 M acetic acid pH 3.4 was radiolabeled with <sup>125</sup>I as above to a specific activity of 0.4 μCi/μg trypsin by the chloramine T method. 20 μl BAL fluid was incubated with 5 μl Tris (0.1M, pH 7.4) and 2 μl <sup>125</sup>I-trypsin containing 0.5 μCi <sup>125</sup>I. After incubation for 10 min at 37°C, the binding of <sup>125</sup>I-trypsin to proteins in the fluid was assessed by autoradiography of SDS-PAGE (below).

**Cleavage of components of the contact and complement systems.** 20 μl BAL fluids were incubated with 2 μl <sup>125</sup>I components (0.1 μg, 0.1 μCi) plus 20 μl 0.05 M Tris-buffered saline, pH 7.4 in plastic vials. After incubation at 37°C for 30 min, the reaction was stopped by addition of SDS and β-mercaptoethanol 10 and 2% respectively, and heating at 100°C for 3 min.

**SDS-PAGE and autoradiography.** The SDS-PAGE system used was performed according to the method of Laemmli (27). Stacking and running gels contained 3 and

<sup>3</sup> Wiggins, R. C. Personal communication.

9% acrylamide, respectively. All samples before application to the gel were boiled for 4 min in the presence of 10% SDS and 2%  $\beta$ -mercaptoethanol and then applied to the gel. Following electrophoresis, gels were fixed and stained using 0.1% Coomassie Brilliant Blue R, 250. They were then destained in 10% glacial acetic acid, dried with a slab gel dryer and placed on Kodak x-omatic film (Eastman Kodak Co., Rochester, N. Y.). The molecular weight of the radiolabeled protein and cleavage fragments were determined from the relative migration of an internal standard protein analyzed on the same gel.

## RESULTS

*Cell count and protein analysis of BAL fluid.* Leukocyte counts and protein concentration of BAL fluid from patients with ARDS are given in Table I. 76%

of the leukocytes (range 57–92%) were neutrophils. Normal subjects had  $\leq 412$  leukocytes  $\text{mm}^3$  BAL, of which  $\geq 87\%$  were macrophages. Protein content averaged 0.4 mg/ml (range 0.1–1.2 mg/ml). Cell count and differential values and protein concentrations in other disease categories were not significantly different from values seen in ARDS.

*Enzymatic activity contained in the BAL fluid.* BAL from patients with ARDS, other pulmonary disease, and from healthy controls were incubated with radiolabeled components of the HF or complement systems as outlined in Methods. Cleavage of the radiolabeled proteins was observed in 17 of 24 patients (71%) with ARDS, none of seven normal individuals, and 29 of 99 (29%) of patients with other forms of

TABLE I  
Enzymes and Inhibitors in BAL Fluids of Patients with ARDS

Patient	Proteolytic cleavage*				Protein conc. †	Leukocytes	$\alpha 1$ -PI ‡	$\alpha 1$ -PI <sup>  </sup> activity	Elastase activity ¶
	HF	PK	PL	HK					
						$\text{mm}^3 \times 10^6$	$\mu\text{g/ml}$		$\mu\text{g/ml}$
1	1	2	1	4	1.8	—	90.6	3	—
2	0	0	0	0	1.1	10.7	<0.3	3	1.0
3	0	0	0	0	1.3	1.2	106.8	3	0.7
4	2	3	3	4	0.3	8.8	<0.3	0	1.4
5	0	0	0	1	0.4	0.2	<0.3	1	0.3
6	4	3	3	4	0.6	6.0	14.6	0	8.1
7	4	3	4	4	0.5	6.1	56.1	0	11.3
8	0	0	0	0	1.7	2.7	60.0	2	0.6
9	1	1	1	0	0.2	0.4	<0.3	2	0.3
10	0	0	0	0	0.7	4.2	28.4	2	0.3
11	1	1	3	1	0.1	0.8	2.3	0	1.1
12	1	1	1	1	0.2	0.3	<0.3	±	0.8
13	3	2	4	4	2.3	0.6	78.1	0	63.5
14	1	3	2	2	0.1	3.8	<0.3	0	0.8
15	1	0	0	0	0.5	0.3	38.9	2	1.6
16	4	3	3	4	0.7	3.0	45.1	0	11.3
17	3	1	3	4	0.7	1.5	46.4	4	—
18	0	0	0	0	1.8	3.4	79.5	2	1.2
19	0	0	0	0	1.9	6.2	196.9	2	1.1
20	1	1	3	1	0.1	0.2	<0.3	0	2.0
21	2	1	3	4	3.1	2.5	107.9	0	11.8
22	3	0	3	4	1.2	—	<0.3	0	7.6
23	3	3	2	4	0.7	0.2	7.3	±	6.2
24	0	0	0	0	1.5	4.4	5.6	2	0.8

\* Proteolytic cleavage of proteins of the contact system was measured as outlined in Methods. The degree of cleavage was assigned an arbitrary number from 0 to 4. In approximate terms 1 = <20% cleavage; 2 = 20–50% cleavage; 3 = >50 but <100% cleavage; 4 = total cleavage of the native  $^{125}\text{I}$ -protein. Grading was performed by two people without knowledge of the patient's clinical history.

† Protein concentration of the BAL fluid was measured as noted in Methods.

‡  $\alpha 1$ -PI concentration of the BAL was measured by quantitative immunoprecipitation technique.

||  $\alpha 1$ -PI activity and  $\alpha_2 = \text{M}$  were measured by the capacity of the  $\alpha 1$ -PI of the BAL fluid to bind  $^{125}\text{I}$ -trypsin (Methods). The results obtained from autoradiography of the  $\alpha 1$ -PI and  $\alpha_2 = \text{M}$ -bound [ $^{125}\text{I}$ ]-trypsin, were given arbitrary numbers from 0 to 4, depending on the relative intensity of antisera deposition on the x-ray film.

¶ Amidolytic activity on methoxysuccinyl-L-ala-L-ala-L-pro-L-val-pNA. Activity with BAL fluid was compared with that of purified elastase to determine the elastase concentration of the fluid. PK, prekallikrein; PL, plasminogen.

pulmonary disease (Methods) (Tables I and II; Figs. 1 and 2). Of the seven ARDS patients not demonstrating proteolytic enzyme, each was found to contain an inhibitor of the proteolytic activity (see below). Plasma obtained from the ARDS patients at the same time as the BAL, and diluted 1:70 in sterile saline (to yield a protein concentration approximating that of the BAL), uniformly failed to cleave components of the HF and complement systems. Analysis of the cleaved fragments of the HF and complement components revealed cleavage of HF into fragments of  $\sim 50,000$  and  $28,000$  and  $31,000 M_r$ . Prekallikrein was cleaved to yield a radiolabeled heavy chain (the light chain was not radiolabeled), while plasminogen and HMWK were cleaved so as to reveal a heavy and light chain on occasions, but more frequently, these latter proteins were cleaved into small fragments that appeared in or near the dye front. Cleavage of C3 occurred in the  $\alpha$ -chain, releasing a fragment of  $\sim 10,000 M_r$  as evidenced by the estimated diminution of size of the  $\alpha$ -chain on SDS-PAGE. Cleavage of C5 resulted in the appearance of only small fragments migrating at the dye front.

*The effect of inhibitors of enzymes on the protein-cleaving capacity of the BAL fluids.* DFP was added to BAL fluids of ARDS patients at a final concentration of 1 mM 60 min before assessment of cleaving capacity in components of the HF system as performed in the previous experiments. In each of 10 cases so assayed, the DFP treatment prevented cleavage of the radioiodinated proteins. BAL fluids treated with DFP solvent maintained activity. Trasylol at 1,500 U/ml final concentration, and soybean trypsin inhibitor (60  $\mu\text{g/ml}$ ) inhibited the enzymatic activity in the lavage fluid in each of four cases of ARDS tested.

Immunopurified antibodies to HF, prekallikrein, and plasmin failed to inhibit the enzymes in each of six of the BAL fluids studied. The antibodies were added in quantities sufficient to prevent completely the cleaving capacity of each active homologous en-

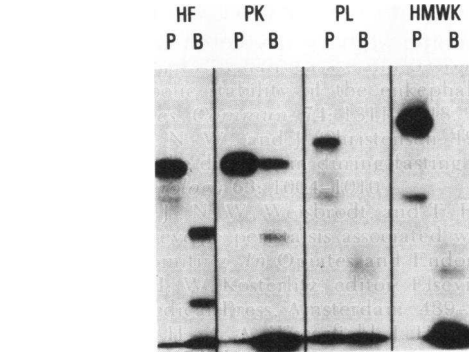


FIGURE 1 SDS-PAGE and  $\beta$ -mercaptoethanol of  $^{125}\text{I}$ -HF, prekallikrein (PK), plasminogen (PL) and (HMWK) after incubation (20 min  $37^\circ\text{C}$ ) with 20  $\mu\text{l}$  of plasma (diluted 1:70 with sterile saline) (P) or 20  $\mu\text{l}$  of bronchoalveolar lavage fluid (B) of patient seven. Cleavage of each protein occurred after incubation with lavage fluid as described in the text. A radiolabeled contaminant is present in the HF preparation in P immediately below the major band of HF, and a small amount of spontaneously cleaved plasminogen and HMWK was observed in the preparations that were untreated or exposed to plasma (P).

zyme in concentrations greater on a basis of activity, than that of the BAL fluid. To determine if plasma contained an inhibitor of the enzymes in BAL fluid, 20  $\mu\text{l}$  of six BAL fluids were mixed with 20  $\mu\text{l}$  normal human first component of complement inhibitor C1-INH-deficient or  $\alpha 1$ -PI-deficient (ZZ phenotype) plasmas diluted 1:5–1:70 in sterile saline. After incubation

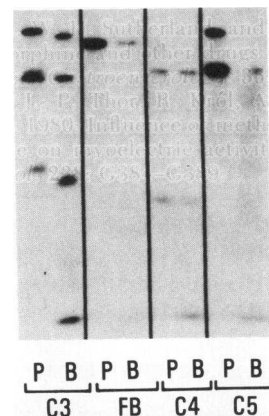


FIGURE 2 SDS-PAGE  $^{125}\text{I}$ -C3, Factor B (FB), C4, and C5 of the complement system after incubation (20 min,  $37^\circ\text{C}$ ) with 20  $\mu\text{l}$  of plasma diluted 1:70 with sterile saline—P) or bronchoalveolar lavage fluid (B) of patient seven. Cleavage of the  $\alpha$ -chain of C3 (uppermost band) is noted with a loss of an  $\sim 10,000 M_r$  weight fragment. Cleavage of FB, the  $\alpha$ -chain of C4 and both  $\alpha$ - and  $\beta$ -chains of C5 are noted. The lowermost band in the preparation of C3 represents either a contaminant or a fragment that is spontaneously dissociated from the parent molecule. This fragment is also partially cleaved by treatment with lavage fluids.

TABLE II  
Protase Activity in BAL Fluids of Patients with Pulmonary Inflammatory Disease

Group	Total no. subjects	Protease activity in BAL fluids
		% positive
ARDS	24	71
Pneumonitis	37	46
Carcinoma	27	7
COPD	8	12
Other	27	33
Normal	7	0

at 22°C for 10 min, the mixtures were assayed for residual enzymatic activity. As noted in Fig. 3, the normal and C1-INH-deficient plasmas blocked activity of the BAL fluids, but  $\alpha$ 1-PI-deficient plasma was not inhibitory. Addition of purified  $\alpha$ 1-PI to the  $\alpha$ 1-PI-deficient plasma in concentrations  $\sim$ 1/5 that of normal plasma, restored full inhibitory capacity of the plasma. Two other  $\alpha$ 1-PI-deficient plasmas have been tested with similar results.

Purified  $\alpha$ 1-PI was then added to BAL fluid of nine cases of ARDS to determine if inhibition of enzymatic activity resulted. Using  $\alpha$ 1-PI at a final concentration of 40  $\mu$ g/ml, complete or nearly complete inhibition of cleavage was evident in each case. Partial or complete inhibition also was noted in BAL fluids from all cases of pneumonia or COPD as well. Because the molar concentration of enzyme was not available in each BAL fluid, the  $\alpha$ 1-PI was added without knowledge of stoichiometric relationships.

*Identification of leukocytic elastase as a principal enzymatic constituent in BAL fluid from patients with ARDS.* With the knowledge that  $\alpha$ 1-PI inhibited the proteolytic capacity of the BAL fluids, attempts were made to determine if leukocytic elastase, known to be sensitive to the action of  $\alpha$ 1-PI, was present in the fluids. Accordingly, BAL fluids from ARDS pa-

tients and controls were assayed for their capacity to lyse elastin in elastin-agar plates. 20  $\mu$ l of each lavage sample were placed in wells of elastin-agar plates and incubated at 37°C up to 72 h. All fluids of ARDS patients containing proteolytic activity, as demonstrated by cleavage of radiolabeled components of the contact system, also revealed elastolytic activity (data not shown). Fluids from patients without demonstrable protease activity uniformly failed to hydrolyse the elastin.

Immunoprecipitation assays on these fluids using monospecific antielastase revealed the presence of elastase antigen in all fluids containing proteolytic enzyme activity and in several fluids of ARDS patients that failed to demonstrate such proteolytic activity (Fig. 4). In each case, the precipitin bands coalesced with bands formed between the antibody and purified elastase. Antibodies to neutrophil elastase were also tested for their ability to inhibit enzyme activity in the lavage fluid of patients with ARDS. In four patients with free protease in the BAL fluid, the amidolytic activity was decreased >90% by antibodies to elastase, but not by normal globulin. The antibodies, along with normal rabbit globulin, were precipitated at 35% ammonium sulfate and then subjected to acidification at pH 3.5 for 1.5 h, followed by exposure to *N*-chlorosuccinamide to inhibit residual  $\alpha$ 1-PI. The antibodies and normal globulin were then brought back to pH 7.4 for use.

Each of the BAL fluids from the ARDS patients was added to the synthetic substrate, methoxysuccinyl-L-Ala-L-Ala-L-Pro-L-Val-pNA, specific for neutrophil elastase. Cleavage of the substrate occurred in each

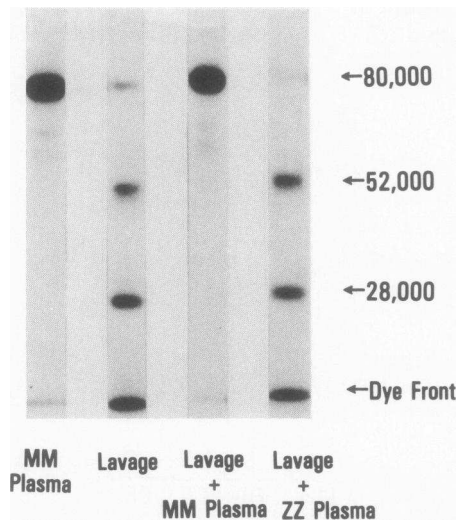


FIGURE 3  $^{125}$ I-HF cleavage by ARDS BAL fluid in normal and  $\alpha$ 1-antitrypsin-deficient plasmas. SDS-PAGE of  $^{125}$ I-HF treated with 20  $\mu$ l normal plasma diluted 1:70 in saline (MM plasma), 20  $\mu$ l BAL, 20  $\mu$ l BAL plus 20  $\mu$ l normal plasma diluted 1:70 (lavage + MM plasma), and 20  $\mu$ l BAL plus 20  $\mu$ l  $\alpha$ 1-PI-deficient plasma 1:70 (lavage + ZZ plasma). The cleavage of the HF by the BAL was inhibited by normal plasma, but not the  $\alpha$ 1-PI-deficient plasma. Reconstitution of the  $\alpha$ 1-PI-deficient plasma with purified  $\alpha$ 1-PI restored the inhibitory activity.

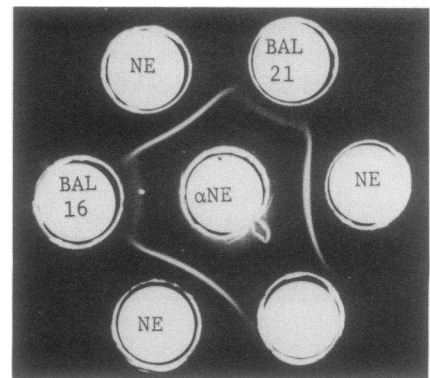


FIGURE 4 Double diffusion immunoprecipitin analysis of the presence of neutrophil elastase (NE) antigen in two cases of BAL from patients with ARDS. Coalescence is noted between these precipitin bands and those developing with purified NE. The center well contains anti-NE ( $\alpha$ NE). The small line of precipitation near the center well and between wells containing BAL 21 and BAL 16 fluids probably represents a complex of elastase and  $\alpha$ 1-PI inhibitor.

case in which proteolytic activity was noted using substrates of the contact system with the exception of patients 1 and 17. The BAL fluid from these two patients had been maintained for several months and had lost their capacity to cleave HF (see below). The quantity of elastase, determined with the synthetic substrate is given in Table I. The activity was unchanged in 1 mM 1,10 phenanthroline.

To examine the numbers and relative activity of enzymes in the BAL fluids of ARDS cases, [ $^3\text{H}$ ]DFP was incubated with the BAL fluids for 30 min at 22°C. Binding of the [ $^3\text{H}$ ]DFP was then determined in SDS-PAGE followed by autoradiography. In each case, binding of [ $^3\text{H}$ ]DFP occurred in a single area as shown in Fig. 5. The molecular size was slightly larger than that of trypsin, having a  $M_r$  of 26,000–30,000. In some cases the zone of radioactivity formed two or three closely grouped bands. Any other zones of radioactivity were associated with large deposits of Coomassie Blue-stained protein and could not unequivocally be designated as evidence of specific DFP binding to a serine protease. Little or no protein could be seen in Coomassie Blue staining that was observed with the major zone of radioactivity.

*Effect of leukocytic elastase on HF.*  $^{125}\text{I}$ -HF was incubated with purified human neutrophil elastase (1:50 wt/wt of elastase to HF) for 10 min at 37°C and then studied using the SDS-PAGE system. Fig. 6 demonstrates that the cleavage of HF by neutrophil elastase was similar, if not identical to that produced by BAL fluids from ARDS patients. The rate of cleavage of HF by neutrophil elastase was measured during a 30-min incubation at 37°C. It was observed that longer incubation periods produced further proteolytic breakdown of  $^{125}\text{I}$ -HF into fragments other than the 50,000- and 28,000-mol wt components. Cleavage of

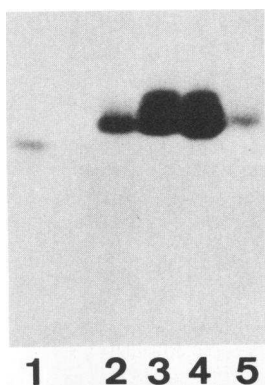


FIGURE 5 SDS-PAGE of BAL or trypsin exposed to [ $^3\text{H}$ ]DFP (Text). Lane 1, trypsin plus [ $^3\text{H}$ ]DFP; lanes 2–5, BAL from four cases of ARDS plus [ $^3\text{H}$ ]DFP. Binding of the [ $^3\text{H}$ ]DFP occurred with protein of  $M_r$  26,000–30,000 in the BAL fluids.

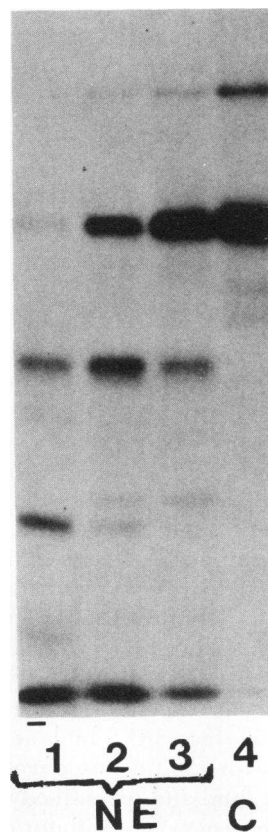


FIGURE 6 SDS-PAGE of  $^{125}\text{I}$ -HF treated with purified neutrophil elastase (NE) or saline (C). Lanes 1–3 contain decreasing quantities of NE and diminishing cleavage of HF. Note the appearance of a 31,000  $M_r$  band in lanes 2 and 3.

$^{125}\text{I}$ -HF by neutrophil elastase could be totally inhibited by pretreatment of neutrophil elastase with monospecific antibody to human neutrophil elastase.

*The relationship of  $\alpha_1$ -PI and leukocytic elastase in BAL fluids.* With the observation that lavage fluids from patients with ARDS contained elastase protein, determined immunologically, but were without proteolytic activity, assays were performed for the presence of inhibitors in these fluids. These BAL fluids were first mixed in equal volume with other BAL fluids, known to contain active enzyme, and tested for inhibition of the proteolytic activity using  $^{125}\text{I}$ -HF as substrate. In each case, inhibition of the proteolytic activity was observed. Analyses were then conducted to identify the inhibitors of the enzyme of the BAL fluids. To accomplish this,  $^{125}\text{I}$ -trypsin (Methods) was added to each BAL fluid and assayed for binding to inhibitors in SDS-PAGE. By combining  $^{125}\text{I}$ -trypsin with purified  $\alpha_1$ -PI and  $\alpha_2$ -macroglobulin ( $\alpha_2$ -M), the complexes of the trypsin and these two inhibitors could be identified in SDS-PAGE. The presence of active  $\alpha_1$ -PI in the BAL fluids is given in Table I. Function-



ally active  $\alpha$ 1-PI was detected in 11 of the cases.  $\alpha$ 2-M was present in only 6/21 BAL fluids. As will be noted below, several fluids that contained  $\alpha$ 1-PI, as determined immunologically, failed to show  $\alpha$ 1-PI activity. Two of the BAL fluids (patients 1 and 17) contained active enzyme when first tested along with active  $\alpha$ 1-PI. In each case the enzymatic activity was lost when assayed either the second or third time, while other enzyme-containing fluids failed to lose enzyme activity.

To analyze further the relationship of elastase enzyme and inhibitor, immunoelectrophoretic analyses were performed using antibodies to leukocytic elastase and  $\alpha$ 1-PI as shown in Fig. 7. The analysis revealed that leukocyte elastase was present, but migrated in an anodal position.  $\alpha$ 1-PI in the fluid was divided between free  $\alpha$ 1-PI that migrated in the anticipated region of the gel, and a more cathodally migrating form of  $\alpha$ 1-PI. The position of the latter  $\alpha$ 1-PI was identical to that of the anodally migrating elastase protein, suggesting a complex of the two molecules existing in these BAL fluids lacking detectable proteolytic activity. This was confirmed in double diffusion immunologic assays: an immunoprecipitin line developing between elastase in the BAL fluid and its antibody coalesced with a precipitin line developing between the  $\alpha$ 1-PI in the fluid and its antibody. This was true in four cases where enzyme-inhibitor complexes were suggested by immunoelectrophoresis.

*Inactive  $\alpha$ 1-PI in the BAL fluid of ARDS patients.* In nine of the ARDS patients with proteolytic activity in the BAL fluid, the simultaneous presence of  $\alpha$ 1-PI was measured immunologically. The ability of the  $\alpha$ 1-PI to bind  $^{125}$ I-trypsin was then assessed as a measure of function of the  $\alpha$ 1-PI. The binding of the  $^{125}$ I-trypsin to  $\alpha$ 1-PI was determined by migration of the  $^{125}$ I-trypsin at an  $R_f$  of the trypsin- $\alpha$ 1-PI complex, i.e., at  $M_r$  65,000. In six of the nine cases, the  $\alpha$ 1-PI did not bind the  $^{125}$ I-trypsin (Table I). Presumably the in-

active  $\alpha$ 1-PI was inactivated by either an interaction with free enzyme or by some other mechanism, or by both. As noted above, immunoelectrophoresis of four of these BAL fluids revealed that free (unbound)  $\alpha$ 1-PI was present as determined by an  $R_f$  typical of free  $\alpha$ 1-PI. Thus inactive  $\alpha$ 1-PI was present in these BAL fluids that migrated with the same  $R_f$  as free  $\alpha$ 1-PI. Plasma of these patients, exposed to  $^{125}$ I-trypsin after 1:70 dilution in sterile saline, exhibited binding of the radiolabeled trypsin that was indistinguishable from that of normal plasmas.

## DISCUSSION

*The presence of elastase and  $\alpha$ 1-PI in ARDS.* We have observed in this study that leukocytic elastase may be recovered from BAL fluids of a great majority of patients (17 of 24) with ARDS. In those patients not exhibiting free enzyme, inhibitor of the elastase was demonstrable. The inhibitor was in greatest part  $\alpha$ 1-PI and to a lesser extent,  $\alpha$ 2-M.

The conclusion that the proteolytic enzyme was in greatest part leukocytic elastase is supported by evidence that the enzyme cleaved elastin, that elastase antigen was detected in the BAL fluids with antibody specific to human neutrophil elastase, that purified neutrophil elastase cleaved components of the contact system similarly, if not identically, to the BAL fluids, and that the enzyme cleaved synthetic peptide bearing close substrate specificity for elastase. The enzyme was inhibited by a spectrum of inhibitors known to block the activity of elastase:  $\alpha$ 1-PI and to a lesser extent,  $\alpha$ 2M, DFP, soybean trypsin inhibitor, and to a lesser extent, Trasylol, while the activity was not inhibited by C1-INH or antibodies to plasma kallikrein or plasmin. That an elastaselike enzyme was the predominant serine protease in the BAL fluids was shown by uptake of [ $^3$ H]DFP principally by enzyme of  $M_r$  26,000 to 31,000, similar to that of neutrophilic elastase. The

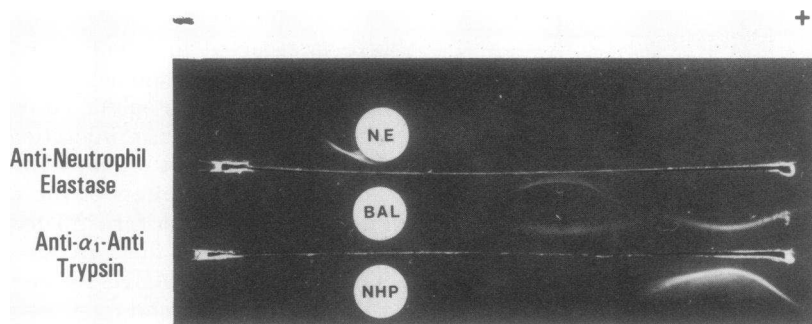


FIGURE 7 Immunoelectrophoresis of purified neutrophil elastase (NE), BAL of patient 18 with early ARDS, and normal plasma (NHP).

sensitivity of this technique in the case of other enzymes at low concentration is uncertain, and their presence and activity cannot be excluded. In addition, it is possible that other enzymes (including the chymotrypsinlike enzyme of neutrophils) could be bound to tissues at the site of injury in the lung and not recoverable in lavage fluid. Neutrophils are the most likely sources of the elastase. As opposed to the present findings, DFP and  $\alpha$ 1-PI do not readily inhibit macrophage elastase, while chelation does (28, 30). In addition, the elastase was detected with antibodies to neutrophil elastase. However, our data do not preclude a partial contribution from macrophages. In addition, one cannot state at present the tissue source of the neutrophil elastase, although since injury initiated primarily in the lung (e.g., with aspiration) can induce ARDS, it is probable that the elastase is released in the pulmonary tissues at least in some cases of ARDS. Neutrophils are present in the lung in ARDS although their numbers are not always great. It is of interest that the presence of free elastase was found to be more frequent in ARDS than in COPD or in bronchopneumonia. While this may relate to sampling error, it may suggest that in ARDS the release of enzymes by the leukocytes is heightened, or that inhibition of the released enzyme is diminished.

In all cases where protease activity was not detected in the BAL fluid of ARDS patients,  $\alpha$ 1-PI inhibitor was found, both immunologically and by function. Complexing of the  $\alpha$ 1-PI and leukocytic elastase was suggested in immunoelectrophoresis in which a comigration of elastase and  $\alpha$ 1-PI appeared in the beta 1 region, i.e., in a position distinct from either enzyme or inhibitor in their free state. The beta 1 position was identical to that occurring when purified elastase and  $\alpha$ 1-PI were mixed before electrophoretic separation.

Our observations on the presence of elastase and  $\alpha$ 1-PI in BAL of patients with ARDS is similar to that reported by Lee et al. (31), which appeared while this manuscript was in preparation. These authors observed that BAL fluid of 12 of 23 patients exhibited elastase activity above background level using an amidolytic assay, and by lysis of elastin. All patients' lavage fluid contained  $\alpha$ 1-PI, and four with low elastase levels possessed high levels of  $\alpha$ 1-PI activity as measured by trypsin binding.

The source of the elastase appeared to be the neutrophil. Nevertheless, total leukocyte (Table II) and differential cell counts (data not presented) of the lavage fluids failed to reveal a clear relationship of the concentration of neutrophils and the levels of elastase. This could be explained by adherence of the neutrophils in the lung tissue or the placement of these cells in the lung parenchyma in different cases.

*Presence of inhibited  $\alpha$ 1-PI in BAL fluid in ARDS.*

Of potential importance in the pathogenesis of the disease was the observation in the present report that in seven cases in which active enzyme and  $\alpha$ 1-PI were found together, the  $\alpha$ 1-PI was inactive. That the inactive  $\alpha$ 1-PI was free, i.e., not bound by enzyme, was revealed in immunoelectrophoresis. The factors responsible for the inactivation of free  $\alpha$ 1-PI are not clear. However among the possible mechanisms that could account for the inactivation is contact with oxidizing agents.  $\alpha$ 1-PI activity is inhibited by oxidation of a methionyl residue at the reactive center of the molecule (32-35). Such a mechanism has been proposed to explain the loss of  $\alpha$ 1-PI in smokers (36-38). Assuming that neutrophils and macrophages are stimulated to release elastase in ARDS, it is altogether possible that they generate superoxide anion,  $H_2O_2$ , and  $^{\circ}OH^-$  as well. In addition, myeloperoxidase, which is released from stimulated leukocytes, inactivates  $\alpha$ 1-PI in the presence of  $H_2O_2$  and chloride ion (39-41). Each of these could bring about the oxidation of the methionyl residue in the reactive center of the  $\alpha$ 1-PI.

*Mechanisms of injury of pulmonary tissue in ARDS.* Injury of the lung tissue in ARDS could result from direct action of the proteinases on the vascular basement membranes (42, 43), elastin (44), collagen (45), and structural elements of the pulmonary tissues (46). Infusion of elastase has been shown to produce severe injury to the lung (47, 48). In addition, the enzyme(s) could cause increased permeability of the blood vessels by activation of components of the contact and/or complement systems (49). In a separate paper, we will offer evidence that activation of HF does follow enzymatic cleavage by BAL fluid of ARDS patients and that C5a can be detected in the fluid. Activation of HF could bring about fibrin formation through the intrinsic and extrinsic clotting systems.

Another possibility that must be considered is that the generation of free radicals derived from molecular oxygen may be responsible for a portion of the injury. Their presence may be reflected in the finding that free  $\alpha$ 1-PI is inhibited in the BAL fluid of ARDS patients as noted above. The capacity of oxygen free radicals and their metabolites to induce injury of the lung of rats has recently been shown by Johnson et al. (50). Superimposition of oxygen therapy in ARDS must also be taken into consideration for its contribution to injury through oxidizing mechanisms.

BAL has been performed by others using volumes ranging from 30 plus 30 plus 40 ml (sequential lavages) (51) to 50 ml or more. How likely is it that our lavage volume of 20 ml, 30-50% of which is recovered, samples the gas exchanging portion of the lung?

We have shown in the rabbit that as little as 0.5 ml of 2% gelatin containing colloidal carbon administered in the mainstem bronchus of either an excised or in

vivo lung reaches the alveolar spaces. Scaling this experiment to human dimensions would suggest that our lavage volume is more than sufficient to reach the alveoli.

The morphometric analysis of K. Horsefield and C. Cumming (52) and information on bronchial and bronchiolar anatomy (53) allow calculation of the anatomic dead space subtended by the segmental bronchus in which our bronchoscope was wedged. A volume of the central airways (lobar and mainstem bronchi) of 12.4 ml, subtracted from an anatomic dead space of 71 ml below the carina, leaves 58.6 ml of dead space beyond the segmental bronchial orifices. Thus, on the average, 3.1 ml of dead space exists beyond the orifice of each segmental bronchus. Recovery of 6–10 ml of lavage fluid administered suggests that 48–69% of the fluid recovered has reached beyond the distal lobular bronchiole. Thus, calculations of the recovered volume indicate that the majority of the fluid has reached at least the level of the respiratory bronchiole. Alveolar structures arising from the respiratory bronchiole (54) are almost certainly bathed by this fluid. Mixing with fluid in more distal alveolar ducts and alveoli will be enhanced by cardiac and ventilatory oscillations.

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