

Interrelationships between the Human Alveolar Macrophage and Alpha-1-Antitrypsin

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ABSTRACT Alveolar macrophages lavaged from human lungs contain protease activity at an optimum pH of 3.0 and possibly a lesser peak of activity at pH 5.5. Protease activity measured at pH 4.1 is inhibited by purified alpha-1-antitrypsin.

Fluorescent antibody studies of human alveolar macrophages showed that alpha-1-antitrypsin is present in normal alveolar macrophages. In addition, macrophages from a patient with a homozygous deficiency of alpha-1-antitrypsin exhibited less fluorescence when incubated in autologous serum than the same macrophages incubated in normal serum. Macrophages from normal subjects showed maximal fluorescence when removed from the lung and additional incubation with serum did not increase fluorescence.

These results implicate the human alveolar macrophage as a possible source of an enzyme that may cause emphysema in patients deficient in alpha-1-antitrypsin. They also show that alpha-1-antitrypsin has access to the alveolus in normal subjects.

INTRODUCTION

Since Eriksson (1) observed that emphysema develops at an early age in people who are homozygotes for the genes that cause deficiency of alpha-1-antitrypsin, investigators have searched for enzymes that might cause emphysema in the absence of the enzyme inhibitor. Most of the enzymes inhibited by alpha-1-antitrypsin, such as trypsin (2), chymotrypsin (2), pancreatic elastase (3), plasmin (4), and thrombin (4), are unlikely to be found in the lung parenchyma. The fibrinolytic enzyme in human neutrophils, which is inhibited by alpha-1-antitrypsin (5), has access to pulmonary tissues in inflammatory states but the pathologic appearance of

emphysema lacks neutrophilic infiltration (6). Mass, Ikeda, Meranze, Weinbaum, and Kimbel (7) demonstrated that an emphysema-like pathologic appearance can be produced in dog lungs by aerosols of enzyme extracts from human and dog neutrophils and of dog alveolar macrophages. They also observed that the ability of an extract to cause the emphysema-like pathologic appearance correlates more closely with the ability of the extracted enzymes to utilize a hemoglobin substrate than calf thymus histone or casein. Janoff (8) observed that an extract of enzymes from human alveolar macrophages produced an emphysema-like appearance in mouse lungs, but previous attempts to locate enzymes that are inhibited by alpha-1-antitrypsin in human alveolar macrophages have failed. This report describes a protease in human alveolar macrophages that is inhibited by alpha-1-antitrypsin. In addition, experiments using fluorescent microscopy indicate that alpha-1-antitrypsin is normally found in human alveolar macrophages.

METHODS

Cell retrieval and preparation. Human alveolar macrophages were lavaged from surgically removed lungs, by the method of Cohen and Cline (9), or from lungs of awake volunteers, by the method of Finley, Swenson, Curran, Huber, and Ladman (10). The cells were lavaged from surgical specimens with Hank's balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum (FCS)¹, 10 U of heparin/ml, 50 U of penicillin/ml, and 50 µg of streptomycin/ml (FCS-Hank's).

Macrophages used for the enzyme studies were obtained by two different methods. Macrophages used in experiments I and II (Table I) were obtained from lung washes that were only minimally contaminated by cells that were not macrophages. Only 4 of 40 lung washes yielded macrophages of sufficient purity to be used without further purification. Greater than 95% of the total cells were alveolar macrophages, judged morphologically and by their ability

¹ Abbreviation used in this paper: FCS, fetal calf serum.

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TABLE I
Effect of Alpha-1-Antitrypsin on Macrophage Cathepsin

Experiment no.	Specific activity of macrophage cathepsin, $\frac{\text{mg non-TCA precipitated protein}}{\text{min} \times \text{mg extracted protein/ml}}$		Average % inactivation of macrophage cathepsin	Average mol of cathepsin/mg protein in macrophage extract	Average mol of cathepsin/10 ⁶ macrophages
	Without α -1-antitrypsin	With α -1-antitrypsin			
I	5.84	1.04	81.7	2.28×10^{-9}	6.67×10^{-10}
	6.25	1.18			
II	2.54	0	100		
	2.47	0			
III	19.20	2.60	89.0	1.20×10^{-8}	2.77×10^{-9}
	19.03	1.60			
IV*	21.65	-0.80‡	100		

* Experiment IV represents several pools of purified alpha-1-antitrypsin. Catheptic activity = mean of 12 samples. Differences between groups are significant at $P < 0.0001$ using unpaired Student's t test. SE = 1.83 in the control and 1.69 in the treated group.

‡ The negative value for enzyme activity results from subtraction of the blank with TCA added before enzyme and hemoglobin.

to phagocytize heat-killed *Candida albicans* (9), and less than 1% of them were neutrophils. In later experiments, III and IV (Table II), enzymes were extracted from macrophages taken from lavages that were initially contaminated by blood cells, and the macrophages were separated from other cells by sedimentation at 1 g for 45 min and erythrocyte lysis using a 40 s pulse with distilled water. More than 96% of the cells in these preparations were alveolar macrophages. The cells were washed three times in sodium phosphate buffer, 0.1 M at pH 7.4, with 0.9% NaCl. They were then centrifuged at 250 g for 15 min and the supernate was discarded and the pellet frozen at -20°C until analyzed. Macrophages from the four patients used in experiments I and II (Table I) were frozen for

3-4 mo before use and macrophages from the three patients used in experiments III and IV (Table I), were frozen less than 7 days before use.

Macrophages used for fluorescent antibody studies were washed three times in FCS-Hank's and then resuspended in McCoy's 5A medium (Grand Island Biological Co.) containing 30% FCS, 50 U of penicillin/ml, and 50 μg of streptomycin/ml. After viable cell counts were performed with 0.4% trypan blue stain, 1 ml containing 10^5 - 10^6 viable macrophages/ml was added to each Leighton tube (Bellco Glass, Inc., Vineland, N. J.) containing a cover slip. The cells were incubated for 4 h at 37°C , non-adherent cells were washed off, and the media was replaced. The following morning the cells were used for the

TABLE II
Results of Different Regimens for Fluorescent Antibody Studies

Regimen* no.	Normal human serum	Rabbit antihuman α -1-antitrypsin	Rabbit antihuman serum albumin	Normal rabbit serum	FCS	Goat antirabbit unlabeled	Goat antirabbit labeled	Results‡
I§	+	+			+		+	+
II	+	+				+	+	-
III§		+			+		+	+
IV		+				+	+	-
V				+	+		+	MF
VI				+		+	+	-

* Reagents for each regimen were added from left to right to macrophages from eight surgical specimens and from lung lavages from two normal subjects. Each reagent was incubated with the macrophages for 30 min at 37°C with washes between reagents.

‡ "+" indicates positive or cytoplasmic pattern of fluorescence as seen in Fig. 2; "-" indicates absence of fluorescence; and "MF" indicates membrane pattern of fluorescence as seen in Fig. 4.

§ When macrophages from the subject with ZZ phenotype for alpha-1-antitrypsin were incubated with regimens I and III, macrophages incubated with regimen I fluoresced clearly brighter than those from regimen III, Fig. 3.

fluorescent antibody experiments except for those cells incubated longer before fluorescent staining in order to follow the time course of positive reactions.

Subjects. Macrophages from seven subjects were used for enzyme studies. The subjects were all over 55 yr of age and all were cigarette smokers except one who had stopped smoking 8 yr before surgery. These patients had bronchogenic carcinoma, but did not have endobronchial obstruction. Their forced vital capacities, lung volumes, and blood gases were within normal limits.

Cells from surgically removed lungs of eight patients and from lavaged lungs of three volunteers with no evidence of lung disease were used for fluorescent antibody studies. The patients ranged from 58 to 65 yr of age and seven were smokers and one had stopped smoking 11 yr before surgery. The three volunteers were nonsmokers and ranged from 25 to 37 yr of age. Their forced vital capacities, lung volumes, and blood gases were within normal limits. Two of the volunteers had the MM phenotype for alpha-1-antitrypsin. The third volunteer, a 37-yr old man, had the ZZ phenotype and a total trypsin-inhibiting capacity of 13.5 mg of trypsin/100 ml of serum. He was asymptomatic and had normal single breath carbon monoxide diffusion capacity, lung volumes, forced expired volume in 1 s expressed as a percent of the measured vital capacity, and airway resistance. Macrophages from all 11 subjects were treated with each of the regimens in Table II. Two tubes were treated with each regimen when macrophages from surgical specimens were employed, and one tube was treated with each regimen when lungs of normal subjects were lavaged.

Enzyme extraction. Macrophage pellets were thawed and 2×10^8 macrophages were suspended in 4.5 ml sodium acetate buffer, 0.01 M at pH 3.8. The suspension was homogenized at 0°C in a homogenizer with a microhomogenizing attachment (Ivan Sorvall, Inc., Newtown, Conn.) and a dial setting of 6 for 60 s. The homogenate was centrifuged for 20 min at 37,400 *g* (Beckman model L-50 preparative ultracentrifuge, Beckman Instruments, Inc., Fullerton, Calif.). The supernate, which contained small and large granules with phase optics at $\times 1,250$ magnification, was used for all enzyme studies.

Cathepsin assay. Cathepsin was measured by the method of Anson (11). The cellular extract, 0.5 ml, was incubated with purified alpha-1-antitrypsin in 0.5 ml in the acetate buffer at 37°C for 60 min. The 0.5 ml contained 0.2, 0.1, 0.7, and 0.9 mg of alpha-1-antitrypsin in experiments I-IV, respectively. 1 ml of hemoglobin substrate, 2 g/100 ml in the acetate buffer, was added and the reagents were incubated again for 60 min at 37°C. An equal volume of 5% trichloroacetic acid (TCA) was added. The precipitated protein was removed by centrifugation and the remaining soluble peptides were measured by the method of Lowry, Rosebrough, Farr, and Randall (12). The pH of the final material with all reactants added except the TCA was 4.0. The final optical densities were corrected for the effects of macrophage enzyme on other reactants and for the degree of TCA precipitability of the native proteins.

The pH titration of macrophage cathepsin was performed in a similar manner, but enzyme inhibition was not tested at other pH values. Analyses at pH 6 and above employed a Tris buffer, 0.1 M.

Fluorescent antibody studies. Fluorescent antibody studies were carried out using a Zeiss microscope (Carl Zeiss, Inc., New York) with a "50" exciter filter, a halogen light source, and a barrier filter "53." Fluorescinated anti-

rabbit gamma globulin (Roboz Surgical Instrument Co., Inc., Washington, D. C.) was absorbed with rat liver powder. Antihuman alpha-1-antitrypsin (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) gave a single precipitin band against both purified alpha-1-antitrypsin and human serum in Ouchterlony analysis. Neither serum formed precipitin bands with FCS in Ouchterlony analysis. The indirect staining technique of Weller and Coons (13) was employed on macrophages fixed for 5 min in cold acetone. All slides were rated by the investigator without knowledge of the group to which the slide belonged. Treated macrophages from each subject were compared only to the simultaneously prepared controls from the same patient.

Alpha-1-antitrypsin was purified by the method of Rimon and Shamash (14) and Shamash and Rimon (15), with some minor modifications. The alpha-1-antitrypsin was eluted from the ion-exchange column with starting buffer instead of a gradient and the last separation was performed with Pevikon block instead of starch block electrophoresis. Ouchterlony analysis showed that it gave a single line of precipitation against antiwhole human serum (Hyland Div., Travenol Laboratories, Inc.). In addition, one of the preparations yielded monospecific antisera when injected into each of two rabbits. The purified protein was used in all of the enzyme inhibition experiments.

RESULTS

In four experiments, 81.7, 100, 89.0, and 100% of the alveolar macrophage proteolytic enzyme or enzymes were inhibited by purified alpha-1-antitrypsin (Table I). Experiments I through III employed two treated and two control samples. Experiment IV included 12 treated and 12 control samples. The differences between groups

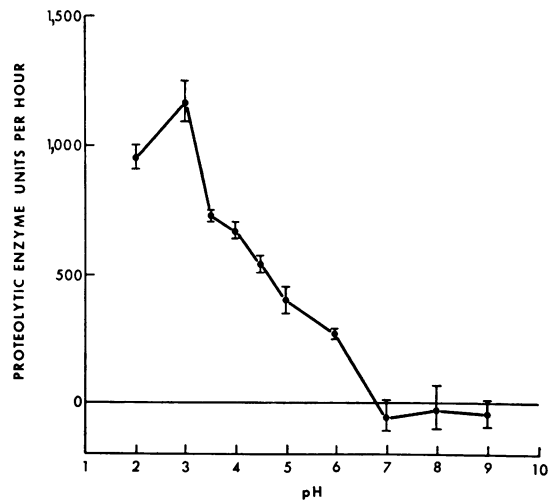


FIGURE 1 Human alveolar macrophage cathepsin:pH profile. A macrophage extract was assayed for proteolytic activity by the method of Anson (11). Proteolytic enzyme units = micrograms protein per milliliter remaining after large proteins are removed by precipitation with TCA. All points were obtained during one experiment on alveolar macrophages. Vertical bars = 1 SE in either direction from the mean value. Extracts from two other subjects show similar pH profiles.

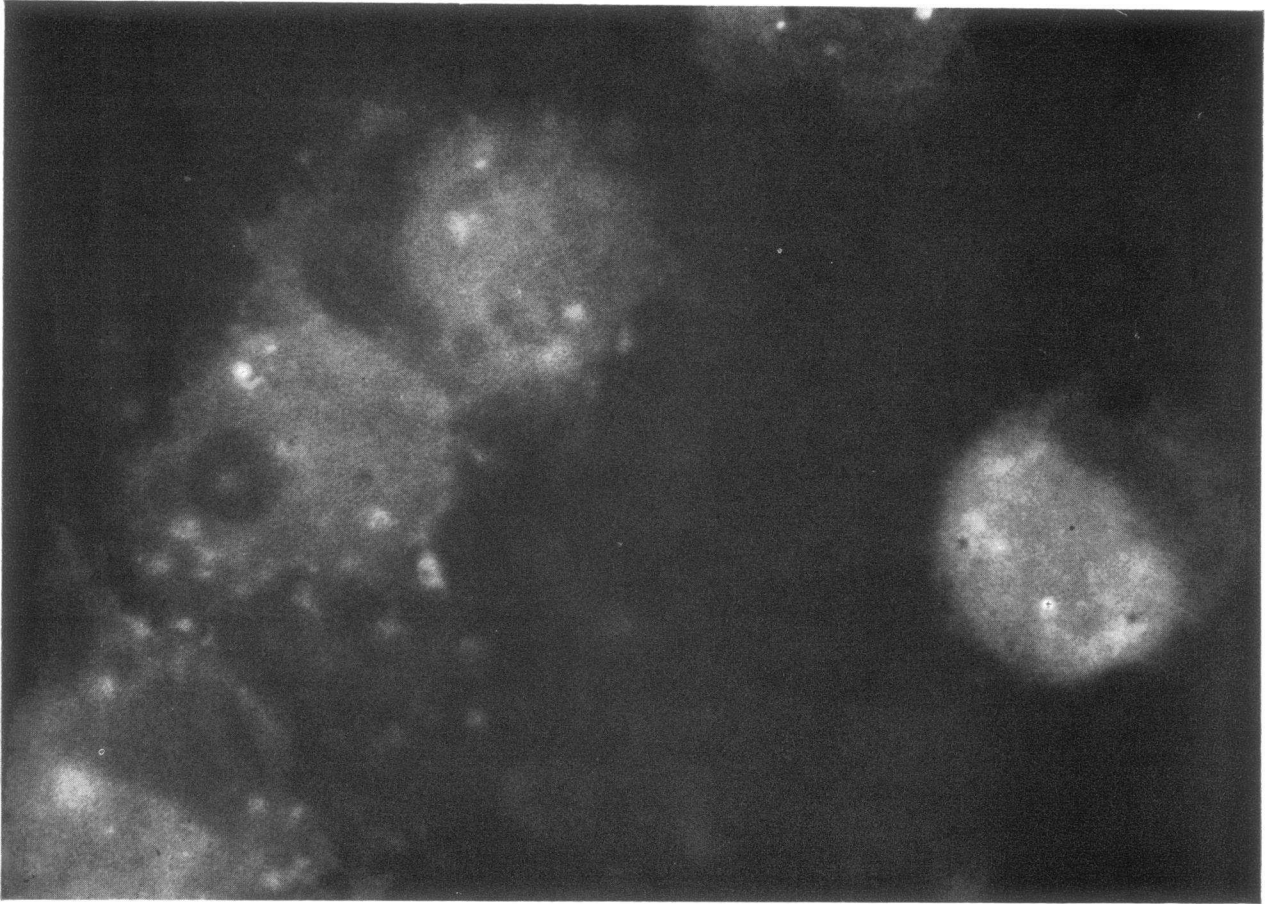


FIGURE 2 Alpha-1-antitrypsin in human alveolar macrophages. Day-old glass-adherent macrophages fluoresce when treated with antihuman alpha-1-antitrypsin (made in rabbits) and subsequently treated with fluorescein-tagged antisera to rabbit gamma globulin. The staining pattern excluded the nucleus suggesting an intracellular localization of the alpha-1-antitrypsin. (Magnification $\times 1,250$.)

in experiment IV were significant at $P < 0.0001$ by Student's *t* test. The specific activities of the enzyme in the extracts, the number of moles of macrophage enzyme per milligram of protein in the extract, and the number of moles of enzyme per million macrophages are listed in Table I. The estimates of the number of moles of macrophage enzyme were based on the assumption that alpha-1-antitrypsin inhibits this enzyme in a stoichiometric manner as it does trypsin and chymotrypsin (2). The denominators were measured directly. The pH profile of the enzyme (Fig. 1) indicates that maximal proteolytic activity for the hemoglobin substrate occurred at pH 3 and a shoulder on the pH curve at pH 4 suggests a second cathepsin. The tests with alpha-1-antitrypsin at pH 4.0 would be likely to measure enzymes from both peaks.

The treatment regimens and results of the fluorescent antibody studies are summarized in Table II. Alveolar macrophages from eight surgical specimens fluoresced

when antisera to alpha-1-antitrypsin was followed by fluorescinated antirabbit gamma globulin (groups I and III). The fluorescence was distributed with a cytoplasmic pattern, excluding the nucleus (Fig. 2). The cells that had human serum added before the antisera (group I) were no brighter than macrophages that had no human serum added (group III). Controls from groups II and IV did not fluoresce.

Alveolar macrophages lavaged from lungs of two normal volunteers with the MM phenotype for alpha-1-antitrypsin showed an identical pattern to macrophages lavaged from surgical specimens; however, macrophages lavaged from one subject with ZZ phenotype for alpha-1-antitrypsin showed a faint diffuse positive fluorescence with indistinct periphery (Fig. 3A) when stained with regimen III (Table II), and a distinctly lesser intensity than the same macrophages stained after incubation with normal serum (group I) (Fig. 3B).

Macrophages treated with normal rabbit serum (group

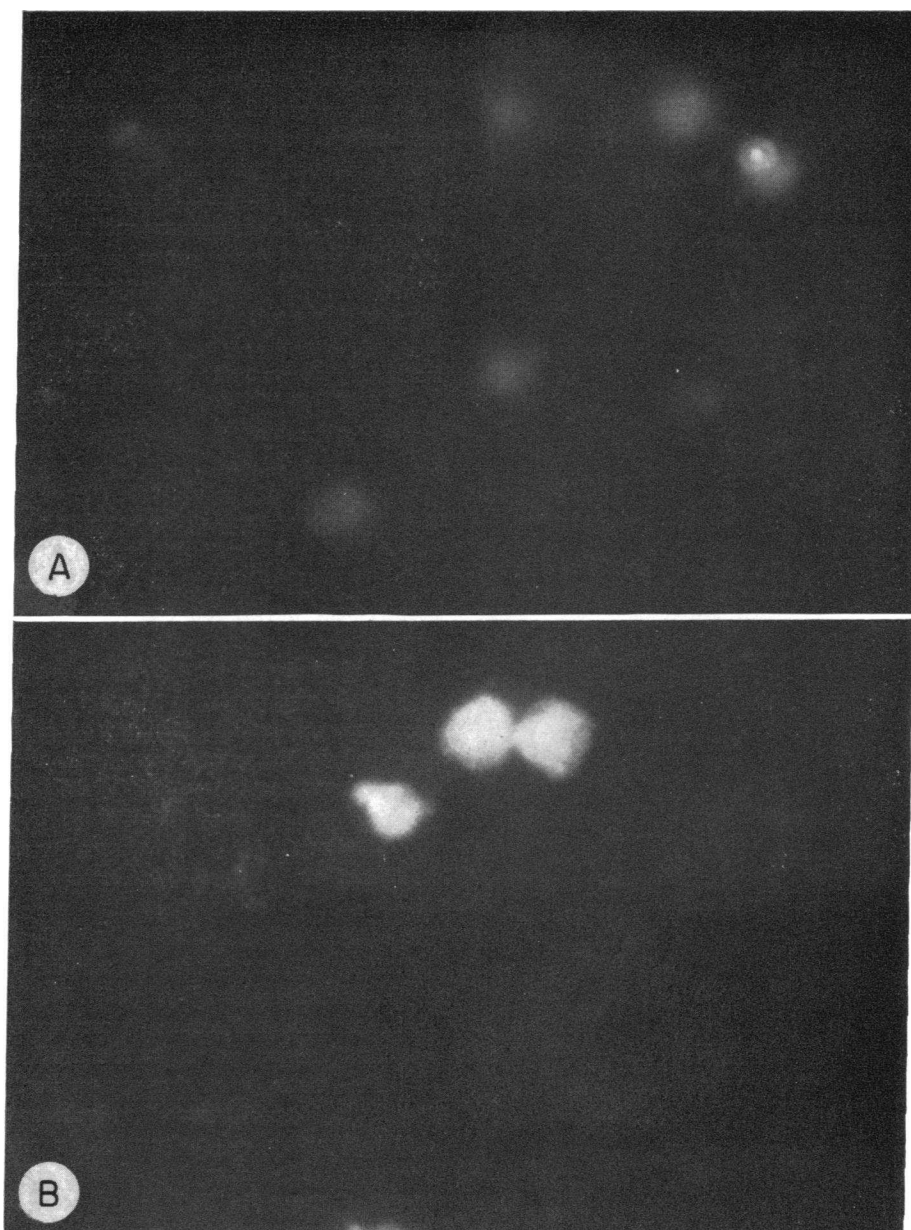


FIGURE 3 Human alveolar macrophages from patient deficient in alpha-1-antitrypsin. (A) Day-old glass-adherent macrophages grown in media with autologous serum fluoresce with low intensity when treated with antihuman alpha-1-antitrypsin and subsequently treated with fluorescein-labeled antirabbit gamma globulin. (B) However, these cells fluoresce brightly when pretreated with normal human serum before staining. (Magnification $\times 450$.)

V) fluoresced with a peripheral ring of membrane-like fluorescence (Fig. 4A) or rarely with a homogeneous fluorescence (Fig. 4B), and the control cells, group VI, failed to fluoresce. After 48–72 h in vitro the fluorescence could no longer be elicited by any regimen in Table II. In addition, when macrophages were incubated for 48–72 h in vitro and then incubated for 48 h with either 30% normal AB Rh-negative human serum or normal

human serum plus enough trypsin to inactivate 50% of available-1-antitrypsin, they failed to regain the capacity to fluoresce when treated with any regimen in Table II.

DISCUSSION

These results show that human alveolar macrophages contain at least one cathepsin that is inhibited by alpha-1-antitrypsin. The optimal pH for proteolytic activity

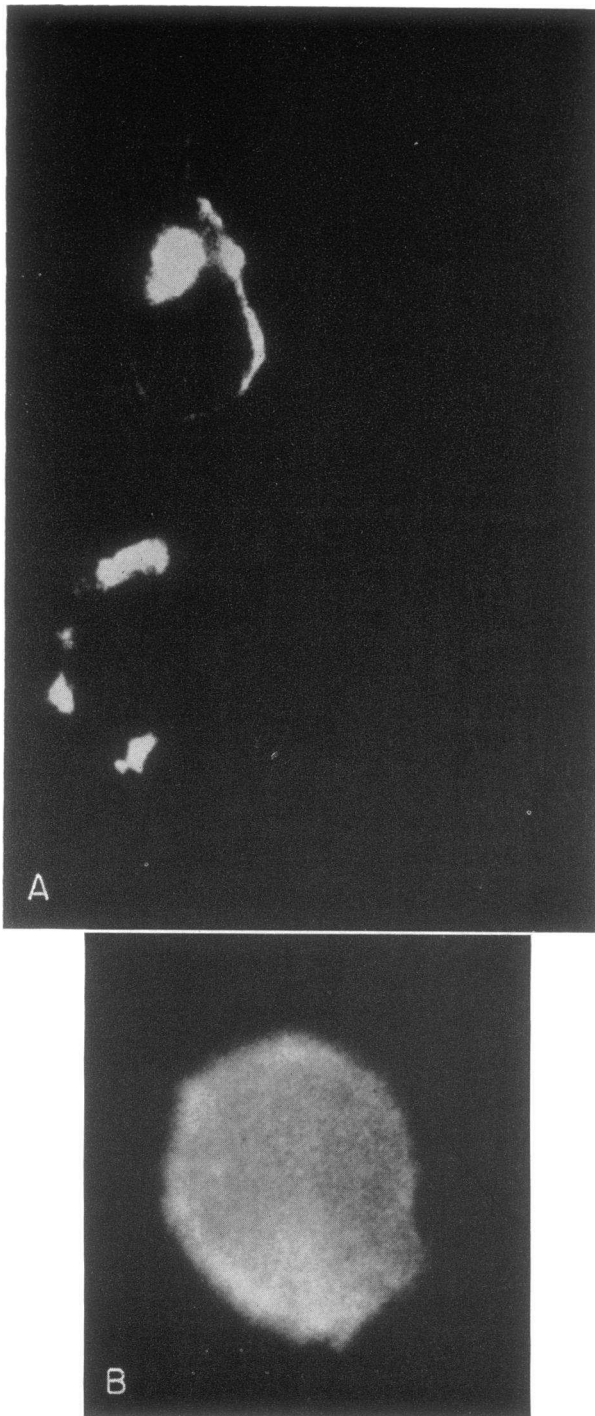


FIGURE 4 Control human alveolar macrophages: (A) Day-old glass-adherent macrophages fluoresce with a peripheral membrane-like pattern when treated by normal rabbit serum and subsequently treated with fluorescein-labeled antirabbit gamma globulin. (Magnification $\times 450$.) (B) About 1 cell in 20 treated by this regimen appeared to have the diffuse staining pattern on examination at a magnification of 1,250, in contrast to the nuclear exclusion pattern seen in Fig. 2. (Magnification $\times 1,250$.)

of the macrophage extract is between 3 and 4, but activity can still be measured up to pH 6.0. Although the pH of alveolar fluid in the adult is unknown, it has a mean value of 6.2 in fetal alveolar fluid and it has a low buffering capacity (16). Therefore, if adult alveolar fluid is similar, this enzyme might have a low level of activity in adult alveoli. Since emphysema develops over many years and "emphysema models" produced by adding enzymes to tracheas occur in shorter time intervals (6-8), it is likely that if proteolysis causes human emphysema then the level of proteolytic activity must be very small in relation to protein repair. Two additional factors favor inclusion of the macrophage cathepsin in the list of possible emphysema-producing enzymes: (a) our method examines proteolysis of hemoglobin over 60 min and may be too insensitive to detect a low level of proteolysis at higher pH, and (b) the pH in the area of the macrophage may be low during phagocytosis. There is evidence to indicate that human alveolar macrophages utilize anaerobic glycolysis (9) for part of their energy requirement during phagocytosis and acid by-products may result.

The significance of the alpha-1-antitrypsin found in human alveolar macrophages is not known but observations suggest that the inhibitor may have some physiologic role in the macrophage, perhaps related to the enzyme in the macrophage that it inhibits. The cytoplasmic-staining pattern suggests that the inhibitor gained access to the interior of the macrophage, and the staining in macrophages lavaged from lungs of normal subjects indicates that the fluorescence did not result from blood contamination at surgery. The reduced fluorescence of alpha-1-antitrypsin in alveolar macrophages from a subject with little alpha-1-antitrypsin in his blood was perhaps to be expected but serves to document the deficiency of the inhibitor in this perhaps critical anatomic area. In addition, when these day-old macrophages were incubated with normal serum they increased their fluorescent staining for alpha-1-antitrypsin. In contrast when macrophages were cultured in vitro for 72 h they lost their staining properties. Subsequently incubating these macrophages in normal serum did not restore their fluorescent staining for alpha-1-antitrypsin. Therefore, macrophages cultured in vitro for 3-5 days lose their ability to take up alpha-1-antitrypsin from their environment. Whereas data from one patient must be considered anecdotal, the rarity of deficient adult patients without lung disease makes it unlikely that we will be able to examine more patients in this category.

While the activity of alpha-1-antitrypsin in alveolar macrophages was not examined, Blondin, Rosenberg, and Janoff (17) have recently described an inhibitor of leukocyte protease, extracted from human alveolar macrophages. The inhibitor is acidic and has an isoelectric point of about 4.0. Alpha-1-antitrypsin shares these char-

acteristics. The coexistence of the inhibitor and the cathepsin it inhibits in the same cell may be related to sequestration of the two agents in separate intracellular compartments or to the inability of alpha-1-antitrypsin to react with the cathepsin at a pH at which the cathepsin is inactive. It is also possible that the enzyme and inhibitor only interact when the cathepsin is released from the macrophage (18).

Finley et al. (10) and others (9) have shown that many more macrophages can be lavaged from lungs of cigarette smokers than from nonsmokers. Therefore, it is possible that emphysema could be caused by an increased level of macrophage enzyme in cigarette smokers or a decreased level of enzyme inhibitor in patients with a deficiency of alpha-1-antitrypsin. If further evidence supports this unifying hypothesis, the therapeutic use of the appropriate enzyme inhibitor might delay or prevent the emphysema that develops in both groups of patients.

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