Alteration of pulmonary structure by *Pseudomonas aeruginosa* exoenzyme S

D. E. WOODS, W. S. HWANG,* M. S. SHAHRABADI and J. U. QUE

Department of Microbiology and Infectious Diseases and *Department of Pathology, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1

**Summary.** Intratracheal administration of purified *Pseudomonas aeruginosa* exoenzyme S elicited extensive, grossly observable damage in the rat lung within 2 h. Light and electronmicroscopy revealed injury and necrosis of bronchial epithelium, type I pneumocytes and capillary endothelial cells after 1 h; associated haemorrhage, fibrinous exudation and released type II cell lamellar bodies in alveolar lumina after 1–12 h; progressively increasing accumulations of polymorphonuclear leucocytes in the bronchi and alveoli and in alveolar septae (interstitial pneumonia) after 1–12 h; collapse of alveolar septal connective tissue and damage to pulmonary arterioles and venules. Treatment of monolayer cultures of bronchial fibroblasts with purified exoenzyme S elicited vacuolation of the cells with apparent membrane damage as revealed by light and electronmicroscopy. In-vivo production and activity of *P. aeruginosa* exoenzyme S may be an important pathogenicity determinant in the necrotising lung injury characteristic of *P. aeruginosa* pneumonia.

**Introduction**

A variety of specific and non-specific defence mechanisms serve to protect the lung from injury by micro-organisms and their products. These include mucociliary clearance, alveolar macrophages, humoral and cell-mediated immunity (Green, 1970). In spite of these usually efficient defence mechanisms, bacterial lung infections do occur. Over the past three decades, gram-negative micro-organisms have become increasingly important as causative agents of serious infections, particularly in hospitalised patients (Centers for Disease Control, 1984). *Pseudomonas aeruginosa* has become especially prominent in this regard (Renner et al., 1972).

Pulmonary infections with *P. aeruginosa* may occur in clinical settings other than nosocomial pneumonias, including community-acquired acute pneumonia (Sullivan et al., 1972), and in chronic lung infections of cystic fibrosis (CF) patients (Reynolds et al., 1976). Pulmonary infection with *P. aeruginosa* may be accompanied by severe tissue damage because this organism produces several virulence factors in mammalian hosts and these contribute to the pulmonary tissue damage (Woods and Sokol, 1986). In addition to cell-associated factors, *P. aeruginosa* produces a number of exoenzymes, including exotoxin A, proteolytic enzymes, phospholipase C, and exoenzyme S, each of which has been implicated as a virulence determinant (Woods and Iglewski, 1983).

Histopathological descriptions of lung injury in acute *P. aeruginosa* infections have varied, depending upon the patient and how the infection was acquired. The lesions seen in nosocomially acquired necrotising pneumonia are characterised by alveolar septal necrosis with necrosis of arterial walls and secondary thrombosis. The alveolar exudate is predominantly mononuclear with some polymorphonuclear leucocytes (Mays et al., 1969). The pathology of community-acquired pneumonia caused by *P. aeruginosa* is characterised by multiple small abscesses and marked alveolar cell necrosis. Arterial wall necrosis, perivascular infiltrate and thrombosis were not noted (Tillotson and Lerner, 1968). The principal microscopic finding in cases of *P. aeruginosa* pneumonia acquired after bacteraemia originating from another site is intra-alveolar haemorrhage with patchy alveolar septal necrosis (Fetzer et al., 1967). The pulmonary histopathology of chronic *P. aeruginosa* infections as seen in cystic fibrosis is dominated by bronchial changes including epithelial metaplasia with loss of cilia, predominance of mucous over serous acini in hyperplastic...
bronchial glands, goblet cell hyperplasia, acute and chronic inflammatory infiltrates, bronchiectasis and mucopurulent plugging of the airways (Bedrosian et al., 1976).

We have proposed that these various pathological pictures might be explained by infection with strains of differing exoproduct phenotypes (Que and Woods, 1987). Indeed, clinical isolates of \( P. \text{aeruginosa} \) are of significantly different exoproduct phenotypes depending upon their site of isolation, indicating that the host environment may select for the phenotypic expression of certain exoproducts in specific infections (Woods et al., 1986). Based upon these studies, we have concluded that elastase and exoenzyme S may play important roles in pulmonary infections with \( P. \text{aeruginosa} \). A similar conclusion has been reached by comparative studies in animal models of lung infections produced by \( P. \text{aeruginosa} \) parent strains and isogenic mutants singly deficient in either elastase or exoenzyme S. The parent strains were shown to be more virulent (Woods et al., 1982; Blackwood et al., 1983; Nicas et al., 1985; Woods and Sokol, 1985).

An alternative approach to the study of the pathogenesis of \( P. \text{aeruginosa} \) pulmonary infection was that of Cash et al. (1982) who instilled purified \( P. \text{aeruginosa} \) exoproteins into the lungs of rats and demonstrated that the resulting pulmonary histopathology was similar to that induced by experimental infection with \( P. \text{aeruginosa} \). Similarly, Gray and Kreger (1979) demonstrated that instillation of purified \( P. \text{aeruginosa} \) proteases into the lungs of rabbits produced changes not unlike those seen in some cases of human pneumonia. The present study was designed to examine the effects of direct instillation of purified \( P. \text{aeruginosa} \) exoenzyme S into rat lungs in order to determine whether the histopathological changes produced by exoenzyme S could be correlated with any of the various pathological pictures described for \( P. \text{aeruginosa} \) pulmonary infections.

**Materials and methods**

**Exoenzyme S preparation**

Exoenzyme S was purified from culture supernates of \( P. \text{aeruginosa} \) strain DG1 grown at 32°C in mineral salts medium containing 110 mM succinate, 100 mM monosodium glutamate, glycerol 1% and 10 mM EDTA until late exponential phase (Woods and Que, 1987). Purification was achieved by sequential ammonium sulphate precipitation, DEAE-Sephalcel anion exchange chromatography, acetone precipitation in the presence of 1 M NaCl, and G100 Superfine gel filtration chromatography. Exoenzyme S was monitored during purification by an assay for ADP-ribosyl transferase activity, mouse and tissue culture cell toxicity and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Woods and Que, 1987).

**Instillation of purified exoenzyme S into rat lungs**

Filter-sterilised (0-2-μm pore size) exoenzyme S preparations were diluted with sterile phosphate-buffered saline (PBS, pH 7.0) to yield solutions containing (0.1 ml) 100, 50 and 1 μg of exoenzyme S per 0.1 ml. A tracheostomy was performed on young adult male Sprague-Dawley rats (200–220 g; Charles River, Canada) under ether anesthesia and 0.1 ml of active exoenzyme S or heat-inactivated exoenzyme S (100°C, 30 min; control) was deposited into a distal bronchus via a bead-tipped curved needle (Cash et al., 1979). Six animals from each group (active versus inactive exoenzyme S, 3 separate concentrations each) were killed by exsanguination under ether anesthesia at intervals of 1, 2, 4 and 12 h after instillation.

**Tissue-culture monolayer treatment**

Dulbecco's Modified Eagle's Medium (DMEM; Flow Laboratories, Mississauga, Ontario) with sodium pyruvate 110 mg/L was supplemented with glutamine 584 mg/L, penicillin 50 IU/ml, streptomycin 50 μg/ml, and fetal calf serum (Myoclone, Gibco, Burlington, Ontario) 10%. Bronchial fibroblasts (CCD 14Br) were obtained from the American Type Culture Collection (Rockville, MD). Tissue-culture monolayers were harvested by rinsing with 5 ml of trypsin-EDTA (Gibco Canada, Inc.), incubation for 10 min with 5 ml of trypsin-EDTA at 37°C in CO₂ 7%, mixing with an equal volume of cold medium and centrifugation (5000 g for 10 min). The cells were resuspended in fresh medium to a concentration of 1 x 10⁵ cells/ml; 200 μl were dispensed into each well of a microtitration plate and incubated overnight at 37°C in CO₂ 7%. Various concentrations of active or heat-inactivated exoenzyme S were filter sterilised, diluted in DMEM and added to the cells.

**Light and electronmicroscopy**

The lungs and heart were removed en bloc from control and exoenzyme S-treated animals, and one-third of the samples were fixed uninflated in buffered formalin (pH 7-0) 10%. Another one-third of the samples were perfused through the trachea.
with buffered formalin (pH 7.0) 10% under 10 cm hydrostatic pressure. All of the above samples were fixed for a minimum of 24 h after which sagittal sections of lungs were embedded in plastic (glycol-methacrylate) for light microscopic histochemical studies. Specimens to be examined by electronmicroscopy (final one-third) were cut into 1-mm cubes and fixed in gluteraldehyde 4% in sodium cacodylate buffer (0.05 M, pH 7.4) for 12 h.

Plastic sections (1 μm) of tissues fixed in the uninflated state were stained with haematoxylin and eosin (H & E), Giemsa, and a combined periodic acid-Schiff-silver stain (Preece, 1972). Plastic sections of tissues fixed in the inflated state were stained with H & E and reticulocyte staining to examine for alveolar septal loss (Woods et al., 1982).

Glutaraldehyde-fixed tissues were washed with cacodylate buffer, postfixed in osmium tetroxide 1% in cacodylate buffer for 2 h, washed with cacodylate buffer, and dehydrated in increasing concentrations of acetone. Specimens were further dehydrated in propylene oxide and embedded in Epon 812. Thick sections (1 μm) were stained with toluidene blue and were examined by light microscopy to evaluate embedding and condition of tissues and to select areas for subsequent electronmicroscopic examination. Thin sections (80 nm) were stained with uranyl acetate and lead citrate. Thin sections were examined in a Hitachi H-600 electronmicroscope.

Light microscopic examination of tissue-culture cells (bronchial fibroblasts, CCD 14Br) was performed directly in microtitration wells after staining with crystal violet. Tissue-culture monolayers of cells treated with active exoenzyme S and heat-inactivated exoenzyme S (control) were rinsed three times with PBS (200 μl), stained with crystal violet (1% in PBS), and washed three further times with PBS to remove excess stain.

Tissue-culture cells from exoenzyme S-treated and control monolayers to be examined by electronmicroscopy were rinsed three times with PBS to remove tissue-culture medium. The monolayer was disrupted by scraping with a “rubber policeman” in the presence of PBS and the cells were transferred to a microfuge tube and pelleted by centrifugation (10,000 g, 5 min; Beckman Microfuge). Cells were fixed in glutaraldehyde 3% in cacodylate buffer (0.05 M, pH 7.2) for 3 h at 4°C. The cells were washed three times with cacodylate buffer and left in this buffer overnight. Cells were post-fixed in osmium tetroxide 1% at room temperature for 3 h. Dehydration was with increasing concentrations of ethanol and the cells were embedded in Epon 812.

Thick sections (80 nm) were stained with uranyl acetate 0.5% in methanol followed by lead citrate 1% and examined in a Phillips 400 electronmicroscope.

Results

Intratracheal instillation of heat-inactivated control preparations did not produce gross or microscopic alterations in pulmonary structure of rats at any of the time points examined. All the rats given active exoenzyme S preparations at concentrations of 100 μg and 50 μg died within 6 h of instillation. The most notable symptom at the time of death was marked respiratory distress. The two highest doses of exoenzyme S (100 and 50 μg) produced numerous, grossly observable confluent haemorrhagic lesions after 1 h and the lesions increased in size progressively and reached maximum size after 4 h. Sagittal lung sections revealed that lesions extended throughout the depth of the lung. In additional experiments, animals were given 1 μg of exoenzyme S and allowed to live for 7 days. Lung structure failed to return to normal in any of the animals examined during this time period (data not shown).

Light microscopy of lungs from animals given intratracheal injections of 50 μg or more of active exoenzyme S revealed almost immediate reactive changes. Airways and parenchyma were completely obscured by the inflammatory response after 1 h (fig. 1). The following histopathological descriptions apply to lungs after instillation of 1 μg of exoenzyme S.

One hour after injection, injury to bronchial epithelium was evident (fig. 2). Increasing accumulations of inflammatory cells and fibrinous exudation, both intrabronchially and peribronchially, continued throughout the 12-h time period until the bronchial epithelial layer was destroyed (fig. 3). Alveolar type-I epithelial cells and capillary endothelial cells showed progressive injury and necrosis from 1 h to 12 h after injection. Electronmicroscopic examination revealed consistent and obvious membrane damage to alveolar type-I epithelial cells. Fig. 4 shows the “lifting-off” of the membrane consistently observed in this cell type from 4 h to 12 h after injection.

Alveolar type-II epithelial cells showed hyperplasia 4 h after injection and this increased progressively throughout the study period. During this time, there was a marked increase in the releases of type-II cell lamellar bodies into alveolar lumina. Membrane damage was also a consistent finding in
Fig. 1. H & E stained section of rat lung 1 h after treatment with 100 μg of purified exoenzyme S. Airways and lung parenchyma are totally obscured by suppurative inflammation consisting of polymorphonuclear leucocytes and proteinaceous exudation. × 50.

Fig. 2. H & E stained section of rat lung 1 h after treatment with 1 μg of purified exoenzyme S. Initial stages of airway injury are indicated by the significant alterations in bronchial epithelium. Initial stages of peribronchial inflammatory cell (polymorphonuclear leucocytes primarily) infiltration are also apparent. × 400.

this cell type after 12 h; fig. 5 shows the vacuolation seen in type-II epithelial cells at this time.

Progressively increased accumulations of polymorphonuclear leucocytes occurred in bronchial and alveolar lumina as well as in alveolar septae (interstitial pneumonia) from 1 h to 12 h after injection. Toluidine blue staining of exoenzyme S-treated lung tissue revealed vacuolation of pulmonary parenchyma reminiscent of that seen in type-II epithelial cells (fig. 6). Collapse of alveolar septal connective tissue and damage to pulmonary arterioles and venules was revealed by reticulocyte staining (fig. 7).

Treatment of monolayer cultures of bronchial fibroblasts with purified exoenzyme S (100 ng) elicited vacuolation of the cells with apparent membrane damage 12 h after treatment (fig. 8). At 24 h after treatment, the monolayer was completely destroyed (fig. 9). Electronmicroscopic examination of bronchial fibroblasts at 12 and 24 h after treatment with exoenzyme S demonstrated progressive vacuolation of cells from 12 h (fig. 10) to 24 h (fig. 11).

Discussion

*P. aeruginosa* produces several extracellular products which have been proposed as virulence determinants in pulmonary infections with this organism (Woods and Iglewski, 1983). Included among these are two distinct ADP-ribosyl transferases—exotoxin A and exoenzyme S—which differ in their molecular structure and substrate specificity (Iglewski et al., 1977, 1978). Recent evidence from several studies indicates that exoenzyme S may play a significant role in pulmonary infection in man. In animal model studies comparing infections produced by parental *P. aeruginosa* strains with
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Fig. 4. Electronmicrograph of alveolar type-I epithelial cell in lung 4 h after instillation of 1 µg of purified exoenzyme S. There is apparent membrane damage characterised by “lifting-off” of the membrane. Uranyl acetate and lead citrate stained, × 5000.

Fig. 5. Electronmicrograph of alveolar type-II epithelial cell in lung 4 h after instillation of 1 µg of purified exoenzyme S. There is vacuolation and lamellar body inclusion and release into alveolar lumina. Uranyl acetate and lead citrate stained, × 6000.

Fig. 3. H & E stained section of rat lung 12 h after instillation of 1 µg of purified exoenzyme S. There are accumulations of inflammatory cells and fibrinous exudation, both intrabronchially and peribronchially with destruction of the bronchial epithelial layer. × 100.

those produced by isogeneic mutants, singly deficient in exoenzyme S, it has been shown that infections with exoenzyme S-positive strains are much more severe (Nicas et al., 1985; Woods and Sokol, 1985). Phenotypic analysis of P. aeruginosa strains isolated from pulmonary infections in man showed that these strains produce significantly more exoenzyme S than strains isolated from other clinical sources such as urine or wound infections (Woods et al., 1986). Further evidence that exoenzyme S may play a role in lung injury during P. aeruginosa infections is provided by the present study, which is the first light and electronmicroscopic characterisation of lung injury after intratracheal instillation of purified exoenzyme S.

Descriptions of the pathology associated with P. aeruginosa lung infections have varied depending upon the patient or upon how the infection was acquired. The aim of the present study was to determine the relationship of those structural
changes induced in lungs by instillation of exoenzyme S with the histopathological changes described for nosocomial, community-acquired, or bacteraemic P. aeruginosa pneumonias, as well as those described for chronic P. aeruginosa lung infections in patients with cystic fibrosis. Surprisingly, the histopathological changes seen in rat lungs after instillation with purified exoenzyme S appeared to overlap with all of the various histopathological descriptions previously published for P. aeruginosa lung infections, regardless of origin.

Alveolar septal necrosis, necrosis of arterial walls, and secondary vessel thrombosis were the morphologic features stressed in the diagnosis of gram-negative bacillary necrotising pneumonias induced by the inhalation of aerosolised P. aeruginosa from positive-pressure nebulisers (Mays et al., 1969). The present study documents clearly that collapse of alveolar septal connective tissue and damage to pulmonary arterioles and venules, as revealed by reticulocyte staining of exoenzyme S-treated lungs, is a basic feature of lung injury caused by exoenzyme S. Thus, histopathological changes during nosocomial pneumonia caused by P. aeruginosa could be explained partially by the elaboration of exoenzyme S in vivo during these infections.

Tillotson and Lerner (1968) described the pathology of ten cases of P. aeruginosa pneumonia that were neither the result of a pre-existing bacteremia, nor nosocomially acquired. Multiple small abscesses were grossly present and marked alveolar cell-wall necrosis was characteristic at microscopic examination. Arterial wall necrosis, perivascular infiltration, and thrombosis were not noted. Similar, though not identical, pathological findings were seen after instillation of purified exoenzyme S into...
the lungs of experimental animals. In comparing our results with the descriptions of Tillotson and Lerner (1968), the differences may not be only in the degree of injury but there may be qualitative differences.

Similar to the findings reported for nosocomial *P. aeruginosa* pneumonias (Mays et al., 1969), we have seen arterial-wall necrosis as a result of instillation of exoenzyme S into the lungs of experimental animals. This is significantly different from the reports of Tillotson and Lerner (1968) and those of Fetzer et al. (1967) that in *P. aeruginosa* pneumonia originating from bacteraemia, organisms and necrosis occurred only in the capillaries. Arterial-wall necrosis was not seen when purified proteases were instilled into the lungs of rabbits (Gray and Kreger, 1979). Based upon these findings, it would appear that strains isolated from cases of nosocomial pneumonia should be examined for the presence of exoenzyme S, or both should be done.

The pulmonary histopathology described for *P. aeruginosa* infections in cystic fibrosis is dominated by bronchial changes. These include epithelial metaplasia with loss of cilia, predominance of mucous versus serous acini in hyperplastic bronchial glands, goblet cell hyperplasia, acute and chronic inflammatory infiltrates, bronchiectasis and mucupurulent plugging of the airways (Bedrosian et al., 1976). The earliest changes noted after instillation of exoenzyme S into rat lungs were disruption of bronchial epithelium and airway inflammation. Additional pathological changes in airways were not seen in our studies, presumably because of the acute nature of the experiments. It is conceivable that chronic exposure to exoenzyme S, as might occur during chronic *P. aeruginosa* infections, could lead to these changes.

It is interesting to note that the most consistent morphologic alteration seen in individual cells of

**Fig. 8.** Monolayer culture of bronchial fibroblasts 12 h after treatment with 100 ng of purified exoenzyme S, showing apparent membrane damage (vacuolation). Crystal violet stained, × 400.

**Fig. 9.** Monolayer culture of bronchial fibroblasts 24 h after treatment with 100 ng of purified exoenzyme S. Monolayer has been completely destroyed. Crystal violet stained, × 400.
both lung tissue and tissue-culture monolayers exposed to exoenzyme S was an apparent membrane disruption which led either to "lifting off" of the membrane or vacuolation. This finding is in agreement with some of our recent studies in which we have demonstrated that exoenzyme S acts at the level of the cytoplasmic membrane (Que, J. U. and Woods, D. E., unpublished observations).

There is little question that the virulence of *P. aeruginosa* is multifactorial. However, results from phenotypic analyses of *P. aeruginosa* strains isolated from a variety of infection sites, indicate that certain exoproducts may play a more important role in certain types of infections (Woods et al., 1986). This hypothesis is confirmed by the present studies which indicate that the pathological changes described for *P. aeruginosa* lung infections in a variety of clinical settings may be reproduced by a single *P. aeruginosa* exoproteins, exoenzyme S.

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