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Microscopic Characterization of Rabbit Lung Damage Produced by *Pseudomonas aeruginosa* Proteases

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The intratracheal administration of highly purified *Pseudomonas aeruginosa* proteases (ca. 10 to 100 μg) elicited extensive, grossly observable rabbit lung damage by 3 h postinjection. Light and electron microscopic characterization of the lesions revealed: (i) progressive injury and necrosis of type I epithelial cells and capillary endothelial cells from 3 h to 1 day postinjection, and progressively increasing accumulations of erythrocytes, plasma proteins, fibrin, and released type II epithelial cell lamellar bodies in alveolar lumina during that time period; (ii) progressively increasing accumulations of macrophages, but not of polymorphonuclear leukocytes, in alveolar lumina from 3 h to 6 days postinjection; (iii) progressive hyperplasia of type II epithelial cells from 12 h to 4 days postinjection; (iv) progressive infiltration of alveolar septa by mononuclear inflammatory cells (interstitial pneumonitis) from 2 to 6 days postinjection; (v) no loss of alveolar septal connective tissue and no damage to pulmonary arterioles and venules; and (vi) almost normal alveolar structure by ca. 8 days postinjection. The study revealed that the intra-alveolar hemorrhage, the injury and necrosis of alveolar septal cells, and the infiltration by mononuclear cells that have been reported to occur during human pseudomonas pneumonia can also be elicited by the experimental administration of pseudomonas proteases. Thus, the results support the idea that in vivo production and activity of *P. aeruginosa* proteases is important, at least in part, in eliciting the lung damage characteristic of pseudomonas pneumonia.

The opportunistic bacterial pathogen, *Pseudomonas aeruginosa*, produces many extracellular enzymes and toxins (20, 21, 24, 30, 31); however, the possible importance of these substances in eliciting lung damage during pseudomonas pneumonia (7, 22, 29, 33, 35) has not been systematically examined or firmly established. Previous results from three different types of studies indicate that in vivo production and activity of *P. aeruginosa* proteases may be important in the pathogenesis of pseudomonas pneumonia. First, Homma et al. (11) and Klinger et al. (17) observed that the sera of humans having *P. aeruginosa* pulmonary infections had elevated antibody titers to pseudomonas proteases. Second, Homma et al. (10) reported recently that vaccination with Formalin-inactivated, purified proteolytic enzymes of *P. aeruginosa* protected mink against *P. aeruginosa*-induced hemorrhagic pneumonia. Third, several investigators have found (5, 16, 23) that intranasal administration of *P. aeruginosa* proteases into mice produced focal and confluent hemorrhagic lung lesions which were grossly identical to those seen in human and experimental *P. aeruginosa* pneumonias (7, 22, 33–35). However, the lung damage produced by the protease preparations was not extensively characterized and compared, by light and electron microscopy, with that produced by the pneumonias.

The present report is the first detailed light and electron microscopic characterization of rabbit lung damage produced by the intratracheal administration of pseudomonas proteases of rigorously documented purity. The main purpose of the study was to determine whether the structural alterations observed in the model refuted or supported the idea of a role for pseudomonas proteases in the production of lung damage during pseudomonas pneumonia. The study revealed that the intra-alveolar hemorrhage, the injury and necrosis of alveolar septal cells, and the infiltration by mononuclear cells that have been reported to occur during human pseudomonas pneumonia (7, 22, 35) can also be elicited by the experimental administration of pseu-

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domonas proteases. Thus, the results support the idea that in vivo production and activity of *P. aeruginosa* proteases is important, at least in part, in eliciting the lung damage characteristic of pseudomonas pneumonia.

MATERIALS AND METHODS

Protease preparations. Highly purified elastolytic protease preparations were obtained from the culture supernatant fluids of *P. aeruginosa*, strains 5-31 and IFO 3455, by sequential ammonium sulfate precipitation, Ultrogel AcA 54 gel filtration, and flatbed isoelectric focusing, as previously described (18). Physicochemical, immunological, and biological activity analyses of the protease preparations did not detect any contaminating medium constituents or other known pseudomonas enzymes and toxins (18). The specific activity of the preparations, with azocasein as substrate, was ca. 450 protease units per mg of protein.

Production of lung damage by purified proteases. Membrane filter-sterilized (0.2-μm pore size) pseudomonas protease preparations were diluted with sterile 0.1 M ammonium bicarbonate to yield solutions containing ca. 2.5, 5, and 25 protease units/ml, and samples (2 ml) of each solution were injected intratracheally into at least 25 unanesthetized New Zealand white rabbits (1.4 to 2.3 kg). The control solutions injected were sterile, heat-inactivated (100°C for 15 min) protease preparations which initially contained 25 protease units/ml. The rabbits were sacrificed at 3, 6, and 12 h and at 1, 2, 4, 6, and 8 days postinjection.

Light and electron microscopy. Experimental animals were sacrificed by intravenous injection of sodium pentobarbital. Tissue specimens from uninflated control lungs and protease-treated lungs were fixed in buffered 10% Formalin (pH 7) before paraffin embedding and subsequent light microscopic histochemical studies. In addition, to monitor lung tissue for alveolar dilatation and alveolar septal loss, control lungs and protease-treated lungs were inflated with 10% buffered Formalin and allowed to fix for 1 day, and tissue specimens containing representative normal areas or lesions were embedded in paraffin. Specimens to be studied by electron microscopy were cut into small cubes and were fixed in 4% glutaraldehyde in sodium cacodylate buffer (0.05 M, pH 7.4) for 6 to 12 h.

Paraffin sections (5 to 7 μm) of tissues fixed in the uninflated state were stained by the alcian blue-periodic acid-Schiff technique (28) to visualize proteoglycan ground substance and by the Verhoeff-Van Gieson technique (28) to visualize elastin and collagen. Paraffin sections of tissues fixed in the inflated state were stained with hematoxylin and eosin.

Glutaraldehyde-fixed tissues were washed with cacodylate buffer, cut into 1-mm cubes, postfixed in 1% osmium tetroxide in *s*-collidine buffer (0.1 M, pH 7.4) for 2 h, washed with *s*-collidine buffer, and dehydrated in ascending concentrations of ethanol. All the above procedures were performed at 4°C. Specimens were further dehydrated at room temperature in propylene oxide and were embedded in Epon 812. Thick sections (1 to 2 μm) were stained with basic fuchsin-alkaline methylene blue (12) and were examined by light microscopy to evaluate embedment and condition of tissues and to select areas for subsequent electron microscopic study. Thin sections (60 to 90 nm) were stained with uranyl acetate and lead citrate. In addition, thin sections were treated with Verhoeff iron hematoxylin (4) to visualize elastin. Thin sections were examined in a Zeiss 9S-2 electron microscope.

RESULTS

Intratracheal injection of heat-inactivated control preparations did not produce grossly observable alterations by 3 h to 1 day postinjection. The only microscopically observable alteration was a slight increase in the number of polymorphonuclear leukocytes (PMN) in the alveolar septa and lumina.

None of the rabbits injected with active protease preparations died. The protease preparations from the two different strains elicited similar structural alterations and, at different times postinjection, the amount of involved lung tissue was directly related to the amount of protease injected. All three doses (5, 10, and 50 protease units) produced numerous, grossly observable, subpleural, focal, and confluent hemorrhagic lesions by 3 h postinjection (Fig. 1), and the lesion size increased progressively with time until reaching maximal intensity at ca. 1 day postinjection. Lung slices showed that the lesions extended deep into the lung tissue. Four to six days postinjection, lungs showed occasional subpleural hemorrhage and moderate to extensive areas of pale discolorations. Eight days postinjection, most lungs appeared grossly similar to

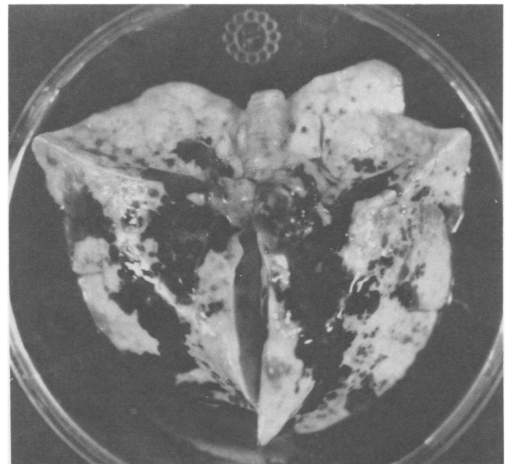


FIG. 1. *Gross lung damage produced by P. aeruginosa proteases 3 h postinjection of 50 protease units. Numerous subpleural, focal, and confluent hemorrhagic lesions are evident.*

lungs which had received control preparations.

Light and electron microscopic examination of the grossly observable lesions revealed the following structural alterations:

(i) Three hours postinjection, alveolar lumina contained accumulations of erythrocytes, plasma proteins, fibrin, and released type II epithelial cell lamellar bodies. This response increased progressively with time until reaching maximal intensity at ca. 1 day postinjection (Fig. 2b and 3a and c). Small numbers of alveolar macrophages and PMN (with PMN predominating) were also observed in alveolar lumina by 3 to 6 h postinjection. After 6 h postinjection, the phagocytic cell population in the lumina was composed primarily of actively phagocytic macrophages and a small percentage of PMN. The macrophage response increased progressively with time until reaching maximal intensity at ca. 4 to 6 days postinjection (Fig. 3b). At 8 days postinjection, alveolar lumina appeared similar to those in control lungs.

(ii) Alveolar type I epithelial cells (Fig. 3c, d, and e) and capillary endothelial cells (Fig. 3f and g) showed progressive injury and necrosis from ca. 3 h to 1 day postinjection. After that time, structurally damaged cells were less frequently observed, and most cells appeared normal by 8 days postinjection. Accumulations of platelets were observed occasionally in capillaries at 4, 6, and 8 days postinjection. No loss or alteration of arteriolar elastin or damage to venules was observed at any time period.

(iii) Alveolar type II epithelial cells showed hyperplasia at ca. 12 h to 1 day postinjection (Fig. 2c), and this reaction increased progressively with time until reaching maximal intensity at ca. 4 days postinjection. During that time, there was also a marked, progressive increase in the number of intracellular lamellar bodies (Fig. 2c), most of which had an atypical whorled or incomplete internal structure when examined by electron microscopy. Eight days postinjection, the number of cells and lamellar bodies was markedly reduced and almost similar to those observed in control lungs.

(iv) Alveolar dilatation and septal loss were not observed, 3 h to 8 days postinjection, in tissue from control lungs (Fig. 2a) and in tissue from representative lesions of protease-treated lungs fixed in expansion (Fig. 2b). In addition, loss or alteration of alveolar septal elastin, collagen, and proteoglycan ground substance was not observed, at any time period, when these extracellular, interstitial components were monitored by (a) electron microscopy (×40,000) of thin sections stained with uranyl acetate and lead citrate (for collagen) and with Verhoeff iron hematoxylin (for elastin), and by (b) light microscopy of paraffin sections stained by the alcian blue-periodic acid-Schiff technique (for proteoglycan) and by the Verhoeff-Van Gieson technique (for elastin and collagen). However,

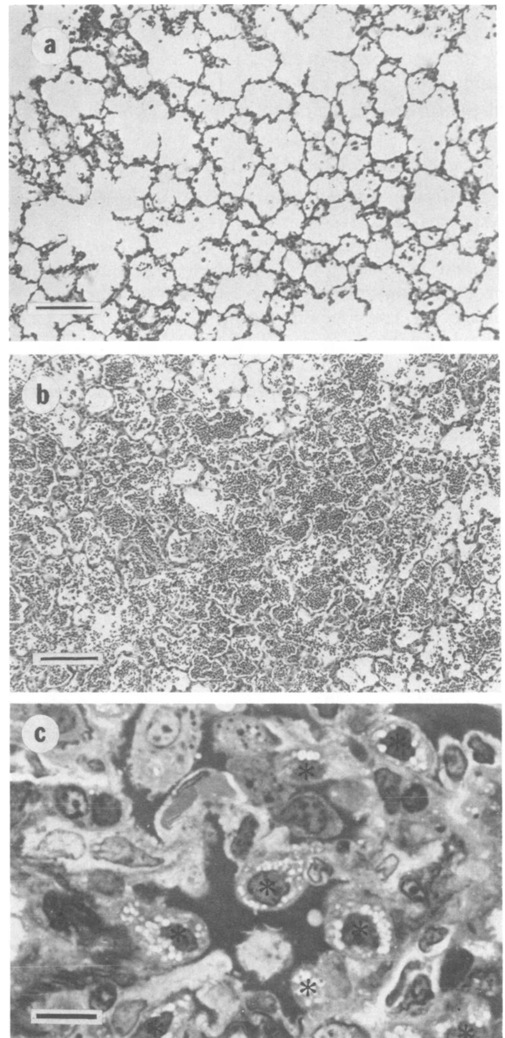


FIG. 2. *Light microscopic observations of alveolar alterations produced by P. aeruginosa proteases. Line markers on micrographs (a) and (b) represent 100 μm; the marker on micrograph (c) represents 10 μm. (a) Alveoli in rabbit lung fixed in the inflated state 6 h postinjection of heat-inactivated protease preparation (control). Paraffin section, hematoxylin and eosin stained. (b) Alveoli in lesion from rabbit lung fixed in the inflated state 6 h postinjection of 50 protease units. The alveoli are filled with erythrocytes, and no loss of alveolar septa is apparent. Paraffin section, hematoxylin and eosin stained. (c) Type II cell hyperplasia 1 day postinjection of 10 protease units. Cells (*) contain numerous lamellar bodies (clear inclusions). Epon section, basic fuchsin-alkaline methylene blue stained.*

ca. 2 to 6 days postinjection (and progressing in parallel with the macrophage response in the alveolar lumina), extensive infiltration and thickening of alveolar septa by mononuclear inflammatory cells (interstitial pneumonitis) were observed (Fig. 3h). The response was most commonly observed in lung tissue exhibiting grossly observable pale discolorations and was almost completely resolved by 8 days postinjection.

DISCUSSION

This study is the first detailed light and electron microscopic characterization of rabbit lung damage produced by the intratracheal administration of pseudomonas proteases of rigorously documented purity (18). Previous light microscopic examinations (7, 35) of lung tissue taken from humans who had died of pseudomonas pneumonia revealed (i) accumulations of erythrocytes, plasma proteins, fibrin, and mononuclear cells in the alveolar lumina, (ii) infiltration of alveolar septa by mononuclear cells rather than by PMN, and (iii) alveolar septal cell injury and necrosis. We observed similar structural alterations in rabbit lungs treated with the purified proteases, and this similarity supports the idea that the ability of *P. aeruginosa* to elicit lung damage during pseudomonas pneumonia is related to the in vivo production by the bacterium of extracellular proteases. This idea is supported further by results from two other types of studies performed in other laboratories. First, in vivo production of *P. aeruginosa* proteases has been demonstrated indirectly in studies showing that the sera of humans having *P. aeruginosa* pulmonary infections had elevated antibody titers to pseudomonas proteases (11, 17). Second, Homma et al. (10) reported recently that vaccination with Formalin-inactivated, purified proteolytic enzymes of *P. aeruginosa* protected mink against *P. aeruginosa*-induced hemorrhagic pneumonia. On the other hand, we should not, at this time, rule out the possibility that other products of the bacterium also are important in producing lung damage and/or death resulting from pseudomonas pneumonia. For example, our observation that the pseudomonas proteases elicited extensive structural alterations typical of pseudomonas pneumonia, but did not cause death of the experimental animals, suggests the possibility that death resulting from pseudomonas pneumonia is a function of pseudomonas products other than the proteases. *P. aeruginosa* exotoxin A is known to be more lethal for mice than are the bacterium's proteases (20), and the observation of elevated exotoxin A antibody titers in cystic fibrosis patients having pulmonary infections due to *P. aeruginosa* (17, 27) indicates that exotoxin A can be produced during the growth of the bacterium in the human lung. In addition, we have observed recently that the intratracheal injection of rabbits with small amounts (10 μg) of highly purified, protease-free and endotoxin-free preparations of exotoxin A (kindly supplied by P. V. Liu) results in death and grossly observable lung damage. Light and electron microscopic examination of the exotoxin A-treated lung tissue revealed, however, that the toxin caused alveolar septal cell necrosis, but did not produce the extensive intra-alveolar hemorrhage and infiltration by mononuclear cells characteristically

FIG. 3. *Electron microscopic observations of alveolar alterations produced by P. aeruginosa proteases. Line marker in each micrograph represents 1 μm. (a) Alveolus 6 h postinjection of 10 protease units. The alveolus contains erythrocytes (E), plasma proteins (P), macrophages (M), released type II cell lamellar bodies (L), and fibrin (F). (b) Macrophage accumulation in an alveolus 6 days postinjection of 10 protease units. Macrophages (M) often contained phagocytized lamellar bodies (L) and completely filled alveoli, thus making it difficult to identify individual septa and alveoli. Portions of an alveolar septum (S) can be seen. (c) Type I epithelial cell alterations 6 h postinjection of 10 protease units. Portions of two type I cells lining an alveolus are shown. The cytoplasmic extensions (*) of the cells are swollen and edematous. The alveolar lumen contains erythrocytes (E), fibrin (F), and an alveolar macrophage (M) containing phagocytized type II cell lamellar bodies. (d) Increased magnification of the nuclear region of the type I cell shown left-center in 3c. Note intracellular edema (E), a disrupted mitochondrion (M), dilated rough endoplasmic reticulum (R), and marginated chromatin (C). (e) Type I epithelial cell alterations 1 day postinjection of 10 protease units. The cell is markedly swollen and has an electron-lucent cytoplasm containing few recognizable organelles. (f) and (g) Capillary endothelial cell alterations 6 h and 1 day, respectively, of 10 protease units. Intracellular edema (E), marginated chromatin (C), a swollen and empty mitochondrion (M), vesicles (V), and the capillary lumen (L) are shown. Not shown but also observed were dilated rough endoplasmic reticulum and disrupted mitochondria. The more severely damaged cell (g) is markedly swollen, contains increased numbers of vesicles, and has an electron-lucent cytoplasm containing few recognizable organelles. (h) Interstitial pneumonitis 2 days postinjection of 50 protease units. The alveolar septum is thickened by a massive infiltration of mononuclear inflammatory cells. The cells are of two types: lymphocytes (L), the majority of which are in various stages of transformation into plasma cells, and peripheral monocytes (M) in various stages of maturation into alveolar macrophages.*

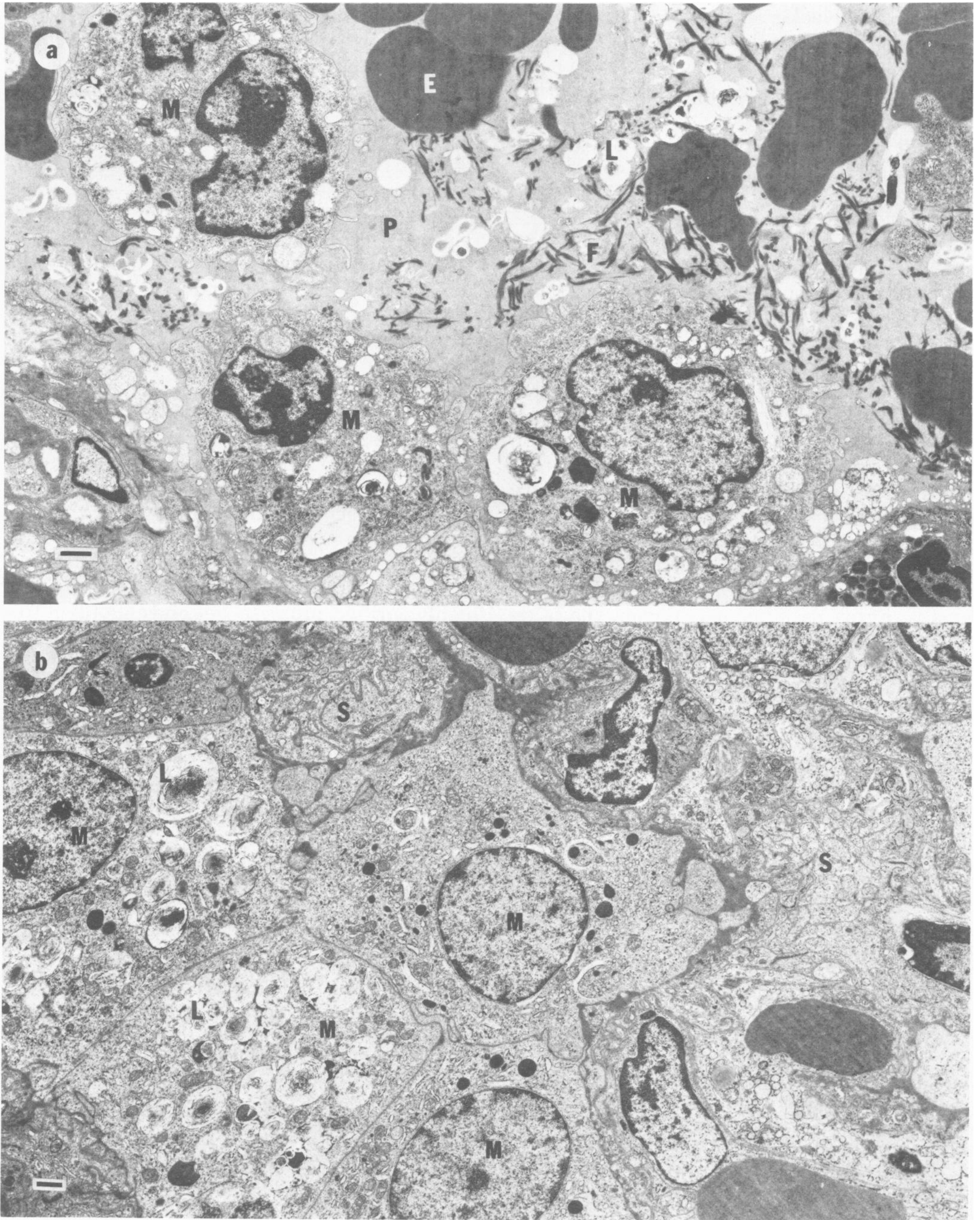
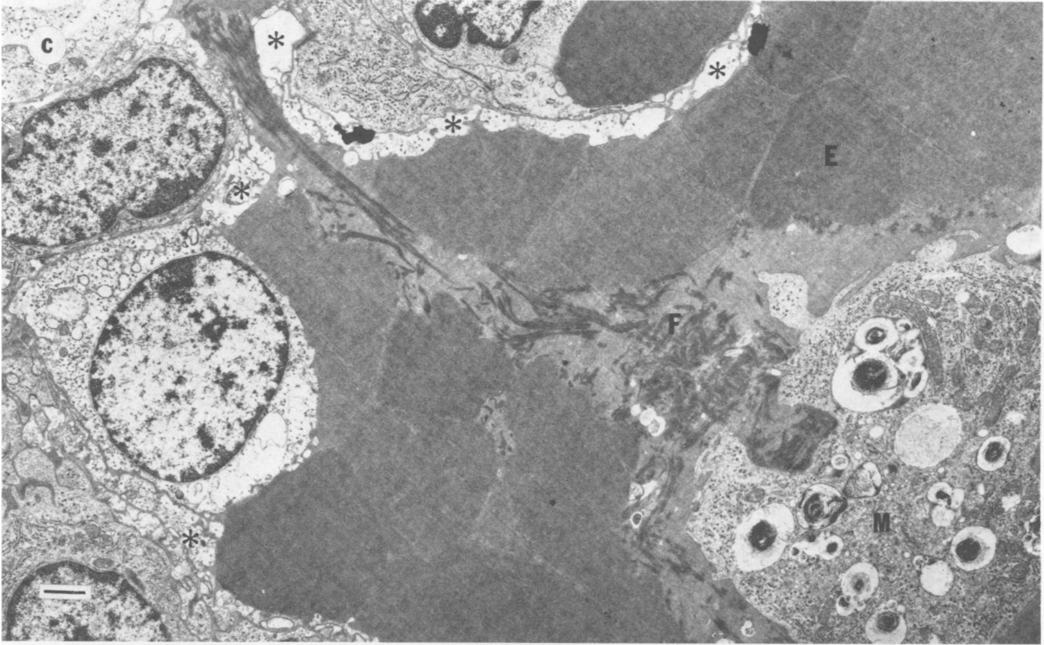


FIG. 3



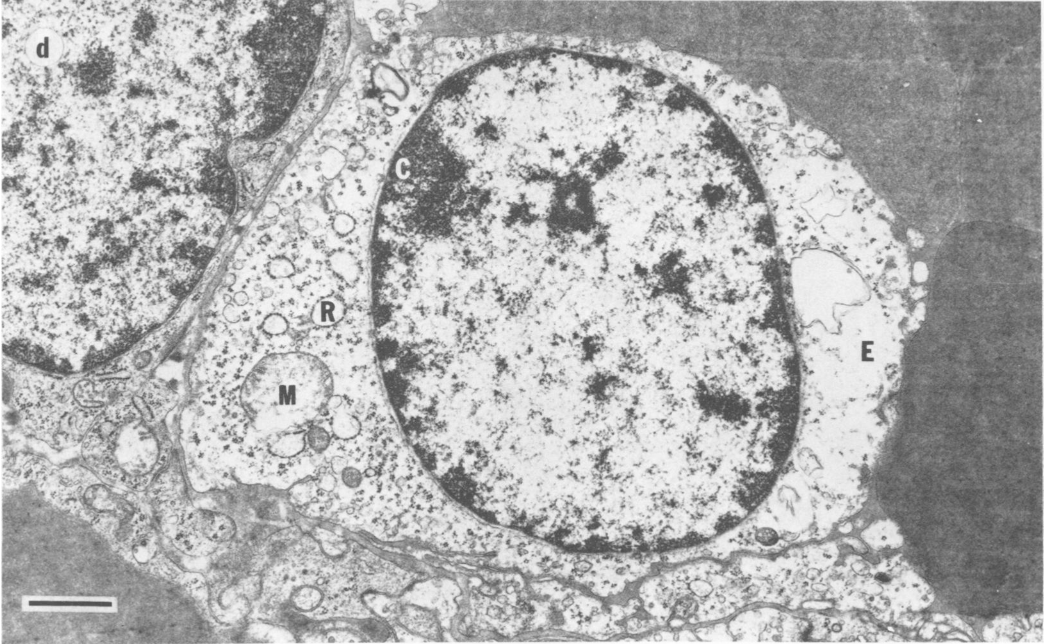
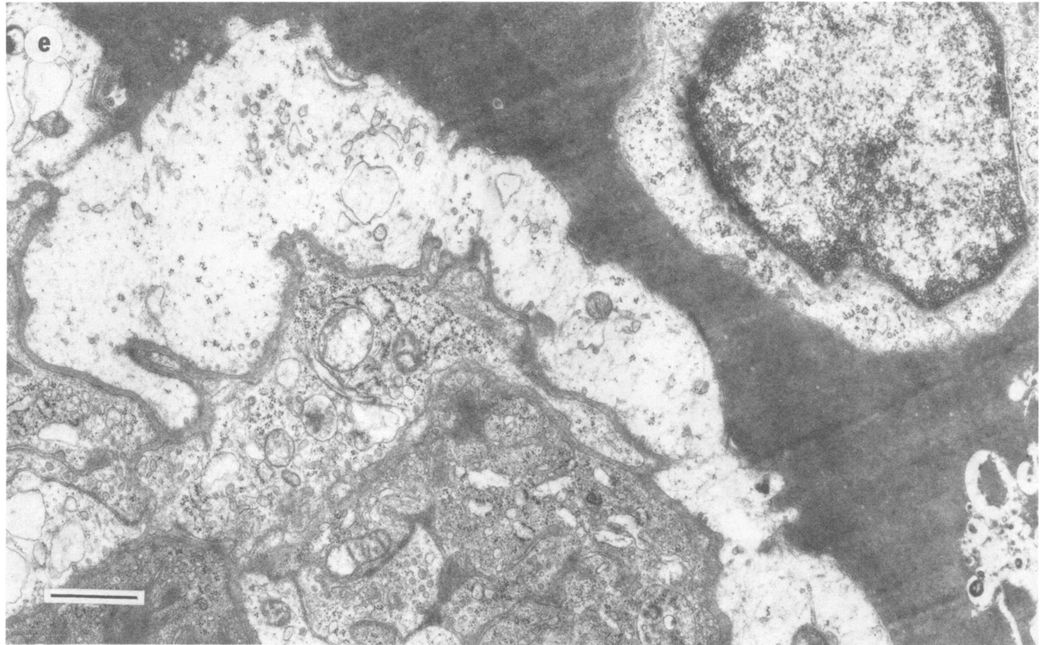


FIG. 3—*cont.*



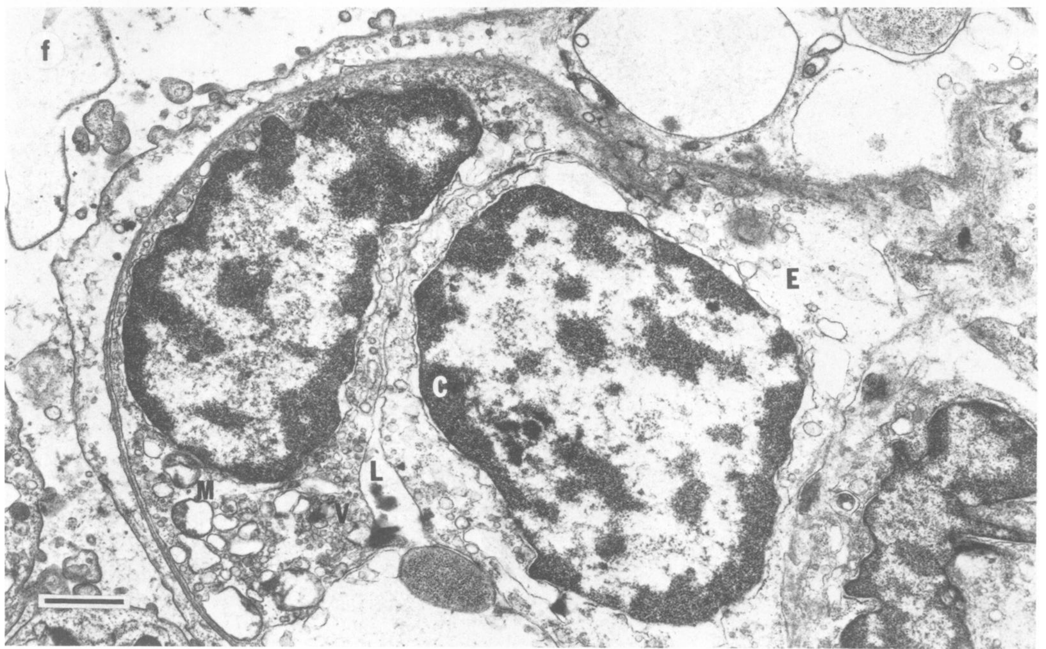


FIG. 3—*cont.*

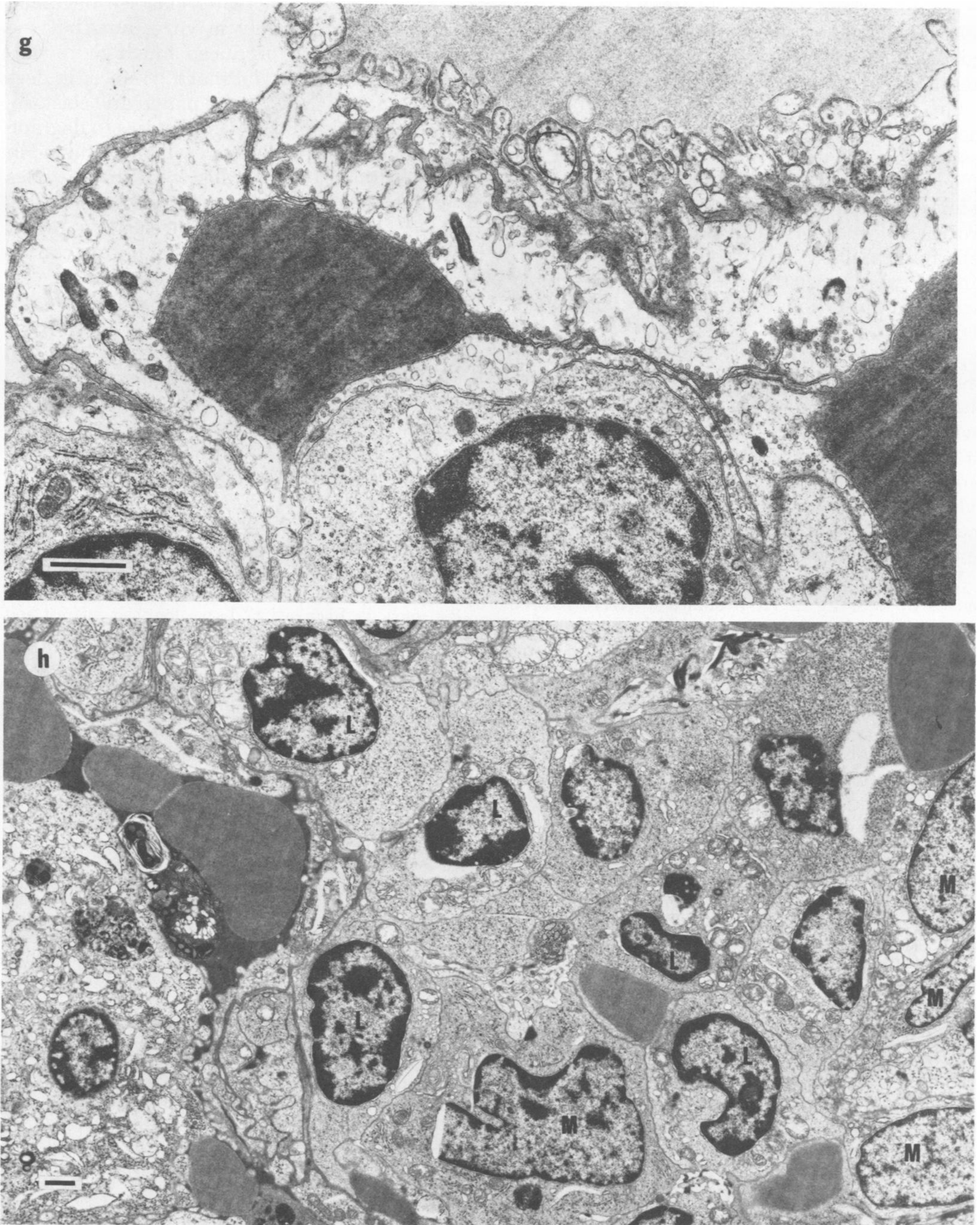


FIG. 3—*cont.*

produced by pseudomonas proteases and by pseudomonas pneumonia. Thus, the bacterium's proteolytic enzymes and exotoxin A may function sequentially or simultaneously during the development of the disease process. To obtain additional data to support or refute the idea of a role for pseudomonas proteases in the pathogenesis of *P. aeruginosa*-induced pulmonary disease, studies are in progress in our laboratory to determine (i) whether the pseudomonas proteases can be detected in lungs during the development of experimental pseudomonas pneu-

monia in rabbits and (ii) whether passive immunization against the proteases can reduce the lung damage normally occurring during experimental pseudomonas pneumonia.

The rapid and extensive pulmonary hemorrhage elicited by the protease preparations suggested, prior to microscopic examination of the affected tissues, that the pulmonary capillaries, venules, and/or arterioles were damaged by the proteases. We observed alveolar type I epithelial cell and capillary endothelial cell damage in lungs treated with the proteases; however, no structural alterations were noted in pulmonary venules or arterioles. Therefore, the simplest explanation for the observed hemorrhage is that the erythrocytes reached the alveolar lumina by diapedesis through the capillary endothelium, basement membranes, and alveolar epithelium. At the present time, it is not known whether the proteases can directly injure capillary endothelial cells or whether they may indirectly damage the vessels by complement activation and kinin generation.

Various plant, microbial, and mammalian proteases possessing in vitro elastase activity have been reported to degrade alveolar septal elastin and to produce emphysema after intratracheal or aerosol administration into experimental animals (3, 13, 14, 19, 32). However, we did not observe this response in our model. There are at least two possible reasons for this difference in response. First, the studies of Oakley and Banerjee (25) and Meinke et al. (23) suggest the possibility that pseudomonas elastases may not readily digest native elastin in vivo. Oakley and Banerjee (25) reported that pseudomonas elastase degraded acid or alkali-treated elastin but not native elastin in aortic arch sections. Furthermore, Meinke et al. (23) did not observe destruction of arterial elastic laminae, loss of alveolar septa, or alteration of alveolar septal elastin after intravenous and intranasal administration of lethal doses of pseudomonas elastase into mice. On the other hand, Kawaharajo et al. (16) claimed severe pulmonary hemorrhage due to destruction of pulmonary arteriolar elastin and alveolar septa after intravenous and intranasal administration of lethal doses of pseudomonas elastase into mice. Second, the observation that normal rabbit serum inhibits pseudomonas protease activity in vitro (8) suggests the possibility that the rapid and extensive alveolar hemorrhage produced after the intratracheal injection of the elastases may have resulted in inhibition of elastase activity before the enzymes reached susceptible interstitial and arteriolar elastin. In that regard, Senior et al. (32) have reported that human neutrophil elastase and porcine pancreatic elastase degraded lung elastin comparably in vitro, but that the neutrophil elastase produced much more pulmonary hemorrhage and much less elastin degradation in vivo than did the pancreatic elastase. The ability of pseudomonas elastases to degrade native alveolar septal elastin will be examined in isolated rabbit lungs instilled with the enzymes after lung lavage and vascular perfusion. This study should tell us whether the elastin in rabbit lungs largely freed of elastase inhibitors can be degraded by the pseudomonas elastases.

The hyperplasia of alveolar type II epithelial cells observed in our model was not an unexpected finding. Type II cell hyperplasia (1, 2, 6, 9, 15, 26) and release of lamellar bodies (26) has been observed in lungs experimentally injured by various agents, and it has been proposed that transition of type II cells into type I cells is an important component in the healing process.

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