Antielastases of the Human Alveolar Structures

IMPLICATIONS FOR THE PROTEASE-ANTIPROTEASE THEORY OF EMPHYSEMA

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ABSTRACT The current concepts of the pathogenesis of emphysema hold that progressive, chronic destruction of the alveolar structures occurs because there is an imbalance between the proteases and antiproteases in the lower respiratory tract. In this context, proteases, particularly neutrophil elastase, work unimpeded to destroy the alveolar structures. This concept has evolved from consideration of patients with α1-antitrypsin deficiency, who have decreased levels of serum α1-antitrypsin and who have progressive panacinar emphysema. To directly assess the antiprotease side of this equation, the lower respiratory tract of nonsmoking individuals with normal serum antiproteases and individuals with PiZ homozygous α1-antitrypsin deficiency underwent bronchoalveolar lavage to evaluate the antiprotease screen of their lower respiratory tract. These studies demonstrated that: (*a*) α1-antitrypsin is the major antielastase of the normal human lower respiratory tract; (*b*) α2-macroglobulin, a large serum antielastase, and the bronchial mucous inhibitor, an antielastase of the central airways, do not contribute to the antielastase protection of the human alveolar structures; (*c*) individuals with PiZ α1-antitrypsin deficiency have little or no α1-antitrypsin in their lower respiratory tract and have no alternative antiprotease protection against neutrophil elastase; and (*d*) the lack of antiprotease protection of the lower respiratory tract of PiZ individuals is a chronic process, suggesting their vulnerability to neutrophil elastase is always present.

INTRODUCTION

Emphysema is a chronic disorder of the alveolar structures associated with enlargement of the distal air spaces, accompanied by destruction of their walls (1). The first major clue to the pathogenesis of alveolar wall destruction in emphysema came from the observation that individuals deficient in serum α1-antitrypsin had severe early-onset emphysema (2, 3). Because α1-antitrypsin is the major serum antielastase (4), this observation led to what is known as the protease-antiprotease theory of emphysema (5–8). This theory holds that the alveolar structures are under constant attack by proteases, particularly elastase, released by inflammatory cells, but are protected from destruction by an antiprotease screen that effectively inhibits this elastase. Because severe deficiency in serum α1-antitrypsin is associated with early-onset, progressive emphysema, the protease-antiprotease theory also predicts: (*a*) α1-antitrypsin must be the major antielastase of the alveolar structures; (*b*) patients with a deficiency of serum α1-antitrypsin would have little or no antielastase activity in their lower respiratory tract; and (*c*) the lack of antielastase screen in the lungs of α1-antitrypsin–deficient individuals is a chronic process.

The present study was designed to evaluate these predictions. To do so, we used the technique of bronchoalveolar lavage to sample the epithelial lining fluid of the lower respiratory tract of a group of nonsmoking, homozygous PiZ individuals with severe emphysema. These findings were compared with the antielastase screen in the lower respiratory tract of two groups: nonsmoking, homozygous PiM individuals with normal lung function; and non-smoking, homozygous PiM individuals with idiopathic pulmonary fibrosis, a chronic inflammatory disorder of the lower respiratory tract associated with interstitial fibrosis, not emphysema (9).

METHODS

Study Population

Normal subjects. Eight healthy, nonsmoking volunteer subjects were admitted to the National Institutes of Health (NIH) Clinical Center for performance of bronchoscopy and

bronchoalveolar lavage. There were five males and three females with a mean age of 32 ± 5 yr.[1] All individuals had normal chest roentgenograms and all had normal vital capacity, total lung capacity, diffusing capacity, forced expiratory volume in 1 s (FEV_1),[2] FEV_1/forced vital capacity, and mid-maximum expiratory flow rate.

Patients with $\alpha1$-antitrypsin deficiency. Eight patients (seven males, one female, mean age 42 ± 5 yr) with severe serum deficiency of $\alpha1$-antitrypsin and advanced emphysema were admitted to the NIH Clinical Center. One never smoked and seven had not smoked for at least 2 yr. Chest films of all eight showed hyperinflation, flattened diaphragms, a small heart, loss of vascular markings, and bilateral basilar bullous disease. As a group, these individuals had a mild decrease in vital capacity ($82\pm10\%$ predicted), normal total lung capacity ($97\pm8\%$ predicted by helium dilution), marked airway obstruction (FEV_1, 1.1 ± 0.2 liters; FEV_1, $54\pm10\%$ predicted, FEV_1/FVC, $42\pm5\%$ predicted; mid-maximum expiratory flow rate $15\pm5\%$ predicted), hyperinflation (residual volume/total lung capacity, 0.52 ± 0.10), and reduced diffusing capacity ($42\pm10\%$ predicted, single breath based on volume and hemoglobin). No individuals in this group had a history of sputum production in the past year.

Idiopathic pulmonary fibrosis. Seven patients (five males, two females, mean age 45 ± 5 yr) with biopsy-proven idiopathic pulmonary fibrosis (IPF) in mid-course were admitted to the NIH Clinical Center. None of these individuals was a cigarette smoker. All had chest films showing small lungs and a diffuse reticulonodular infiltrate. As a group, these individuals had reduced lung volumes (vital capacity, $66\pm7\%$ predicted; total lung capacity, $61\pm5\%$ predicted; normal $FEV_1\%$, ($98\pm2\%$ predicted); and reduced diffusing capacity ($56\pm10\%$ predicted). No individual in the group had a history of sputum production.

Serum $\alpha1$-antitrypsin phenotyping and $\alpha1$-antitrypsin levels

Sera of all patients were analyzed by isoelectric focusing (10) in precast, ampholine-containing polyacrylamide slab gels (pH 4–6; LKB Instruments, Inc., Rockville, Md.). The nine normal subjects all had the PiM phenotype, the seven patients with severe serum $\alpha1$-antitrypsin deficiency all had the PiZ phenotype, and the seven patients with IPF all had the PiM phenotype.

Serum levels of $\alpha1$-antitrypsin in all subjects were determined using radial immunodiffusion plates obtained from Calbiochem-Behring Corp., American Hoechst Corp., (San Diego, Calif.) (11). The $\alpha1$-antitrypsin levels were confirmed by "rocket" electroimmunodiffusion using monospecific goat antiserum to human $\alpha1$-antitrypsin (Atlantic Antibodies, Westbrook, Maine) (12).

Bronchoalveolar lavage

All patients subject to lavage had an FEV_1 of ≥0.75 liters. Five, 20-ml aliquots of sterile 0.9% saline were used to lavage the right middle lobe or the lingula segment of the left upper lobe; potential contamination of the sample by central airway secretions was prevented by advancing the bronchoscope into the "wedge" position. The distal tip of the Olympus

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fiberoptic bronchoscope (model BF/B3, Olympus Corporation of America, New Hyde Park, N. Y.) used in this study has a diameter of 5 mm. When extended to a wedge position, this instrument permits the isolation and lavage of the lower respiratory tract at the level of airways whose caliber is 4 mm and less. The lavage fluid was recovered in a sterile vacuum trap, and the cellular and fluid phases immediately separated by centrifugation at $500 g$ for 5 min. The fluid phase was then concentrated by nitrogen pressure dialysis (Amicon UM2 membrane, Amicon Corp., Scientific Systems Div., Lexington, Mass.) to a final volume of 1.0 ml, buffered with 1.0 M Tris-HCl, pH 7.4 (5% by volume), and stored in liquid nitrogen vapor until analyzed (13).

Immunochemical evaluation of antielastases in lavage fluid

$\alpha1$-Antitrypsin and $\alpha2$-macroglobulin levels in bronchoalveolar lavage fluid were determined with radial immunodiffusion (Calbiochem-Behring), and rocket electroimmunodiffusion using monospecific goat antiserum to $\alpha1$-antitrypsin and $\alpha2$-macroglobulin, respectively (Atlantic Antibodies). As previously described, the levels of $\alpha1$-antitrypsin and $\alpha2$-macroglobulin in lavage fluid were compared to lavage fluid albumin levels determined by radial immunodiffusion. Serum $\alpha1$-antitrypsin, $\alpha2$-macroglobulin, and albumin levels were simultaneously determined to provide comparison data for the lavage antielastase/albumin levels.

The state of the $\alpha1$-antitrypsin in concentrated lavage fluid of normal subjects was evaluated using two-dimensional immunoelectrophoresis in agarose at pH 8.1 (14). Purified, normal $\alpha1$-antitrypsin and $\alpha1$-antitrypsin complexed with human neutrophil elastase were used as standards.

Enzyme-linked immunoassay (ELISA) for human $\alpha2$-macroglobulin was performed as described (15). Briefly, wells of polystyrene microelisa plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 1.6 μg of purified human $\alpha2$-macroglobulin (16) in Voller's buffer (17). Monospecific goat anti-human $\alpha2$-macroglobulin (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.) diluted 1:2,000 was mixed with an equal volume of sample or standard and 0.2 ml of the mixture was transferred to the rinsed coated plate. $\alpha2$-Macroglobulin in the sample blocked binding of the antibody to the antigen-coated plastic well. The amount of antibody that did bind was quantitated by adding a second antibody, 1:1,500 dilution of horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (IgG) followed by a colorless substrate (*o*-phenylenediamine, Aldrich Chemical Co., Inc., Milwaukee, Wis.) for the peroxidase which is converted into a chromophore and measured spectrophotometrically (15). The test was sensitive to 25 ng/ml and was able to reliably detect $\alpha2$-macroglobulin in complex biological fluids such as plasma or bronchoalveolar lavage fluid (16).

Counterimmunoelectrophoresis (18) in agarose was used to detect the bronchial mucous inhibitor in lavage samples. Using a monospecific antibody (kindly provided by Dr. Harvey Carp; State University of New York at Stony Brook), this immunoassay was able to detect the inhibitor when present at concentrations as low as 2 μg/ml. Central airway secretions obtained from saline lavage of human subjects via endotracheal tubes were used as a positive control for the immunochemical detection of the bronchial mucous inhibitor.

Functional activity of lung antiproteases

To evaluate the functional activity of the antiproteases of the lower respiratory tract of the three study groups, concentrated

bronchoalveolar lavage fluid was tested for inhibitory activity against purified human neutrophil elastase. The neutrophil enzyme was used throughout this study, because it represents the elastase which bears physiologic relevance to the emphysematous process (19, 20) and because, unlike porcine pancreatic elastase, it is inhibited by the bronchial mucous inhibitor (21) whose role in the lower respiratory tract we sought to clarify.

Human neutrophil elastase was purified by the procedure of Taylor and Crawford (22); sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified product showed a single protein band with a molecular weight of ~30,000. The 1.0 μg of purified enzyme degraded 200 μg of insoluble elastin in 24 h. The insoluble [³H]elastin substrate used in these studies was prepared as described by Takahashi (23); the substrate had a specific activity of 500 dpm/μg.

To test lavage fluid antielastase activity, the concentrated lavage fluid was incubated with 1.0 μg purified neutrophil elastase at 23°C for 45 min. For all assays, the volume of lavage fluid added was that which contained 50 μg of albumin. This mixture was then added to 200 μg of the [³H]elastin substrate in the presence of 100 mM Tris-HCl, pH 7.4 (final volume 300 μl) and incubated at 37°C for 18 h. For each group of patients evaluated, lavage fluid from at least 3 PiM normal controls were assayed in parallel. The data for the controls were averaged, and that value was considered to represent 100% functional antielastase activity. The functional antielastase activity of the patient groups was then expressed as the percentage of elastase inhibition afforded by the lavage fluid of the average normal controls. Correction for differences in dilution of the lavage fluid samples was made on the basis of albumin concentration in the sample (13).

To determine the importance of α1-antitrypsin as an antielastase in the lower respiratory tract, α1-antitrypsin was removed from concentrated bronchoalveolar lavage fluid of normal individuals using immunoprecipitation with anti-α1-antitrypsin. To accomplish this, the IgG fraction of monospecific goat antihuman α1-antitrypsin was added to the lavage sample in a quantity which removed all antigenic α1-antitrypsin from the sample; the efficacy of the process was confirmed by radial immunodiffusion. The lavage fluid was then evaluated for residual antielastase activity using the assay described above.

To insure that the α1-antitrypsin in the lavage fluid of the PiM normal controls had not lost antielastase activity during processing, the amount of α1-antitrypsin in lavage fluid was determined by rocket immunoelectrophoresis and expressed as moles of α1-antitrypsin/moles of lavage fluid albumin. This sample was then tested in the functional antielastase assay described above and the micromoles of lavage fluid α1-antitrypsin required to inhibit 1 μmol purified neutrophil elastase was determined (24).

Analysis of the influence of alveolar macrophages on the recovery of antielastases from the human lower respiratory tract

Normal human alveolar macrophages were obtained by bronchoalveolar lavage (13) and cultured in sterile plastic plates (Costar; Cambridge, Mass.) at a concentration of 10⁶/ml in Gey's solution (Microbiological Associates, Walkersville, Md.). The buffer was diluted with sterile 0.15 M NaCl to obtain an albumin content approximating that of lavage fluid (1 mg/ml). Purified human α1-antitrypsin and α2-macroglobulin (16) were added to the macrophage cultures to provide a final concentration of these antielastases of 200 μg/ml. Purified human neutrophil elastase was then added to the macrophage

cultures (supplemented with α1-antitrypsin and α2-macroglobulin) in amounts ranging from 1 to 100 μg/ml (final concentration in culture). The alveolar macrophage cultures, containing varying amounts of neutrophil elastase and constant amounts of α1-antitrypsin and α2-macroglobulin, were then incubated at 37°C with constant mixing for 60 min. After this incubation the suspensions were centrifuged (1,500 g for 10 min) and the supernates were analyzed for the content of α1-antitrypsin (rocket immunoelectrophoresis), α2-macroglobulin (ELISA), the uninhibited neutrophil elastase activity ([³H]elastin substrate), and the formation of neutrophil elastase-antielastase complexes (immunoelectrophoresis).

Molecular sieve chromatography

To determine the molecular weight of the antielastases in the lavage fluid of normal individuals, concentrated bronchoalveolar lavage fluid (0.5 ml) was chromatographed on a 40- × 2.5-cm column of G-75 Sephadex (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) in 0.5 M NaCl–0.03 M Tris-HCl, pH 7.4 (4°C). 0.5-ml fractions were collected and assayed directly for functional activity against human neutrophil elastase as well as antigenic activity for α2-macroglobulin and α1-antitrypsin. The column was calibrated with dextran blue (mol wt, 1,000,000), ovalbumin (45,000), and cytochrome c (12,500).

Sequential evaluation of PiZ lung antielastase screen

To establish the chronicity of the lack of antielastase protection of the lower respiratory tract of PiZ individuals, three patients with PiZ α1-antitrypsin deficiency were lavaged several times over a 6- to 12-mo period. The lavage fluid was handled as described above and the antielastase activity determined. Three PiM individuals with idiopathic pulmonary fibrosis were evaluated in a similar fashion for comparison. The antielastase activity in the lavage fluid was expressed as a percentage of antielastase activity of lavage fluid of a group of PiM normal individuals as described above.

RESULTS

Immunochemical determinations of antiproteases in serum and bronchoalveolar lavage in normal subjects. The mean serum level of α1-antitrypsin in the nine normal subjects was 187±32 mg/dl (Fig. 1). The bronchoalveolar lavage α1-antitrypsin in these individuals was 51±10 μg/mg albumin. This value for lavage fluid α1-antitrypsin per milligrams of albumin was in close agreement with the serum α1-antitrypsin expressed as a function of serum albumin in these subjects (serum α1-antitrypsin 53±11 μg/mg albumin, $P > 0.3$), suggesting that the α1-antitrypsin present in the normal lower respiratory tract is not produced or concentrated within the lung but rather represents a filtrate of plasma. The serum α2-macroglobulin in these normal subjects was 254±45 mg/dl (Fig. 1). In contrast, α2-macroglobulin was below detectable limits in the concentrated bronchoalveolar lavage fluid obtained from normals. Since the minimal detectable levels of α2-macroglobulin in the rocket electroimmunodiffusion assay was 3 μg, this
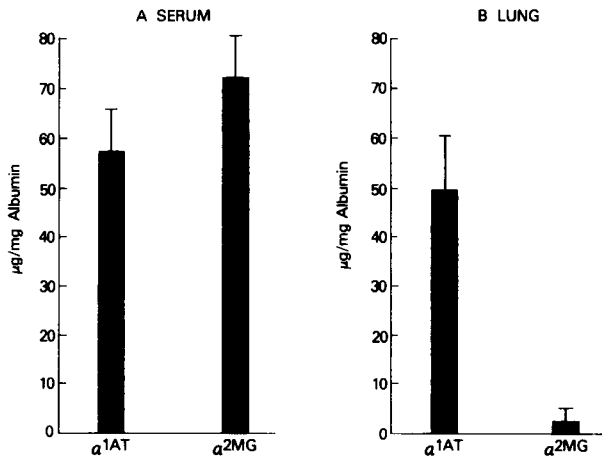
FIGURE 1 Immunochemical quantitation of α1-antitrypsin and α2-macroglobulin in the serum and lung of normal, non-smoking individuals. (A) Serum α1-antitrypsin and α2-macroglobulin; and (B) lung α1-antitrypsin and α2-macroglobulin. The data is presented as micrograms of antiprotease compared to albumin levels (see Methods for details). α2-Macroglobulin content of normal human lower respiratory tract fluid was 2.1±0.2 μg/mg albumin by ELISA. All data are presented as mean±SEM.

finding suggests that if present, α2-macroglobulin must represent <0.3 nmol/μmol albumin. In contrast, α1-antitrypsin is present in normal lavage fluid at 60 nmol/μmol albumin, a minimum of 200-fold higher molar concentration than α2-macroglobulin.

In order to verify the low values of α2-macroglobulin in the human lower respiratory tract suggested by the electroimmunodiffusion assay, an ELISA was adapted for the detection of α2-macroglobulin in broncho-alveolar lavage (15). Using both purified human α2-macroglobulin and normal human serum as standards, the mean α2-macroglobulin content of bronchoalveolar lavage was 2.1±0.2 μg/mg albumin in four normal subjects, and 2.4±0.1 μg/mg albumin in five PiZ individuals ($P > 0.2$). These values confirm that, although detectable in lavage samples, α2-macroglobulin represents <0.5% of the molar content of lower respiratory tract antielastases when compared to the other major serum antielastase, α1-antitrypsin.

Functional antielastase activity in the normal human lower respiratory tract. Lavage fluid obtained from the lower respiratory tract of normal PiM subjects was an effective inhibitor of human neutrophil elastase activity against an insoluble elastin substrate. In the assay used, 1 μg elastase released 100,000 dpm from 200 μg of elastin substrate in 24 h. When lavage fluid (equivalent to 50 μg lavage fluid albumin) from a typical PiM normal subject was added, <5,000 dpm elastin was released by 1 μg of neutrophil elastase.

To verify that α1-antitrypsin represents the principal form of functional antielastase activity within the nor-

mal lower respiratory tract, monospecific, IgG antibody to α1-antitrypsin was used to deplete the normal PiM lavage fluid of its α1-antitrypsin. After addition of this antibody, the lavage fluid was again adjusted to an albumin content of 50 μg/ml, and its elastase inhibitory activity measured (Fig. 2). Addition of this antibody to the PiM normal lavage fluid removed nearly all the functional antielastase activity present. Neither addition of buffer nor antibody to albumin affected the antielastase activity of the fluid (data not shown). If antielastases other than α1-antitrypsin (e.g., α2-macroglobulin, bronchial mucous inhibitor) were present in the lower respiratory tract of normal individuals, removal of α1-antitrypsin would have left antielastase activity in the lavage fluid. We can conclude, therefore, that α1-antitrypsin is the major antineutrophil elastase of the normal human lower respiratory tract.

Further evidence that α1-antitrypsin is the principal source of functional antielastase protection of the normal human lower respiratory tract was provided by
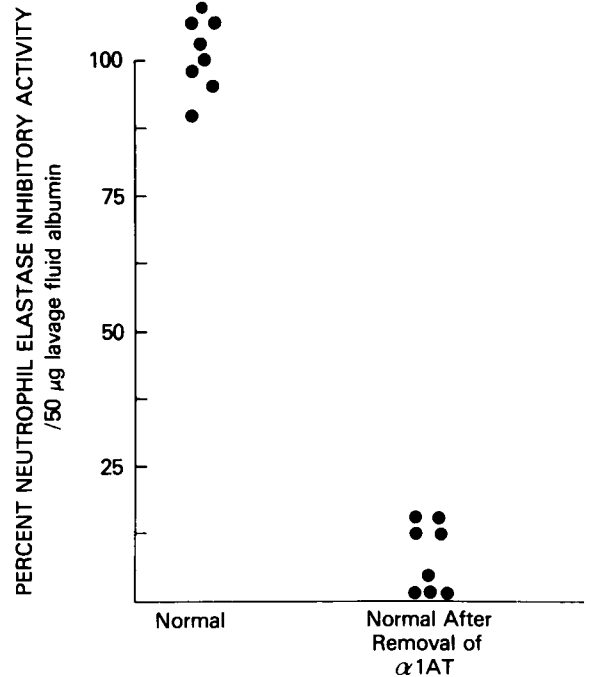

FIGURE 2 Antielastase activity in the lower respiratory tract of normal, nonsmoking PiM individuals. Normal broncho-alveolar lavage fluid is an effective inhibitor of human neutrophil elastase. After removal of α1-antitrypsin by immunoprecipitation, there is an eightfold loss of antielastase activity. Lavage fluid containing 50 μg albumin was evaluated for its ability to inhibit the proteolysis of 200 μg of insoluble elastin in a standard assay using 1 μg purified human neutrophil elastase. The mean inhibitory value for eight normal individuals was considered to represent 100% inhibition. The antielastase inhibitory activity after removal of the α1-antitrypsin was then expressed relative to this 100% mean value.

chromatographic analysis of the lavage fluid. Sephadex G-75 molecular sieve chromatography of normal lower respiratory tract fluid demonstrated that a single peak of functional antielastase activity eluted between the void volume and 45,000-molecular weight markers in the fractions containing the antigenic α1-antitrypsin activity (Fig. 3). There was no antielastase activity in those fractions eluting in the 10,000- to 15,000-molecular weight range nor in the >60,000-molecular weight range, further suggesting that significant amounts of bronchial mucous inhibitor or α2-macroglobulin do not have access to the normal lower respiratory tract.

The absence of significant quantities of bronchial mucous inhibitor in the human lower respiratory tract was also demonstrated by counterimmunoelectrophoresis of lavage samples vs. monospecific antibody to this low molecular weight inhibitor. Whereas counterimmunoelectrophoresis readily demonstrated the inhibitor in central airways' secretions and purulent sputum, there was no precipitin line formed when this antibody was electrophoresed vs. concentrated normal bronchoalveolar lavage obtained via fiberoptic bronchoscopy (data not shown).

The amount of α1-antitrypsin in the normal PiM lower respiratory tract was present at an amount equivalent to 60 nmol/μmol albumin. Evaluation of the antielastase activity of this lavage fluid demonstrated that it inhibited 25 nmol of purified neutrophil elastase per micromoles of albumin, close to the 1:1 molar combining ratio for α1-antitrypsin and neutrophil elastase (since the molecular weight of elastase is approximately one-half that of α1-antitrypsin) (24). In addition, crossed
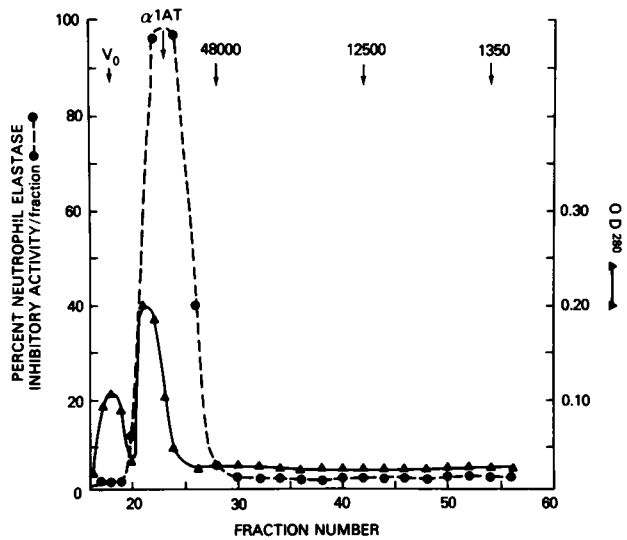

FIGURE 3 Molecular weight distribution of antielastases of bronchoalveolar lavage fluid obtained from the lower respiratory tract of normal, PiM nonsmoking individuals. Lavage fluid was concentrated and applied to a G-75 column, eluted with 0.5 M NaCl, 0.03 M Tris, pH 7.4, and 0.5-ml aliquots evaluated for antielastase activity against human neutrophil elastase using an insoluble elastin substrate. The elution position of various markers of different molecular weight are shown, including α1-antitrypsin. The peak of antielastase activity in the lavage fluid (●) coincided with the peak of α1-antitrypsin detected by an α1-antitrypsin antibody. Optical density at 280 nm is also shown (▲).

immunoelectrophoretic evaluation of the concentrated lavage fluid from normal PiM individuals demonstrated that the α1-antitrypsin present migrated with a free

TABLE I
Analysis of the Effect of Neutrophil Elastase on Fluid-phase α2-Macroglobulin: Assessment of the Clearance of α2-Macroglobulin-elastase Complexes by Alveolar Macrophage Receptors

Alveolar macrophage*	α2-Macroglobulin‡	α1-Antitrypsin§	Neutrophil elastase‖	Recoverable α2-macroglobulin¶	Residual elastase activity**
	$\mu g/ml$	$\mu g/ml$	$\mu g/ml$	$\mu g/ml$	
10^6	200	200	0	180	—
10^6	200	200	1	215	0
10^6	200	200	10	220	0
10^6	200	200	25	197	0
10^6	200	200	100	220	0
10^6	200	0	100	195	85
0	0	0	100	—	100

* Normal human alveolar macrophages obtained by bronchoalveolar lavage (13).
‡ α2-Macroglobulin = purified human α2-macroglobulin.
§ α1-Antitrypsin = purified human α1-antitrypsin.
‖ Purified neutrophil elastase.
¶ α2-Macroglobulin recovered from macrophage culture after incubation with neutrophil elastase; α2-macroglobulin content as quantitated by ELISA (15).
** Uninhibited elastase remaining in culture after a 60-min incubation; elastolytic activity determined by solubilization of [^{3}H]elastin substrate (22). 100 μg/ml of purified neutrophil elastase degrades 1,000 μg of native elastin per hour.
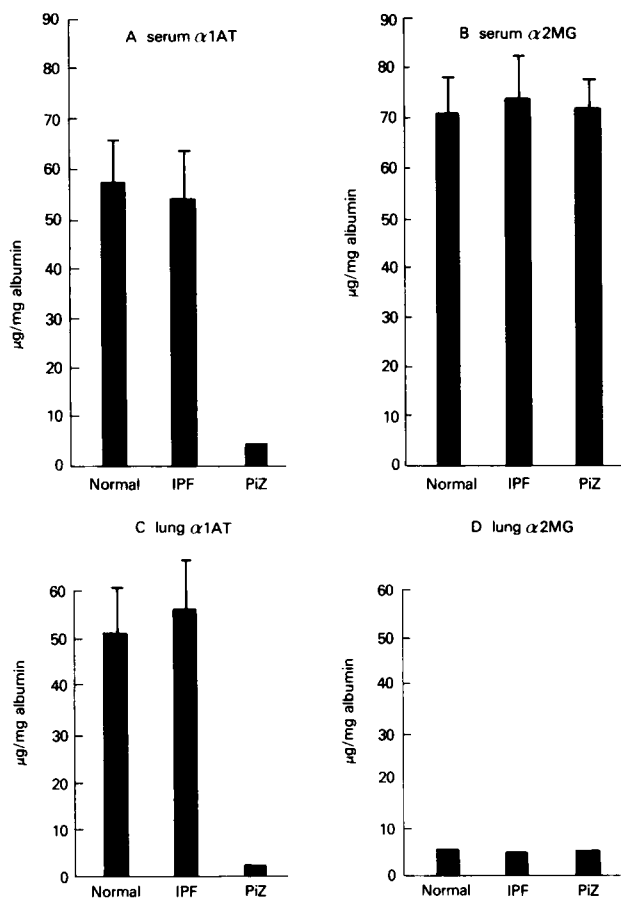
FIGURE 4 Antigenic levels of α1-antitrypsin and α2-macroglobulin in the serum and lower respiratory tract of individuals with homozygous PiZ α1-antitrypsin deficiency. For comparison, values for nonsmoking normals and patients with IPF are shown. The data is expressed per milligram of albumin to afford a comparison between serum and lung (see Methods for details). (A) Serum α1-antitrypsin; (B) serum α2-macroglobulin; (C) lung α1-antitrypsin; and (D) lung α2-macroglobulin. All data are presented as mean±SEM.

(i.e., uncomplexed) α1-antitrypsin standard (data not shown). Thus, not only is α1-antitrypsin the only antielastase of the normal lower respiratory tract, but the majority of it is active and in an uncomplexed form.

Assessment of the effect of alveolar macrophage-mediated clearance of protease-antiprotease complexes on the content of fluid-phase α2-macroglobulin in the lower respiratory tract. To assess a possible effect of macrophage-mediated clearance of protease-antiprotease complexes on the concentration of antielastase species in bronchoalveolar lavage fluid, a simulation of the alveolar milieu was constructed. Normal human alveolar macrophages were cultured in the presence of equivalent amounts of purified serum antielastases, α1-antitrypsin, and α2-macroglobulin (Table I). After incubation with increasing amounts of neutrophil elas-

tase up to the expected saturation of α1-antitrypsin (assuming a 1:1 molar-combining ratio), there was no reduction of α2-macroglobulin detectable by ELISA assay (Table I). In the presence of α1-antitrypsin, all of the added neutrophil elastase was inhibited and formation of elastase-α1-antitrypsin complexes was demonstrated by the cathodal shift of the immunoelectrophoretic pattern of α1-antitrypsin (data not shown). Thus, although macrophages have been shown to bind α2-macroglobulin-protease complexes via cell surface receptors, in the presence of α1-antitrypsin, this macrophage-complex interaction does not alter the content of α2-macroglobulin in the extracellular environment.

Confirmation of α1-antitrypsin as the primary alveolar antielastase: there is no alternative antielastase within the lower respiratory tract of PiZ individuals. The mean serum antigenic level of α1-antitrypsin in the eight PiZ individuals was 39±7 mg/dl as compared to 187±32 mg/dl for the normal subjects. Expressed relative to the amount of albumin present, the mean serum α1-antitrypsin of the PiZ individuals was 11±2 μg/mg albumin in contrast to the normal value of 51 ± 10 μg/mg albumin (Fig. 4A). There was no significant difference in the mean serum α2-macroglobulin levels between the PiZ and PiM individuals, whether expressed as amount α2-macroglobulin per milliliter or amount α2-macroglobulin per milligrams of albumin (Fig. 4B, $P > 0.4$, all comparisons).

Immunochemical analysis of lower respiratory tract lavage fluid demonstrated that no detectable α1-antitrypsin was recoverable from the lungs of the PiZ individuals (<2 μg/mg albumin) (Fig. 4C). In contrast, an average of 55±15 μg of α1-antitrypsin per milligrams of albumin was recovered from the lungs of PiM individuals with IPF (another chronic lung disease in which proteases may play a pathogenetic role) (35), a value similar to that of normals ($P > 0.25$). As was the case for the normal subjects, α2-macroglobulin does not have free access to the lower respiratory tract of PiZ individuals or patients with IPF (Fig. 4D). Thus, immunochemical studies suggest that individuals with serum α1-antitrypsin deficiency have no detectable α1-antitrypsin in their lower respiratory tract and that, within their alveolar structures, there is no "compensatory" increase of α2-macroglobulin to provide an alternative form of antielastase. (PiZ individuals possess a mean of 2.4±0.1 μg of α2-macroglobulin per milligram of albumin in their lavage fluid, see above.)

If α1-antitrypsin is the principal antielastase at the level of the alveolar structures, then analysis of functional antielastase activity in the lower respiratory tract of α1-antitrypsin-deficient individuals would be predicted to be negligible. In fact, this was the case (Fig. 5). The fluid recovered from the lower respiratory tract of PiZ individuals demonstrated ninefold less antielastase activity than was recovered from the lungs of normal

individuals and sevenfold less than that of individuals with IPF. In addition to confirming the primary role of serum α1-antitrypsin in the antielastase screen of the human respiratory tract, these data suggest that there is a potential elastase-antielastase imbalance within the alveolar structures of the PiZ individual.

Analysis of bronchoalveolar lavage obtained from individuals with serum α1-antitrypsin deficiency also serves to demonstrate the relatively unimpeded passage of α1-antitrypsin from intravascular to alveolar space. After the intravenous administration of 4 g of α1-antitrypsin, lavage fluid α1-antitrypsin levels in these individuals rises from >2 μg/mg albumin to 30 μg/mg albumin (25). In addition, studies using [^{125}I]-α1-antitrypsin have demonstrated that the access of intravascular α1-antitrypsin to the alveolar structures is comparable to that of serum albumin, a molecule of similar size.[3]

Chronicity of lack of antielastase screen in the lower respiratory tract in PiZ individuals. α1-Antitrypsin deficiency is a chronic disease in which there is slowly progressive destruction of the alveolar structures. The protease-antiprotease theory of emphysema would predict, therefore, that the lack of antielastase screen in the lower respiratory tract of the PiZ subject would be a chronic condition. To insure that this was the case, three PiZ individuals and three PiM patients with IPF were sequentially lavaged over a 6- to 12-mo period. As predicted by the protease-antiprotease theory, the PiZ individuals had little change in their diminished antielastase screen when they were evaluated at each time point. In contrast, the patients with IPF had adequate antielastase protection in their lower respiratory tract throughout the study period (Fig. 6).

DISCUSSION

Because α1-antitrypsin is the major serum antielastase (4) and individuals deficient in serum α1-antitrypsin have early-onset, progressive destruction of their lower respiratory tract, the protease-antiprotease theory of emphysema predicts that α1-antitrypsin provides the major antielastase screen for the human alveolar structures. The present study confirms that prediction and demonstrates that individuals with α1-antitrypsin have no alternative antielastase in their lower respiratory tract.

α1-Antitrypsin is the major antielastase of the human lower respiratory tract. The current concepts of the pathogenesis of emphysema suggest that alveolar destruction is mediated, at least in part, by the unimpeded action of neutrophil elastase (5-8, 26). The role of alveolar macrophage elastase in the pathogene-
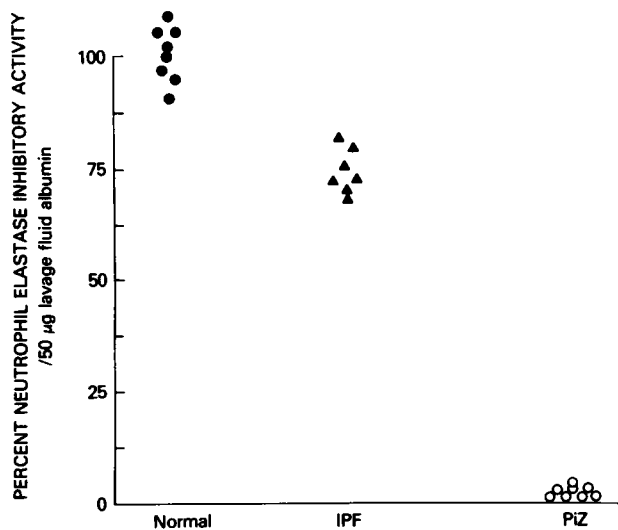
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[3] Jones, E. A., J. E. Gadek, and R. G. Crystal Unpublished observations.

FIGURE 5 Antielastase activity in the lower respiratory tract of individuals with PiZ α1-antitrypsin deficiency. The antielastase activity of bronchoalveolar lavage fluid was treated in a standard system using purified neutrophil elastase and an insoluble elastin substrate (see legend to Fig. 2 and Methods for details). The average value for eight normal, nonsmoking PiM individuals was taken as 100% antielastase activity of bronchoalveolar lavage fluid. Data is shown for eight normal individuals, seven patients with IPF, and eight PiZ individuals.

sis of emphysema is less certain (27). The fact that this elastase is not inhibited by α1-antitrypsin (27) and that experimental models do not exist make it difficult to integrate macrophage elastase into the protease-antiprotease theory. This theory draws heavily from the clinical model of serum α1-antitrypsin deficiency and is challenged by the discovery of an alternative, locally produced, respiratory tract antielastase, the bronchial mucus inhibitor (21). Thus, validation of this concept requires demonstration that the major functional antielastase within the lower respiratory tract is α1-antitrypsin. Several pieces of evidence support this concept:

(*a*) Immunochemical quantitation of the major serum antielastases (α1-antitrypsin and α2-macroglobulin) in bronchoalveolar lavage fluid of normal individuals demonstrated that while lung α1-antitrypsin concentration (relative to albumin) is similar to serum, lung α2-macroglobulin concentration is 25 times less than its serum concentration. Since α1-antitrypsin and α2-macroglobulin inhibit neutrophil elastase in equimolar amounts, the observation suggests that α1-antitrypsin contributes at least 200-fold more antielastase protection to the lower respiratory tract than could α2-macroglobulin. This is not to suggest that α2-macroglobulin is not important to the human lung, but rather that its presence in the alveolar structures, at a level some 25 times lower than the serum level, is not sufficient to maintain elastase-antielastase homeostasis in the

alveolar structures. In this context, perhaps the primary role of α2-macroglobulin in the human lower respiratory tract concerns the modulation of proteases for which α1-antitrypsin cannot serve as an effective antiprotease (e.g., neutrophil collagenase or the proteases of the coagulation and fibrinolytic pathways) (28).

(b) Removal of α1-antitrypsin from lavage fluid of normal individuals results in virtually complete loss of the functional antielastase activity recovered from the lower respiratory tract.

(c) Molecular sieve chromatography of bronchoalveolar lavage fluid of normal individuals demonstrated that the only functional antielastase activity retrievable from the normal human lower respiratory tract coeluted with α1-antitrypsin.

(d) Analysis of the influence of alveolar macrophages on the local clearance of α2-macroglobulin suggests that, in the presence of α1-antitrypsin, receptor-mediated clearance does not alter local α2-macroglobulin concentration.

Clearly, therefore, α1-antitrypsin is the primary antielastase of the lower respiratory tract of normal man. Since α1-antitrypsin has a molecular weight of 52,000 (6), it diffuses through the alveolar structures from the endothelial to the epithelial surface with relative ease. In this context, metabolic studies of radioiodinated α1-antitrypsin show that the labeled protein is recovered in bronchoalveolar lavage fluid in quantities comparable to the activity in plasma when normalized to the albumin content of the fluid.[3] In contrast, α2-macroglobulin is of large (725,000 daltons) molecular weight, a molecular size that appears readily excluded from diffusion by alveolar-capillary structures. While α2-macroglobulin may be produced locally by alveolar macrophages (27), the present study suggests that the level of production in vivo must be quite low.

Even though another antielastase (the so-called "bronchial mucous inhibitor") has been found in human lung (21, 29–32), the present study suggests that the bronchial mucous inhibitor must be confined to more central airways rather than in those regions of lung sampled by the fiberoptic bronchoscope. This data is consistent with the findings of Tegner (31), who has shown that the bronchial mucous inhibitor accounts for ~80% of the neutrophil elastase-inhibitory activity in epithelial fluid recovered from the large airways. Unlike plasma-derived α1-antitrypsin, which diffuses through the alveolar structures, the bronchial mucous inhibitor is likely secreted by the epithelial cells and/or mucous acinar cells of the upper respiratory tract (33). Since α1-antitrypsin seems to contribute little to the antielastase screen of the central airways, we may conclude from Tegner's and the present study that α1-antitrypsin is the major antielastase of the lower respiratory tract while the bronchial mucous inhibitor affords antielastase protection of the upper respiratory tract.
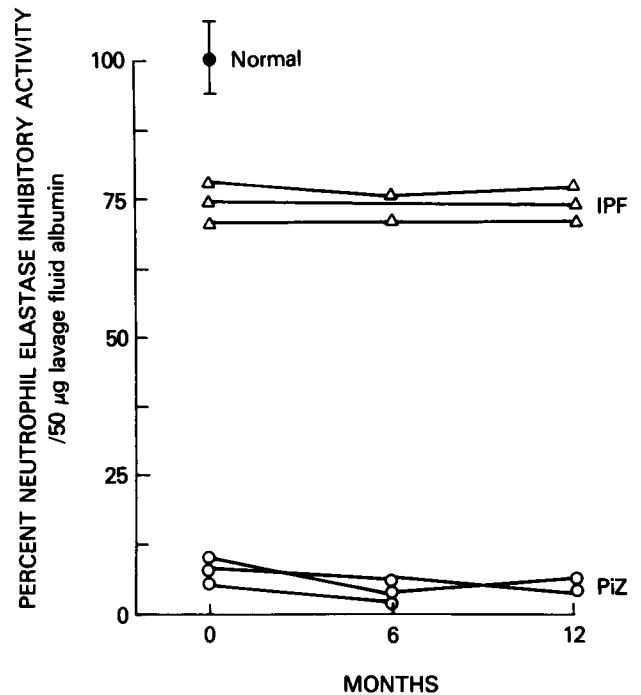

FIGURE 6   Chronicity of the antielastase screen of the lower respiratory tract of patients with PiZ α1-antitrypsin deficiency. Three individuals with PiZ α1-antitrypsin deficiency were lavaged two to three times each over a 6- to 12-mo period. For comparison, three individuals with IPF were lavaged three times over the same period. The antielastase activity of their bronchoalveolar lavage fluid is expressed as elastase inhibitory activity per 50 μg lavage fluid albumin (see legend to Fig. 2 and Methods for details). The average values for eight normal individuals are shown (●).

Nonsmoking PiM individuals with IPF provide a relevant experimental group, permitting the study of the effect of a chronic inflammatory disease of the alveolar structures on antielastase activity (9). These individuals had a slight (25% less than normal) deficiency of antielastase activity in their lower respiratory tract. The reason for this is not clear, but it has been demonstrated that smoking PiM individuals have normal antigenic levels of α1-antitrypsin in their lower respiratory tract, yet have ~50% functional antielastase activity compared to normal nonsmoking individuals (24). In an interesting series of studies to evaluate the effect of oxidants on α1-antitrypsin, Carp and Janoff have shown that a variety of oxidants, including oxidants produced by neutrophils, as well as those in cigarette smoke, are capable of rendering α1-antitrypsin functionally inactive (34). Since IPF is a disease associated with chronic accumulation of neutrophils in the lower respiratory tract, it is reasonable to hypothesize that the slightly reduced function of antielastase activity in the lower respiratory tract of PiM nonsmoking individuals with IPF results from the release of oxidants from these

neutrophils (24, 34). Although active collagenase can be recovered from the lungs of individuals with IPF, the antielastase activity in their lower respiratory tract appears sufficient to preclude the expression of neutrophil elastase and may thus regulate the character of the pathologic lesion (35).

Implications for the protease-antiprotease theory of emphysema. Because αl-antitrypsin is the major antielastase of the lower respiratory tract, the protease-antiprotease theory of emphysema predicts that patients with serum αl-antitrypsin deficiency should have little, if any, antielastase activity in their alveolar structures. Direct analysis of epithelial fluid derived from the lower respiratory tract of PiZ individuals confirms this hypothesis: these individuals have no antigenic αl-antitrypsin in their lower respiratory tract nor do they have any alternative antielastases to replace it. This seems to be true for the same PiZ individual evaluated sequentially over a 6- to 12-mo period, confirming the prediction that the lack of antielastases in the lower respiratory tract of these individuals occurs on a chronic basis. Thus, individuals with PiZ αl-antitrypsin deficiency have no means to protect their alveolar structures from elastolytic attack. Although the neutrophil burden of the lower respiratory tract in αl-antitrypsin-deficient individuals is not known, the fact that these subjects have almost no antielastase screen suggests that any neutrophils present likely would have a devastating effect on the alveolar structures in these individuals. Further evidence may be provided by the recent experimental model devised by Cohen, in which serum αl-antitrypsin has been rendered functionally inactive (36).

Implications for the therapy of αl-antitrypsin deficiency. Although it is estimated that ~20% of homozygous individuals with αl-antitrypsin deficiency do not develop emphysema, the disease is fatal in the majority of deficient individuals (5, 7). Since cigarette smoking appears to greatly accelerate this destructive lung disease (37), presumably by increasing the influx of elastase-containing neutrophils into alveolar structures (38), cessation of cigarette use becomes the initial point of attack in this disease. Because αl-antitrypsin deficiency is clearly associated with the deficiency in the antielastase screen in the lower respiratory tract, a rational approach to the therapy of this disease would be to increase the serum αl-antitrypsin levels (hence the lung antielastase screen) (39). In this regard, two therapeutic strategies for these patients are under study.

First, PiZ patients are being treated with danazol, an impeded androgen that increases serum levels of Cl esterase inhibitor, another liver-produced antiprotease (40, 41). Preliminary studies in seven αl-antitrypsin-deficient individuals treated for 1 mo showed that danazol administration increased serum αl-antitrypsin levels by an average of 40% (42). While this is not the serum αl-antitrypsin level thought to completely protect the lower respiratory tract, it does bring the αl-antitrypsin-deficient individual closer to the theoretical protective threshold. Presently, we are evaluating this treatment over a 1-yr period to determine if chronic administration of this drug can maintain increased αl-antitrypsin levels in these patients.

Second, a small group of PiZ patients with severe emphysema have been treated with direct intravenous infusion with partially purified αl-antitrypsin (25). Preliminary studies suggest that, with a once weekly administration, serum and lung αl-antitrypsin levels can be maintained at or above the protective threshold level. Thus, it may be possible to directly replenish the antielastase protection of the alveolar structures of such individuals by intermittent, direct parenteral administration.

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