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Effects of *Pseudomonas aeruginosa* Elastase on Alveolar Epithelial Permeability in Guinea Pigs

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Elastase-deficient mutants of *Pseudomonas aeruginosa* are less virulent than the wild type and are easily cleared from the lungs of guinea pigs. The effect of *P. aeruginosa* elastase on lung epithelium, however, is not yet understood. We addressed the hypothesis that breach of the epithelial barrier by elastase from *P. aeruginosa* allows invading organisms and toxic substances to penetrate the interstitium. We measured the clearance of aerosolized technetium-labeled albumin (molecular weight, 69,000) from the lungs of anesthetized guinea pigs with the aid of a gamma camera and a dedicated computer. Aerosols of the elastase (0.1 to 5 μg) increased the rate of clearance of labeled albumin from the lungs in proportion to the elastase dose. Electron microscopic studies using horseradish peroxidase as a tracer revealed that elastase interrupts intercellular tight junctions of the epithelial lining, thereby increasing the permeability to macromolecules. The amounts of elastase used in this report did not cause interstitial or alveolar edema, as determined by both postmortem extravascular lung water volume measurement and morphological examination. The data indicate that the elastase is a potentially important virulence factor in acute lung infection.

Pseudomonas aeruginosa, an opportunistic gram-negative bacterium, causes infection in patients with underlying diseases. It can cause severe pneumonia in cystic fibrosis patients and in acquired immunodeficiency syndrome victims. The lack of effective antibiotics and the absence of specific vaccines make infections with this organism particularly difficult to treat.

P. aeruginosa produces two distinct proteases, elastase and alkaline protease (8, 9). *P. aeruginosa* elastase contributes, directly or indirectly, to the pathogenicity of this bacterium. The elastase can inactivate certain complement and coagulation factors in vitro, and it may be responsible for the destruction of the arterial lamina in septicemia (3, 13). Protection against *P. aeruginosa* infection in mink by toxoids of elastase further implicates this protease in *Pseudomonas* infection (4).

The respiratory epithelium normally protects the lungs against invasion by microorganisms and macromolecules. Proteolytic damage to the epithelium, however, may permit entry of *P. aeruginosa* and its toxins into the interstitium. To address this hypothesis, we investigated the effects of *P. aeruginosa* elastase on the epithelial permeability to technetium-labeled human serum albumin in guinea pig lungs. The objective was to explore the mechanism(s) by which elastase modifies the barrier function of the epithelial lining.

MATERIALS AND METHODS

Animal preparation. Mature male Hartley-Duncan guinea pigs (800 to 1,000 g; Sasco, Omaha, Nebr.) were used. Each animal was anesthetized by intramuscular injection of a mixture of xylazine (1.5 mg/kg) and acepromazine (0.5 mg/kg). Lidocaine (1%) was used subcutaneously as a local anesthetic during the surgical procedure. Halothane (1%) maintained anesthesia during the surgery and throughout the experiment. A catheter was placed in the carotid artery to measure arterial pressure and arterial blood gas partial pressures. A tracheal cannula was installed for mechanical ventilation and instillation of *P. aeruginosa* elastase and the tracer. All protocols were approved in advance by the Animal Research Committee of the University of Texas Health Center at Tyler.

Instillation of the elastase. The elastase (Nagase Biochemicals, Fukuchiyama, Japan) was suspended in 6 ml of phosphate-buffered saline (PBS) and aerosolized with an ultrasonic nebulizer (Ultraneb 100; DeVilbiss). Aerosolized particles (approximately 1.0 μm in diameter, as measured with an Aerodynamic Particle Sizer; TSI Inc., St. Paul, Minn.) entered the lungs during mechanical ventilation for 20 min. The amount of elastase deposited by this method was estimated in two animals by adding technetium pertechnetate ($^{99m}TcO_4^-$) to the elastase solution prior to nebulization. After 20 min, the radioactivity of the marker in the entire animal was measured with a gamma camera and compared with the initial radioactivity in the elastase solution. It was necessary to measure the radioactivity in the whole body rather than just that in the lungs because although the $^{99m}TcO_4^-$ cleared rapidly from the lungs, it did not leave the body or the field of the camera in 20 min. This technique showed that approximately 0.1% of the elastase deposited in the lungs over the 20-min period.

Aerosolization and imaging of the tracer. Aerosols of technetium-labeled human serum albumin (^{99m}Tc-HSA; molecular weight, 69,000) were used to detect changes in the permeability of the pulmonary epithelium. The tracer was prepared as recommended by the manufacturer (Medi+ Physics, Paramus, N.J.). Briefly, 25 to 30 mCi of fresh $^{99m}TcO_4^-$ obtained from a molybdenum generator was mixed with 7 mg of human serum albumin. After 20 min of incubation at room temperature, the mixture was first diluted to 2 ml in saline and then concentrated with a disposable microconcentrator (Amicon; M_r cutoff > 30,000). We repeated this process three times to ensure removal of unbound technetium. The rate of clearance of ^{99m}Tc-HSA from the lungs as measured with a gamma camera was used to

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quantitate changes in epithelial permeability to proteins and fluids caused by injury (12).

After anesthesia, surgical preparation, and deposition of elastase or PBS alone (control animals), each animal was placed under a gamma camera (Seimens Pho/Gamma). The imaging procedure began by the recording of background activity for 4 min. The animal was then ventilated with the aerosol of ^{99m}Tc-HSA for 6 min or until sufficient radioactivity (about 10,000 cpm) collected in the lungs to form a clear image on the monitor. Aerosolized tracer that did not deposit in the respiratory tract was vented to a laboratory hood. The data were collected for 80 min with a dedicated computer.

To determine whether the ^{99m}Tc label was removed from the albumin complex by the proteolytic activity of *P. aeruginosa* elastase, we measured the amount of free technetium in samples of an elastase–^{99m}Tc-HSA mixture and compared it to that in the control (PBS–^{99m}Tc-HSA). The treated and untreated samples were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

At the end of each experiment, the animals were sacrificed by increasing the fraction of inspired halothane from 1% to 5%, followed by intravenous injection of saturated KCl. The lungs were either fixed for histologic studies (see below) or removed, weighed, and dried in a microwave oven for measurement of lung water content (11).

Animal study groups. Aerosols containing various amounts of purified *P. aeruginosa* elastase were administered to four groups of animals to determine the effect on lung epithelial permeability. According to our estimates, each group (minimum of five animals per group) received 0.1, 0.5, 1, or 5 μg of elastase in PBS. Three animals treated with 5 μg of elastase also received Sigma type II horseradish peroxidase (HRP) (300 mg in 3 ml of saline; molecular weight, 40,000) intravascularly 10 min before sacrifice for ultrastructural study.

Five control animals received aerosols of sterile PBS alone to establish whether the surgery or the vehicle affected the permeability of the pulmonary epithelium. Three additional animals were treated with aerosols of heat-inactivated (98°C, 10 min) *P. aeruginosa* elastase to assess the importance of the proteolytic activity of the elastase.

Data analysis. A computer (Microdelta) stored lung imaging data obtained by the camera every 2 min during the 80 min imaging period. The region of interest was identified with the aid of an image-processing software package (Computer Design Associates). The radioactivity in the region for each 2-min period was tabulated and corrected for background and decay by a Vax computer. After the peak value was identified, the corresponding time was designated as time zero and the data were expressed as a percentage of that value. The data were then fitted by a least-mean-squared-error subroutine (International Mathematical and Statistical Libraries, Houston, Tex.) to a single exponential model to determine the rate of clearance of labeled albumin from the lungs (12).

Lung histopathology and electron microscopy. Fixed samples of lung tissue were examined by light and electron microscopy to determine whether *P. aeruginosa* elastase caused alveolar edema and whether intravascularly injected HRP crossed the airway epithelium into the airspace. Alveolar edema was scored as follows: 0, 0 to 1% of alveoli flooded; 1, 2 to 10% flooded; 2, 11 to 25% flooded; 3, 26 to 50% flooded; and 4, >50% flooded (7).

The lung structure in three control animals was compared with that in three animals treated with 5 μg of *P. aeruginosa* elastase. HRP was injected intravascularly 10 min prior to death to investigate the integrity of the alveolar epithelial lining. All animals were perfused via the right ventricle with 2.5% buffered glutaraldehyde, and the lungs were removed and placed in the same fixative for 45 min. Tissue samples were collected from peripheral lung sites, fixed in 2.5% buffered glutaraldehyde for 30 min, transferred to 0.05 M Tris buffer, and left overnight at 4°C. The following day, tissue samples were incubated in 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/ml) for 10 min before further (10 min) incubation in DAB containing 0.3% H_2O_2. Finally, the samples were placed in vials containing DAB-H_2O_2 and shaken for 50 min to complete the reaction. After being washed in Tris buffer, the samples were processed through osmium tetraoxide, uranyl acetate, ethanol, and acetone and embedded in epoxy resin. Semi-thin sections (0.5 μm) were cut and stained with toluidine blue, and suitable areas were selected by light microscopy for thin sectioning and electron microscopy.

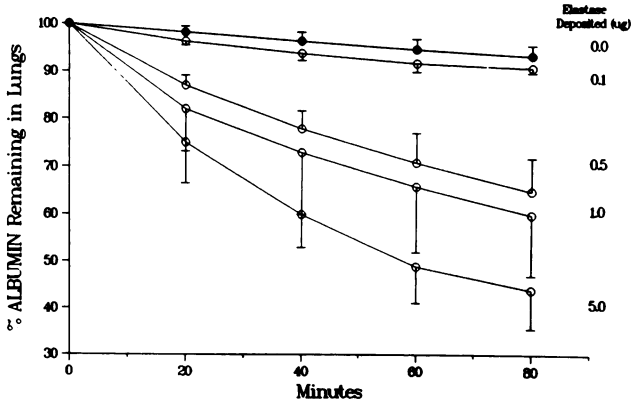

FIG. 1. Activity of ^{99m}Tc-HSA (ALBUMIN) in the lungs of five groups of guinea pigs during 80 min following deposition of the albumin. The radioactivity level at each data point presented is the percentage of the peak value at time zero. Results are the mean ± standard deviation of five or more experiments. The estimated amount of elastase deposited in the lungs of animals in each group is shown on the right.

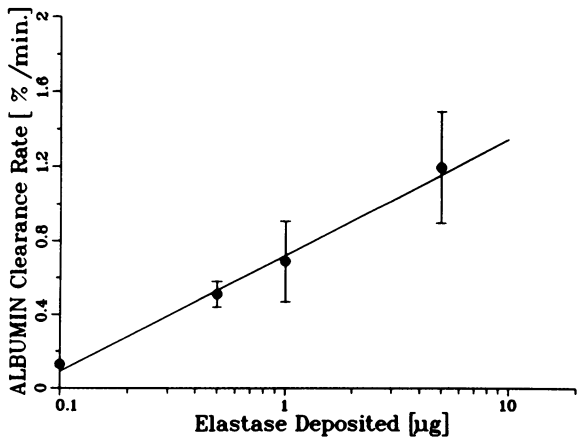

FIG. 2. Effect of *P. aeruginosa* elastase dose on clearance rate of ^{99m}Tc-HSA (ALBUMIN) from the lungs. The clearance rate, determined by a one-compartment model, correlated well with the logarithm of elastase dose ($r = 0.98$, $P < 0.01$). Values are means ± standard deviation of five or more experiments.

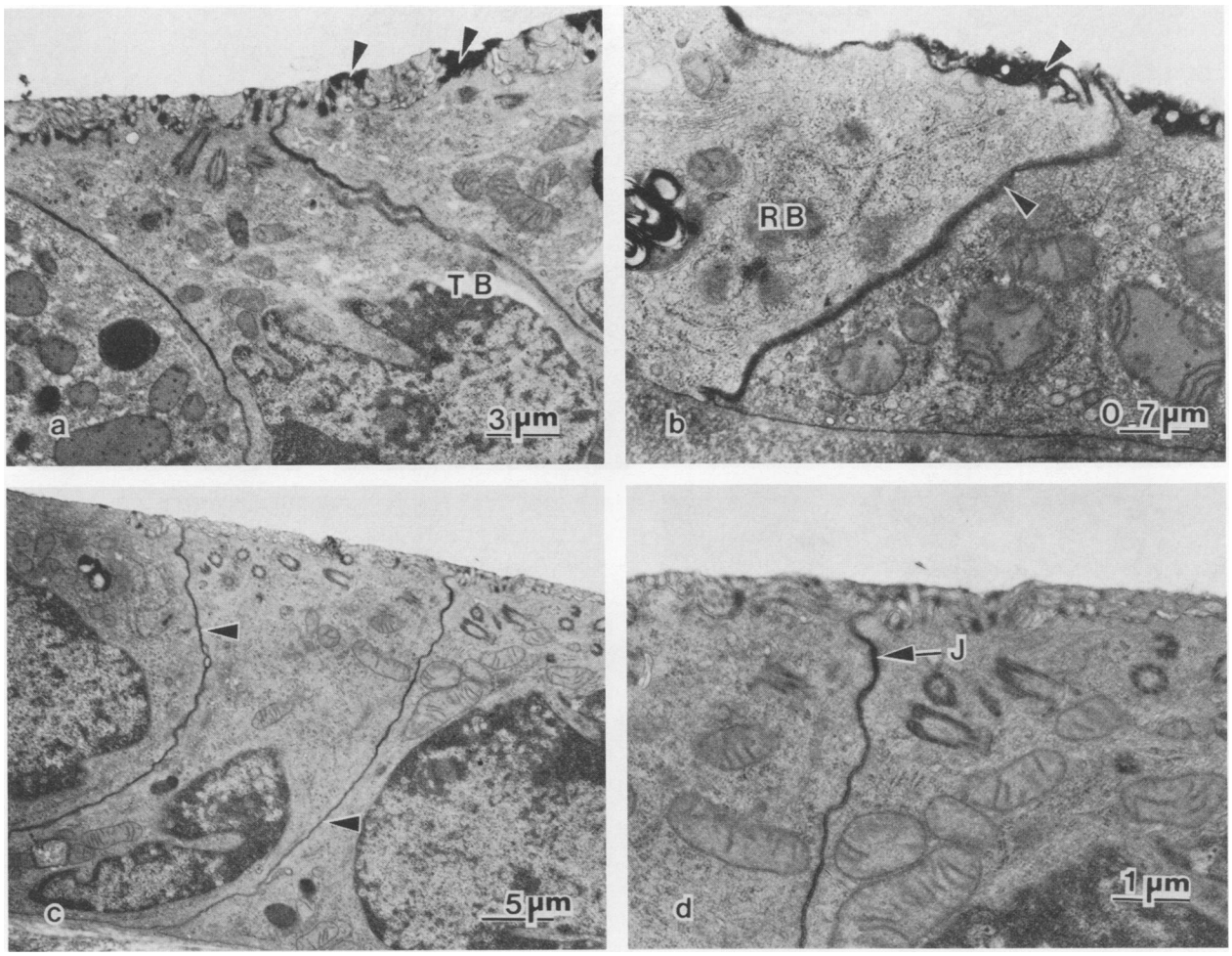

FIG. 3. HRP tracer studies in bronchioles. (a) HRP (arrowheads) on the surface of the terminal bronchioles (TB) in lung tissues of animals exposed to 5 μg of *P. aeruginosa* elastase. (b) HRP (arrowheads) in the intercellular space and past the tight junction on the respiratory bronchiole (RB) surface in elastase-exposed tissue. (c) HRP (arrowheads) only in the intercellular space in control tissue. (d) Higher magnification of panel c showing HRP penetrating only to the tight junction (J) in control tissue.

Statistical analysis. We analyzed the differences in the clearance rates among the groups by a one-way analysis of variance, assuming a completely randomized design with six treatment groups, and used Tukey's multiple comparison procedure after the overall F test to test pairwise differences in the treatment groups (Statistical Analysis System, Cary, N.C.). All data were expressed as mean ± standard deviation. The correlation coefficient and significance of the dose response were calculated by linear regression analysis of the clearance rate and the logarithm of the elastase dose.

RESULTS

Clearance of ^{99m}Tc-HSA from the lungs. As shown in Fig. 1, most of the ^{99m}Tc-HSA remained in the lungs of control animals during the 80-min measurement period. *P. aeruginosa* elastase increased the rate of clearance of the ^{99m}Tc-HSA from the lungs in a dose-dependent manner. The fraction of ^{99m}Tc-HSA remaining in the lungs of animals treated with 0.5 to 5 μg of the elastase was significantly less than that remaining in the lungs of control animals. The highest dose of elastase decreased the amount of ^{99m}Tc-HSA in the lungs of control animals from 93.6 ± 2.2% to 44 ± 8.4% at the end of the 80-min measurement period.

We were concerned that the increased clearance rate might be due to proteolytic cleavage of ^{99m}Tc-HSA, resulting in either free ^{99m}Tc or smaller labeled molecules that could clear more rapidly than the intact complex. Gel electrophoresis of elastase-treated ^{99m}Tc-HSA, however, revealed no degradation products of human serum albumin, and the difference in the free-technetium level between elastase-treated ^{99m}Tc-HSA and control samples was not significant (data not shown). We also addressed this issue in other studies by showing that elastase increases the pulmonary clearance of ^{131}I-human serum albumin from the lungs of sheep (Peterson et al., Am. Rev. Respir. Dis. **139:**A294, 1989).

Relationship between clearance rate and dose and activity of the elastase. The rate of clearance (K; percent per minute) of ^{99m}Tc-HSA from the lungs correlated well ($r = 0.98$, $P < 0.05$) with the logarithm of the estimated dose of *P. aeruginosa* elastase delivered to the lungs in each group (Fig. 2). The clearance rate increased from 0.08 ± 0.03%/min in control animals to 1.2 ± 0.3%/min after administration of 5 μg of the elastase. This change constituted a 15-fold increase in the ^{99m}Tc-HSA clearance rate due to the effects of the elastase on epithelial permeability.

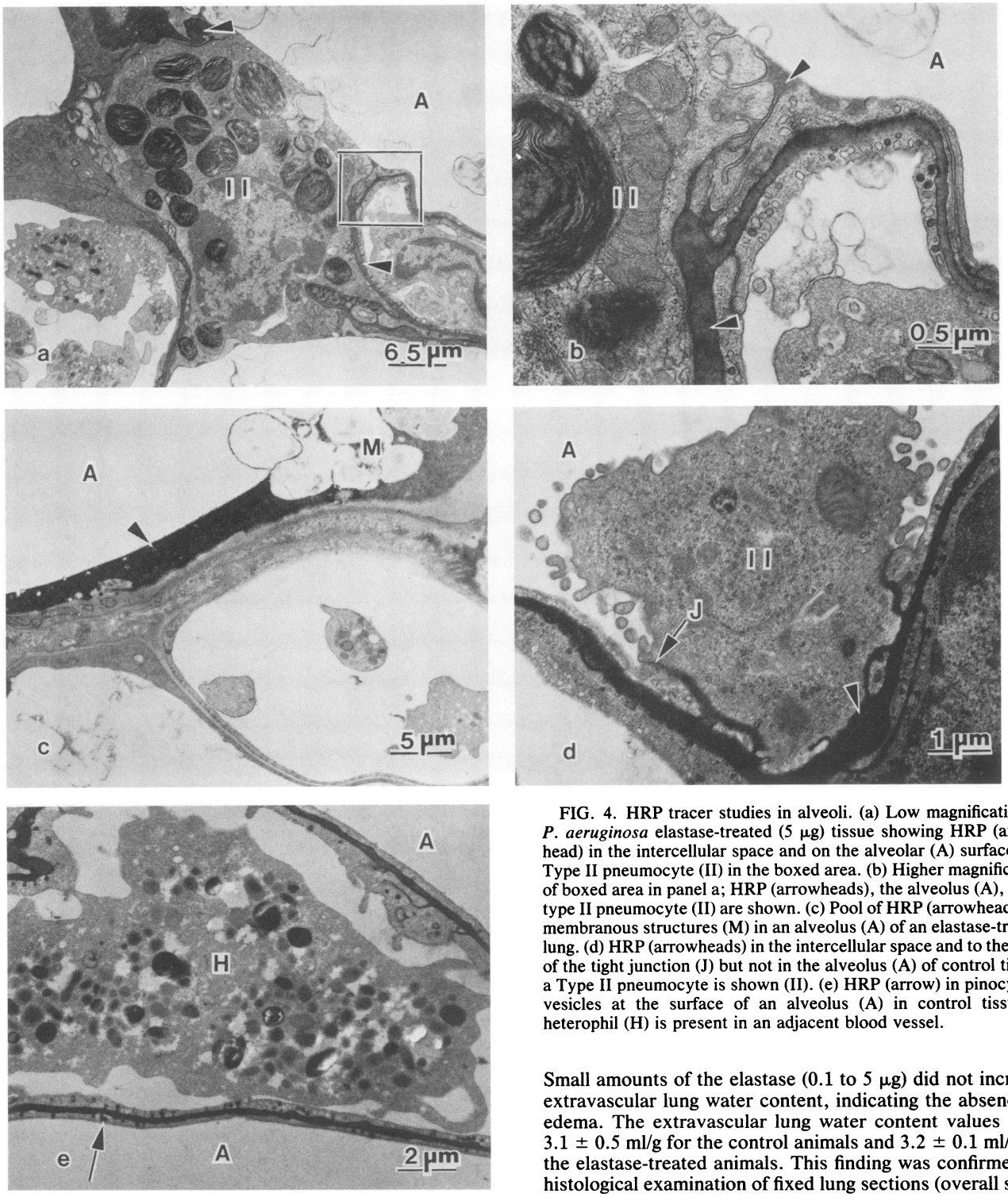

FIG. 4. HRP tracer studies in alveoli. (a) Low magnification of *P. aeruginosa* elastase-treated (5 μg) tissue showing HRP (arrowhead) in the intercellular space and on the alveolar (A) surface of a Type II pneumocyte (II) in the boxed area. (b) Higher magnification of boxed area in panel a; HRP (arrowheads), the alveolus (A), and a type II pneumocyte (II) are shown. (c) Pool of HRP (arrowhead) and membranous structures (M) in an alveolus (A) of an elastase-treated lung. (d) HRP (arrowheads) in the intercellular space and to the level of the tight junction (J) but not in the alveolus (A) of control tissue; a Type II pneumocyte is shown (II). (e) HRP (arrow) in pinocytotic vesicles at the surface of an alveolus (A) in control tissue; a heterophil (H) is present in an adjacent blood vessel.

The requirement for proteolytic activity was demonstrated in experiments in which heat-inactivated *P. aeruginosa* elastase was compared with that of the native protein. The rate of clearance of albumin from the lungs of the animals treated with inactivated elastase (1 μg; $K = 0.12 \pm 0.4\%$/min) did not differ significantly ($P < 0.05$) from the rate in control animals ($K = 0.09 \pm 0.04\%$/min).

***P. aeruginosa* elastase and postmortem lung water content.** Small amounts of the elastase (0.1 to 5 μg) did not increase extravascular lung water content, indicating the absence of edema. The extravascular lung water content values were 3.1 ± 0.5 ml/g for the control animals and 3.2 ± 0.1 ml/g for the elastase-treated animals. This finding was confirmed by histological examination of fixed lung sections (overall score of 0.3).

Histology and ultrastructure. Electron microscopic assessment of *P. aeruginosa* elastase-treated animal lungs revealed that HRP traversed intercellular tight junctions at the levels of terminal (Fig. 3a) and respiratory bronchioles (Fig. 3b) and also in the alveoli (Fig. 4a and b). Large pools of the tracer were found in the alveoli (Fig. 4c). In control animals, the HRP stopped at the junctions and did not penetrate into the airspace (Fig. 3c and 3d and Fig. 4d). A small amount of HRP in the alveoli of controls was probably deposited

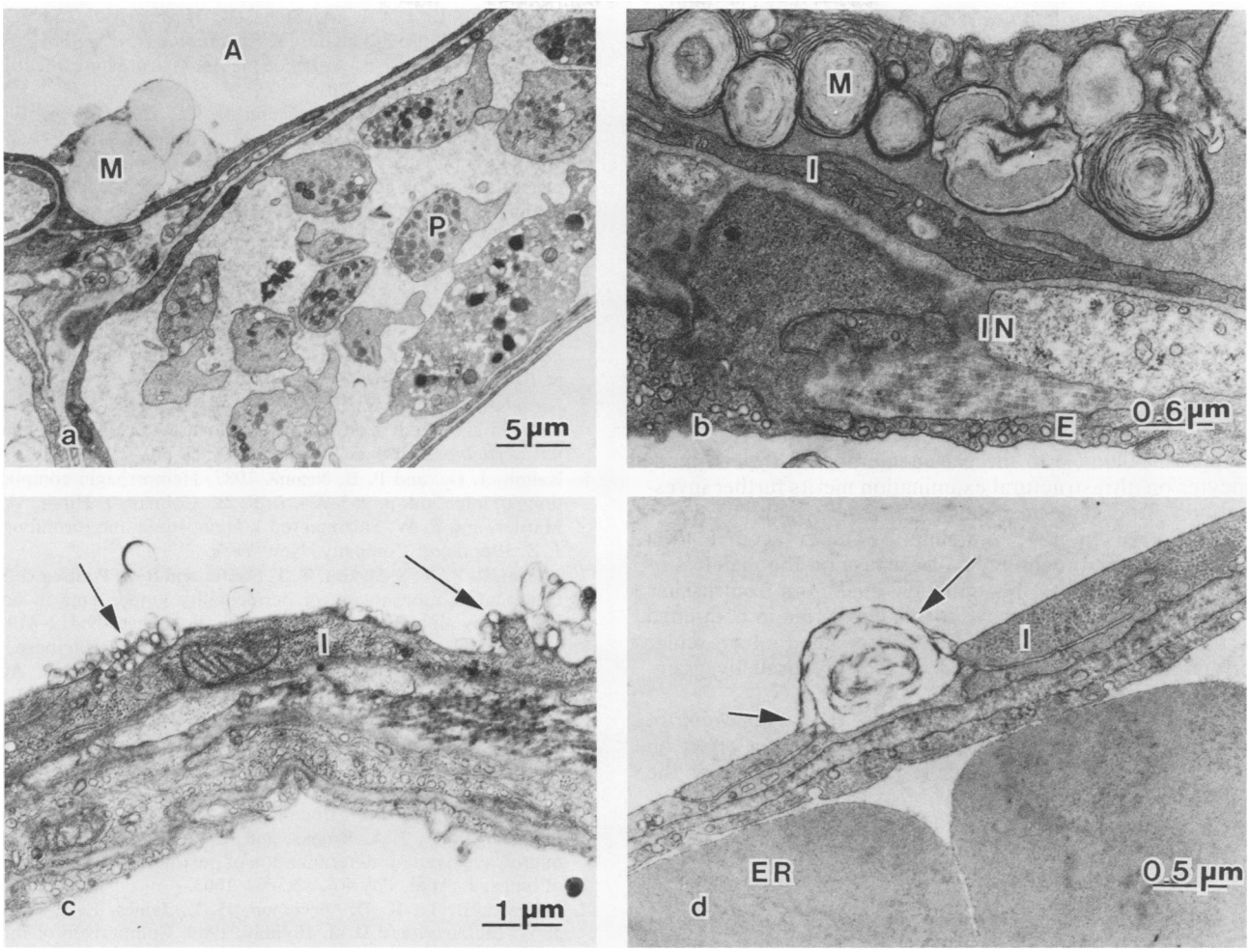

FIG. 5. Pathologic features of *P. aeruginosa* elastase-treated (5 µg) lung tissue. (a) Membranous structures (M) in an alveolus (A) and platelet (P) accumulation in blood vessel. (b) Membranous structures (M) adjacent to the alveolar wall; a Type I pneumocyte (I), the interstitium (IN), and endothelium (E) are shown. (c) Vesicular malformations (arrows) on a Type I cell (I) plasma membrane. (d) Blebbed area (arrows) on a type I cell (I) membrane; an erythrocyte (ER) is shown in an adjacent vessel.

through vesicular transport (Fig. 4e) since there was no leakage at the junctions (Fig. 4d). The most striking features of elastase-exposed lungs were membranous structures that appeared to be similar to lamellar bodies. These structures were located adjacent to the alveolar epithelium and often were associated with pools of HRP (Fig. 5a). Figure 5b shows these structures at a higher magnification. The elastase also caused vesiculation and blebbing of the Type I cell membranes (Fig. 5c and d). Platelets accumulated in many blood vessels of the treated lungs but did not appear aggregated (Fig. 5a).

DISCUSSION

The respiratory epithelium is ideally situated for protection against invasion by microorganisms and their toxins. Tight junctions between epithelial cells normally prevent passage of macromolecules across this layer and regulate passage of phagocytes into alveolar spaces (1). Breach of this barrier by bacterial proteases would permit bacteria and their metabolites to reach the lung interstitium.

Several extracellular products of *P. aeruginosa* contribute to the pathogenicity of this organism. Although specific metabolites have been isolated, no single factor can be designated as the cause of the destructive tissue changes. Elastase and exotoxin A are the most widely studied of the known virulence factors (2, 10). Proteases of *P. aeruginosa* are thought to be aggressins, and exotoxin A, which arrests protein synthesis in target cells, is probably a major cause of cell death. However, a number of animal studies suggest that strains producing both active elastase and exotoxin A are more virulent in chronic lung infection and that elastase, rather than exotoxin A, is the most important virulence factor in acute *P. aeruginosa* pneumonia (2, 14).

P. aeruginosa elastase causes pathologic changes in the lungs of laboratory animals similar to those in human lungs with *Pseudomonas* pneumonia, but the underlying mechanisms of injury have yet to be understood. Most investigators who studied lung injury by bacterial proteases instilled relatively large doses of elastase into the tracheas of experimental animals and studied changes in lung structure (5). The design of these studies and the limited histologic observations made it difficult to discriminate direct epithelial injury from indirect injury caused by inflammatory changes within the lung. Our experiments were designed to determine whether low doses of elastase could increase permeability of the epithelium without causing lung edema. We

found that elastase had a direct effect on the epithelium, as indicated by an increased rate of clearance of ^{99m}Tc-HSA from the lungs. The clearance rate of the tracer correlated with the amount of elastase and occurred only when proteolytically active elastase was administered. In contrast to studies in which larger doses (>40 μg) were used (5), small amounts of elastase (0.1 to 5 μg) did not increase extracellular water content and did not disrupt lung structure.

Experiments with an electron-dense protein tracer (HRP) showed that alteration in epithelial permeability was due to changes at the epithelial tight junctions. Lungs of control animals examined by electron microscopy clearly showed retention of HRP at the epithelial junctions, but in elastase-treated lungs HRP passed through epithelial junctions and collected on the surfaces of bronchiolar and alveolar epithelia. These findings are consistent with the measured changes in the rate of clearance of radiolabeled albumin.

Apparent damage to the cell membrane of Type I pneumocytes on ultrastructural examination merits further investigations of possible mechanisms through which *P. aeruginosa* elastase injures epithelium. Platelet accumulation might be due to direct effects of elastase on the platelets or might be triggered by inflammatory mediators from tissues or blood. Platelets may have a significant role in continued exposure to *P. aeruginosa* since disseminated intravascular coagulation has been reported in sepsis caused by gram-negative bacteria in humans (6).

The present study shows that elastase from *P. aeruginosa* increases epithelial permeability through a direct effect on the epithelial junctions. This is probably an initial step in the injury caused by this organism. Measurement of the ^{99m}Tc-HSA clearance rate should be useful for further exploration of the mechanism of lung injury by other metabolites of *P. aeruginosa* or inflammatory mediators.

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LITERATURE CITED

1. **Bachofen, M., and E. R. Weibel.** 1982. Structural alterations of lung parenchyma in the adult respiratory distress syndrome, p. 35–56. *In* R. C. Bone (ed.), Clinics in chest medicine. The W. B. Saunders Corp., Philadelphia.
2. **Blackwood, L. L., R. M. Stone, B. H. Iglewski, and J. E. Pennington.** 1983. Evaluation of *Pseudomonas aeruginosa* exotoxin A and elastase as virulence factors in acute lung infection. Infect. Immun. **39**:198–201.
3. **Heck, L. W., K. Morihara, and D. R. Abrahamson.** 1986. Degradation of soluble laminin and depletion of tissue-associated basement membrane laminin by *Pseudomonas aeruginosa* elastase and alkaline protease. Infect. Immun. **54**:149–153.
4. **Homma, J. Y., C. Abe, K. Tanamoto, Y. Hirao, K. Morihara, H. Tsuzuki, R. Yanagawa, E. Honda, Y. Aoi, Y. Fujimoto, M. Goryo, N. Imazeki, H. Noda, A. Goda, S. Takeuchi, and T. Ishihara.** 1978. Effectiveness of immunization with single and multi-component vaccines prepared from a common antigen (OEP), protease and elastase toxoids of *Pseudomonas aeruginosa* on protection against hemorrhagic pneumonia in mink due to *P. aeruginosa*. Jpn. J. Exp. Med. **48**:111–133.
5. **Kawaharajo, K., J. Y. Homma, Y. Aoyama, and K. Morihara.** 1975. In vivo studies on protease and elastase from *Pseudomonas aeruginosa*. Jpn. J. Exp. Med. **45**:89–100.
6. **Kelton, J. G., and P. B. Neame.** 1987. Hemorrhagic complications of infection, p. 965–974. *In* R. W. Colman, J. Hirsh, V. J. Marder, and E. W. Salzman (ed.), Hemostasis and thrombosis. J. B. Lippincott Company, New York.
7. **Michel, R. P., T. S. Hakim, T. T. Smith, and R. S. Poulsen.** 1983. Quantitative morphology of permeability lung edema in dogs induced by alpha-naphthylthiourea. Lab. Invest. **49**:412–419.
8. **Morihara, K.** 1963. *Pseudomonas aeruginosa* proteinase. I. Purification and general properties. Biochim. Biophys. Acta **73**:113–124.
9. **Morihara, K.** 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. J. Bacteriol. **88**:745–757.
10. **Morihara, K., and J. Y. Homma.** 1985. *Pseudomonas* proteases, p. 42–75. *In* I. A. Holder (ed.), Bacterial enzymes and virulence. CRC Press, Inc., Boca Raton, Fla.
11. **Peterson, B. T., J. A. Brooks, and A. G. Zack.** 1982. Use of a microwave oven for determination of postmortem water volume of lungs. J. Appl. Physiol. **52**:1661–1663.
12. **Peterson, B. T., K. D. Dickerson, H. L. James, E. J. Miller, J. W. McLarty, and D. B. Holiday.** 1989. Comparison of three tracers for detecting lung epithelial injury in anesthetized sheep. J. Appl. Physiol. **66**:2374–2383.
13. **Schultz, D. R., and K. F. Miller.** 1974. Elastase of *Pseudomonas aeruginosa*: inactivation of complement components and complement-derived chemotactic and phagocytic factors. Infect. Immun. **10**:128–135.
14. **Woods, D. E., S. J. Cryz, R. L. Friedman, and B. H. Iglewski.** 1982. Contribution of toxin A and elastase to virulence of *Pseudomonas aeruginosa* in chronic lung infections of rats. Infect. Immun. **36**:1223–1228.