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Toxicity of Pneumolysin to Pulmonary Alveolar Epithelial Cells

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Mortality during the first several days of pneumococcal pneumonia has not decreased appreciably over the past 30 years, despite the widespread use of antibiotics. Disruption of the alveolar epithelial barrier is likely an initial step in the pathogenesis of pneumococcal pneumonia. We report that soluble factors from *Streptococcus pneumoniae* can directly injure isolated rat alveolar epithelial cells. Using biochemical and immunological techniques, we identified pneumolysin as a major soluble *S. pneumoniae* toxin for alveolar epithelial cells. Alveolar epithelial cells at 24 or 72 h after isolation were equally sensitive to injury by purified pneumolysin. Purified pneumolysin substantially increased alveolar permeability in an isolated perfused rat lung model. Electron microscopy revealed that instilled pneumolysin caused widespread lung injury, primarily to type I alveolar epithelial cells. Pneumolysin toxicity to alveolar epithelial cells may be important in the pathogenesis of acute lung injury during pneumococcal pneumonia and may facilitate pneumococcal bacteremia.

Streptococcus pneumoniae is one of the most common etiologic agents of community-acquired pneumonia, causing approximately 50,000 deaths annually in the United States (25). Overall adult mortality remains as high as 30%, and mortality in patients requiring intensive care may reach 60 to 75% (15, 22). Despite the use of potent antibiotics and aggressive intensive-care support, fatality rates during the first 5 days of bacteremic infections have not changed significantly over the past 30 years (22, 26). Consequently, a better understanding of the early events in the pathogenesis of pneumococcal pneumonia is needed to control this prevalent and serious disease.

Physiological studies indicate that the alveolar epithelium is the principal limiting membrane for alveolar capillary water and solute transport in the normal lung (5, 34). The lung alveolar epithelium is composed of two distinct populations of cells. Thin, elongated type I pneumocytes cover about 95% of the alveolar surface and are connected by tight junctions to form the alveolar barrier. Widespread damage to type I epithelial cells is a common morphological feature of acute lung injury from a variety of inhaled and blood-borne toxins (1, 2, 10, 12, 30). In contrast, type II pneumocytes cover only 4 to 5% of the alveolar surface and appear relatively more resistant to injury. These cells appear to repopulate the alveolar epithelium after acute lung injury and serve the additional important function of synthesizing and secreting the lipoproteinaceous surfactant that prevents alveolar collapse (1, 12, 14, 30).

Histologically, the initial phase of pneumococcal pneumonia is characterized by flooding of the alveoli with protein-rich fluids (35), implying injury to the alveolar epithelium. Although numerous bacteria are present, few inflammatory cells are seen in this serous exudate, suggesting that alveolar epithelial cell injury may be caused directly by pneumococcal toxins rather than by leukocyte products. Of the several pneumococcal toxins that have been identified, pneumolysin, an oxygen-labile intracellular cytolysin, has been implicated in the virulence of *S. pneumoniae* and has been shown to be cytotoxic to several different types of pulmonary cells in vitro. We have shown that pneumolysin is a major soluble pneumococcal cytotoxin for pulmonary artery endothelial cells (32), and others have demonstrated pneumolysin toxicity to human nasal and tracheobronchial ciliated epithelia (33). However, the toxicity of pneumolysin to the pulmonary alveolar epithelium has not been described. Consequently, we have identified pneumolysin as a major cytotoxin in *S. pneumoniae* bacterial autolysate supernatants (BAS) and have studied its effects on pulmonary alveolar epithelial cells, using both cell cultures and a unique model, the isolated perfused rat lung preparation.

MATERIALS AND METHODS

Materials. Native pneumolysin purified as described previously (17) and rabbit antipneumolysin antisera were the generous gifts of Mary K. Johnson, Tulane University, New Orleans, La. Two different preparations of purified pneumolysin were used in these studies; they had specific activities of approximately 3×10^5 and 3×10^6 hemolytic units (HU) per mg of protein, assayed as described previously (17) with human erythrocytes. Unless otherwise specified in the text, all commercially available reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

Endothelial and epithelial cell cultures. Bovine pulmonary artery endothelial cells were purchased (CCL 209; American Type Culture Collection, Rockville, Md.) and cultured as previously described (32). Cells were replaced every 3 months (at approximately passage 30) and used at confluence (5×10^5 cells per cm^2 in 2-cm^2 polystyrene wells [Costar, Cambridge, Mass.]). Primary cultures of type II rat alveolar epithelial cells (RAEC) were prepared by a modification of the method of Dobbs et al. (9). Specific-pathogen-free male Sprague-Dawley rats (180 to 200 g; Harlan Sprague-Dawley, Indianapolis, Ind.) were anesthetized with sodium pentobarbital (75 mg/kg) and exsanguinated by severing of the inferior

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vena cava. After cannulation of the trachea and the pulmonary artery, the lungs and heart were carefully removed en bloc. The pulmonary artery was perfused with nominally calcium-free phosphate-buffered saline (PBS), containing 140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 15 mM Na_2HPO_4, and 3.3 U of heparin sulfate per ml. The lungs were lavaged via the tracheal catheter with buffer containing 140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 6 mM glucose, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and then with calcium-free PBS. The lungs were continuously filled with porcine pancreatic elastase solution (21.5 U/ml; Elastin Products Co., Inc., Owensville, Mo.) for 20 min at 37°C before being minced in the presence of DNase (250 μg/ml), stirred, and then incubated in fetal bovine serum (BioProducts, Indianapolis, Ind.) in calcium-free PBS for 30 min at 37°C. The suspension was sequentially filtered through two- and four-ply sterile gauze and then 100 and 20 mesh. The mixture of cells was plated in minimal essential medium (CellGro; Fisher Scientific, Itasca, Ill.) on bacteriologic plastic dishes coated with rat immunoglobulin G (500 μg/ml; Calbiochem, San Diego, Calif.) to remove contaminating alveolar macrophages. After 1 h, the nonadherent cells were recovered by centrifugation, and cultured in minimal essential medium containing 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at a density of 1.5×10^6 cells per cm^2 in 2-cm^2 polystyrene tissue culture wells (Costar). When seeded, type II RAEC showed a purity of 90 to 95%, as assessed by staining with tannic acid (24) and alkaline phosphatase (11). RAEC were incubated at 37°C in 5% CO_2–95% air and studied at either 24 or 72 h after isolation. At 72 h, >95% of the cells stain positively with an epithelial cell-specific PKK-1 cytokeratin monoclonal antibody (31).

Bacterial cultures and BAS. Stock bacteria (type 14 *S. pneumoniae*; ATCC 6314) were stored frozen on glass beads at −70°C. BAS was prepared as previously described (32). In brief, bacteria were cultured for 16 h on Trypticase soy agar supplemented with 5% sheep erythrocytes, washed twice with Dulbecco's PBS (GIBCO Laboratories, Grand Island, N.Y.), resuspended in PBS at 10^8 CFU/ml, and incubated at 37°C in 5% CO_2 for 4 h. During this time, marked autolysis occurred, as determined by an approximately 70% reduction in the optical density at 600 nm and an approximately 99% reduction in bacterial cell viability (32). After centrifugation at 17,000 × *g* for 15 min at 4°C in a Sorvall SS34 rotor, the supernatant (BAS) was filtered through a 0.2-μm-pore-size membrane and stored at 4°C. Before BAS was used, its sterility was confirmed by culturing on blood agar plates.

Cytotoxicity assay. Cellular injury was assayed as the leakage of ^{51}Cr-labeled cytosolic proteins as previously described for endothelial cells (32). In brief, cells were radiolabeled by incubation with medium containing 2 μCi of sodium [^{51}Cr]chromate (Dupont, NEN Research Products, Boston, Mass.) per ml for 16 h. Wells were washed three times with PBS, and the contents were incubated with 500 μl of incubation buffer (PBS, 0.5% bovine serum albumin, 0.2% glucose) and BAS or purified pneumolysin at 37°C in 5% CO_2–95% air for 4 h. The medium was centrifuged at 500 × *g* for 10 min, and the radioactivity in an aliquot of the supernatant was counted by liquid scintillation to determine ^{51}Cr release. For determination of the remaining cellular ^{51}Cr, 2 N NaOH was added for 30 min at 37°C, wells were scraped with a rubber policeman, the solution was mixed, and the radioactivity in an aliquot was counted. The percent ^{51}Cr release was determined as the percentage of total disintegrations per minute in the medium divided by the total disintegrations per minute in the medium and the cell layer.

Gel filtration. Gel filtration of BAS was performed as previously described (32). BAS was concentrated approximately 10-fold by ultrafiltration with a 10,000-nominal-molecular-weight-limit membrane (Centriprep 10; Amicon, Beverly, Mass.). Concentrated BAS was applied to a Sephacryl S-200 (Pharmacia, Uppsala, Sweden) gel filtration column (1.6 by 120 cm; void volume, 72 ml) and eluted with PBS into 3-ml fractions with continuous monitoring of the UV A_{280}. Aliquots (450 μl) of alternate fractions were assayed for ^{51}Cr release activity by use of endothelial cells and RAEC as described above and for hemolytic activity as described previously (17). The void volume was determined as the elution volume for blue dextran (molecular weight, 2,000,000). The elution volume for pneumolysin was determined by chromatography of purified pneumolysin (25 μg) and assay of cytotoxic and hemolytic activities.

Oxidation and reduction of BAS. Dithiothreitol (DTT) was oxidized by incubation with hydrogen peroxide (10:1 molar ratio) for 60 min at 4°C, frozen, and lyophilized to remove residual hydrogen peroxide. As a control, reduced DTT was incubated with water, frozen, and lyophilized. BAS was pretreated with either reduced or oxidized 5 mM DTT before use in the cytotoxicity assays.

Binding of cholesterol to BAS. Cholesterol binding to BAS was accomplished essentially as described for pneumolysin (18). Cholesterol was dissolved in chloroform (100 mM) and then diluted in propylene glycol-dioxane (2:1) to a final concentration of 65 nM. BAS (500 μl) was incubated with 50 μl of dissolved cholesterol for 2 min at 4°C, separated from free cholesterol on a Sephadex G-25 desalting column, and assayed for cytolytic and hemolytic activities. As a control, BAS (500 μl) was incubated with 50 μl of propylene glycol-dioxane vehicle, eluted through Sephadex G-25, and assayed for cytolytic and hemolytic activities.

Measurement of alveolar epithelial cell permeability in isolated intact rat lungs. The alveolar epithelial cell permeability-surface area (PS) product for [^{14}C]sucrose was measured in isolated perfused rat lungs as described previously (27). In brief, lungs from Sprague-Dawley rats weighing 180 to 200 g were removed and suspended from a force transducer to measure changes in lung weight during perfusion via the pulmonary artery at a constant rate (4 ml/min) with perfusion buffer (12 mM Tris-HCl, 136.8 mM NaCl, 2.68 mM KCl, 1.25 mM $MgSO_4$, 1.82 mM $CaCl_2$, 5.55 mM glucose, 2.92 mM sucrose [pH 7.4]) warmed to 23 to 25°C. After perfusion of the lungs for 5 to 10 min, 3 ml of perfusion buffer containing 135 μCi of [^{14}C]sucrose (Dupont) and pneumolysin or control buffer was instilled into the trachea over 30 to 60 s. Lung perfusion was then maintained for 60 min while timed samples of the venous efflux were collected. The radioactivity in aliquots of the instilled fluid and of the timed samples of the venous efflux was counted by liquid scintillation. Transport of [^{14}C]sucrose between airspaces and venous efflux was calculated as the PS product. The assumptions in making this calculation and the possible sources of error have been discussed (27).

Lung morphology. The effects of pneumolysin on the ultrastructure of the blood gas barrier were assessed with several isolated perfused lung preparations. Lungs were mounted and perfused as described above. After 20 min, the airspace fluid was withdrawn and the lungs were fixed by instillation of half-strength Karnovsky's fixative (0.1 M sodium phosphate [pH 7.4], 1.25% glutaraldehyde, 1%

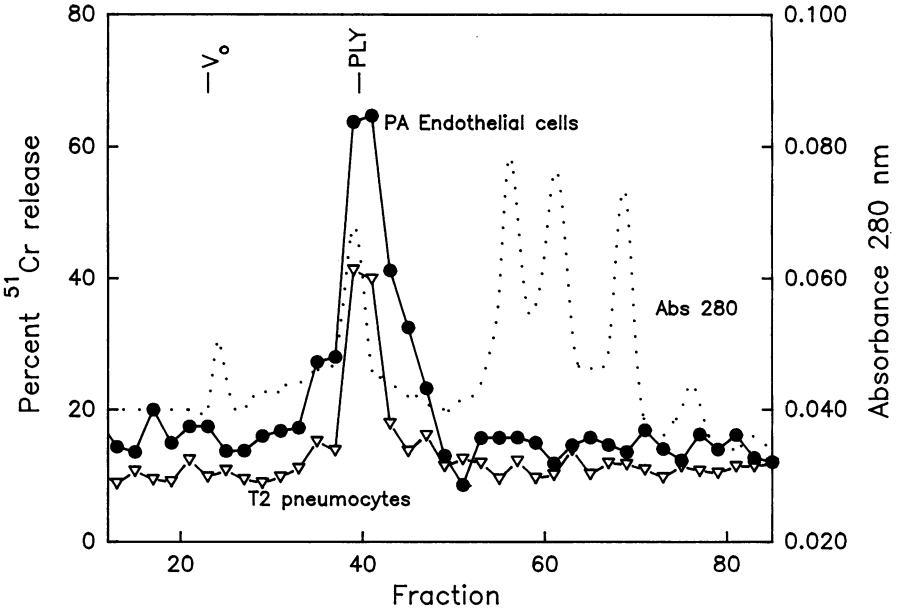

FIG. 1. Gel filtration of BAS. Type 14 *S. pneumoniae* BAS was applied to a Sephacryl S-200 column and eluted with PBS (3-ml fractions) as described in Materials and Methods. The A_{280} of the BAS fractions (dotted line) was continuously monitored with an in-line UV monitor. Aliquots (450 μl) of alternate fractions were added to incubation buffer, and the percent ^{51}Cr release from endothelial cell (●) and epithelial cell (▽) cultures after 6 h of incubation was determined as described in Materials and Methods. The void volume (V_o) and the elution volume for purified pneumolysin (PLY) were determined as described in Materials and Methods.

paraformaldehyde) into the trachea. After complete fixation, 1- to 2-mm-thick lung sections were cut from multiple sites, postfixed in aqueous 1% osmium tetroxide–1.5% potassium ferrocyanide, and embedded in 2% agar. Sections were then stained en bloc with 1% aqueous uranyl acetate, dehydrated, and embedded in Polybed 812 resin (Polysciences, Warrington, Pa.). Thin sections from several sites were stained with uranyl acetate and lead citrate and viewed by transmission electron microscopy.

Statistics. In cell culture experiments, datum points represent the means ± standard errors of the means for at least three cell culture wells. All results reported were verified by one to three repeat experiments. In experiments with intact rat lungs, datum points in figures represent the means ± standard errors of the means for three lung preparations.

RESULTS

Identification of BAS cytotoxicity for alveolar epithelial cells. For identification of soluble pneumococcal toxins for RAEC, type 14 *S. pneumoniae* BAS was fractionated by gel filtration on a Sephacryl S-200 column and samples were assayed for pulmonary artery endothelial cell and RAEC cytotoxicity (Fig. 1). RAEC were studied 72 h after isolation, when they had assumed a flattened morphology and had reached a confluent monolayer. The major peak of ^{51}Cr release activity was identical for pulmonary artery endothelial cells and RAEC, eluting in fractions 33 to 43 (Fig. 1). In addition, these same fractions showed hemolytic activity against human erythrocytes (data not shown).

Identification of pneumolysin as a major BAS toxin for RAEC. In our studies of endothelial cells (32), the major peak of cytolytic activity was identified as pneumolysin, a thiol-activated, heat-sensitive hemolytic toxin released by *S. pneumoniae* during autolysis. Our inference that the BAS RAEC cytotoxin was pneumolysin was tested in several ways. First, gel filtration of purified pneumolysin (25 μg) on the same column produced a single peak of ^{51}Cr release activity and hemolytic activity that coeluted with the major peak of BAS activity (Fig. 1). Second, BAS cytolytic and hemolytic activities were sensitive to heat, thiol activation, and cholesterol binding (Table 1). Incubation of BAS at 56°C for 30 min completely abolished hemolytic activity and significantly reduced ^{51}Cr release activity, although not to control levels. Also, incubation of BAS with the thiol-reducing agent DTT (5 mM) increased ^{51}Cr release activity by approximately 10% and hemolytic activity by approximately 160%, whereas incubation of BAS with 5 mM oxidized DTT did not increase these activities. In addition, BAS cytolytic and hemolytic activities were totally inhibited by incubation with 6.5 nM cholesterol, as is characteristic for pneumolysin and other thiol-activated toxins (18). Finally,

TABLE 1. Characterization of type 14 *S. pneumoniae* BAS cytotoxicity to RAEC

Exptl conditions	Cytotoxicity (% ^{51}Cr release, mean ± SEM)	Hemolytic activity (HU/ml)
Control	19.7 ± 1.0	0
BAS		
Alone	61.1 ± 1.1	3,000
+ Heat (56°C, 30 min)	32.8 ± 0.7	0
+ DTT (5 mM, reduced)	67.6 ± 2.3	5,000
+ DTT (5 mM, oxidized)	59.5 ± 0.8	3,000
+ Cholesterol (6.5 nM)	17.0 ± 2.8	0
+ Vehicle[a]	64.5 ± 2.5	3,000
+ Antipneumolysin (1:100)	20.9 ± 0.9	0
+ Preimmune serum (1:100)	62.1 ± 1.1	3,000

[a] Cholesterol vehicle (propylene glycol-dioxane [2:1]).

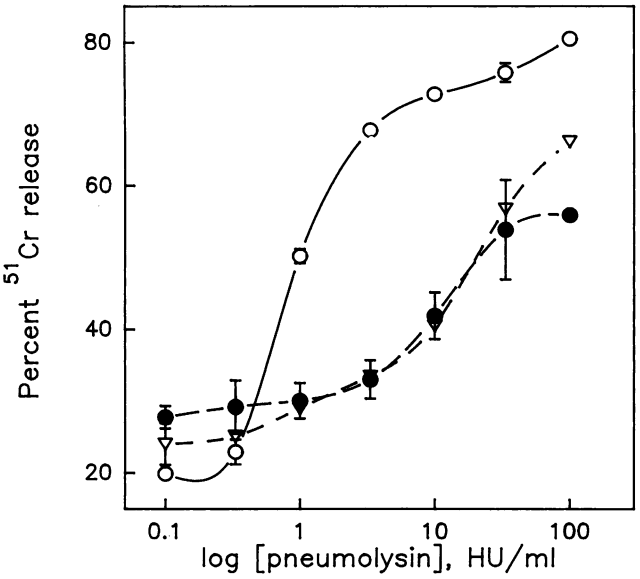

FIG. 2. Concentration dependence of pneumolysin cytotoxicity to endothelial cells and RAEC. Radiolabeled endothelial cells (○) and RAEC at either 24 h (●) or 72 h (▽) were incubated for 4 h with the indicated concentrations of purified pneumolysin, and the percent ^{51}Cr release was determined as described in Materials and Methods. The control percent ^{51}Cr release was 20 ± 0.9.

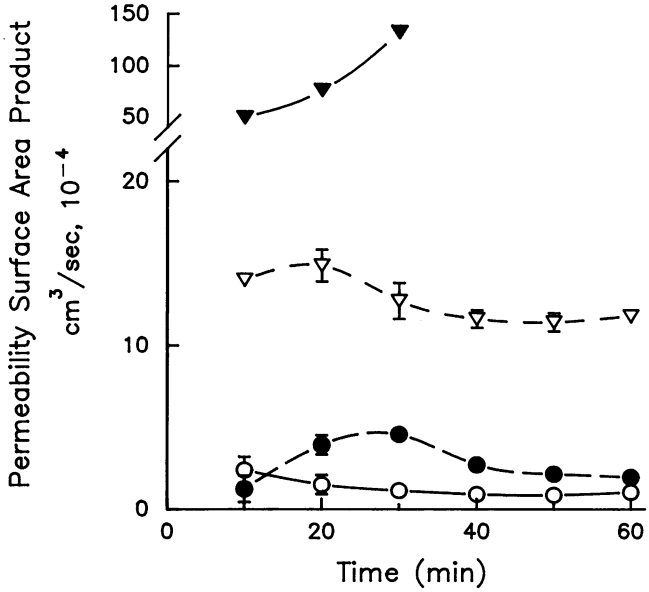

FIG. 3. Pneumolysin injury to the alveolar barrier of isolated perfused lungs. Changes in the PS product for [^{14}C]sucrose were calculated as described in Materials and Methods as a function of time after the intratracheal instillation of pneumolysin at concentrations of 0 (○), 10 (●), 100 (▽), and 1,000 (▼) HU/ml. Datum points represent the mean ± standard error of the mean (n = 3).

^{51}Cr release and hemolytic activities were specifically inhibited after preincubation of BAS with rabbit antipneumolysin antibody (1:100) for 1 h at 25°C, whereas preincubation with preimmune rabbit serum had no effect on either activity (Table 1). Taken together, these data suggest that pneumolysin is a major soluble cytotoxin for RAEC in vitro.

Comparison of pneumolysin toxicities to different epithelial cell phenotypes. Injury to type I alveolar epithelial cells is a common morphological feature of acute lung injury caused by a variety of toxins. The toxicologic responses of type I cells in vitro have not been studied because methods are not available for the isolation of these cells with sufficient purity. However, type II cells "dedifferentiate" in culture from a type II pneumocyte phenotype at 24 h after isolation to assume many of the characteristics of type I pneumocytes by 72 h, including a characteristic morphology, intercellular tight junctions, and type I cell-specific antigens (6–8, 14). Consequently, alveolar epithelial cells 72 h after isolation frequently have been studied as surrogates for type I cells.

For investigation of whether sensitivity to pneumolysin injury correlated with apparent alveolar epithelial cell phenotype, RAEC (1.5×10^6 cells per cm^2) were radiolabeled with ^{51}Cr at 8 or 56 h after isolation and used in cytotoxicity assays at 24 or 72 h after isolation. Both types of RAEC were injured by purified native pneumolysin in a concentration-dependent manner (Fig. 2). Both type I-like cells (72 h) and type II-like cells (24 h) appeared equally sensitive to pneumolysin injury. Pneumolysin caused half-maximal ^{51}Cr release from both cell types at a concentration of approximately 15 HU/ml, although maximal ^{51}Cr release was higher for 72-h RAEC. By comparison, bovine pulmonary artery endothelial cells (5×10^5 cells per cm^2) were appreciably more sensitive to pneumolysin injury, with half-maximal ^{51}Cr release occurring at a pneumolysin concentration of approximately 1 HU/ml.

Effects of pneumolysin on alveolar epithelial cell permeability and structure in isolated perfused rat lungs. The artifacts inevitably introduced by cell culturing and the uncertainty about the true phenotype of RAEC maintained in culture limit the utility of RAEC studies. In contrast, isolated perfused rat lungs provide a singular model to study the early effects of toxins that produce acute lung injury. In particular, direct pneumolysin injury to the intact alveolar epithelium within 60 min of exposure, in the absence of inflammatory cells, can be studied by physiological determinations of alveolar permeability and by ultrastructural studies with this model.

Purified pneumolysin substantially increased the alveolar epithelial cell PS product for [^{14}C]sucrose in a concentration-dependent manner. In lungs not exposed to pneumolysin, the average [^{14}C]sucrose PS product remained relatively constant at between 1×10^{-4} and 3×10^{-4} cm^3/s over the 60-min perfusion period (Fig. 3), during which time the lungs lost an average of 580 ± 90 mg in weight. When purified pneumolysin (1,000 HU/ml) was added to the alveolar fluid, the average [^{14}C]sucrose PS product increased approximately 100-fold and substantial lung weight gains were evident within the first few minutes of exposure. At this concentration of pneumolysin, the lungs could not be perfused beyond 30 min because of massive alveolar flooding. A pneumolysin concentration of 100 HU/ml caused an approximate 10-fold increase in the average [^{14}C]sucrose PS product, along with smaller lung weight gains that became evident after about 30 min of exposure. A pneumolysin concentration of 10 HU/ml appeared to represent the threshold for alveolar epithelial cell injury.

These physiological effects of pneumolysin on the alveolar epithelium were paralleled by ultrastructural changes. A 20-min exposure to purified pneumolysin (100 HU/ml)

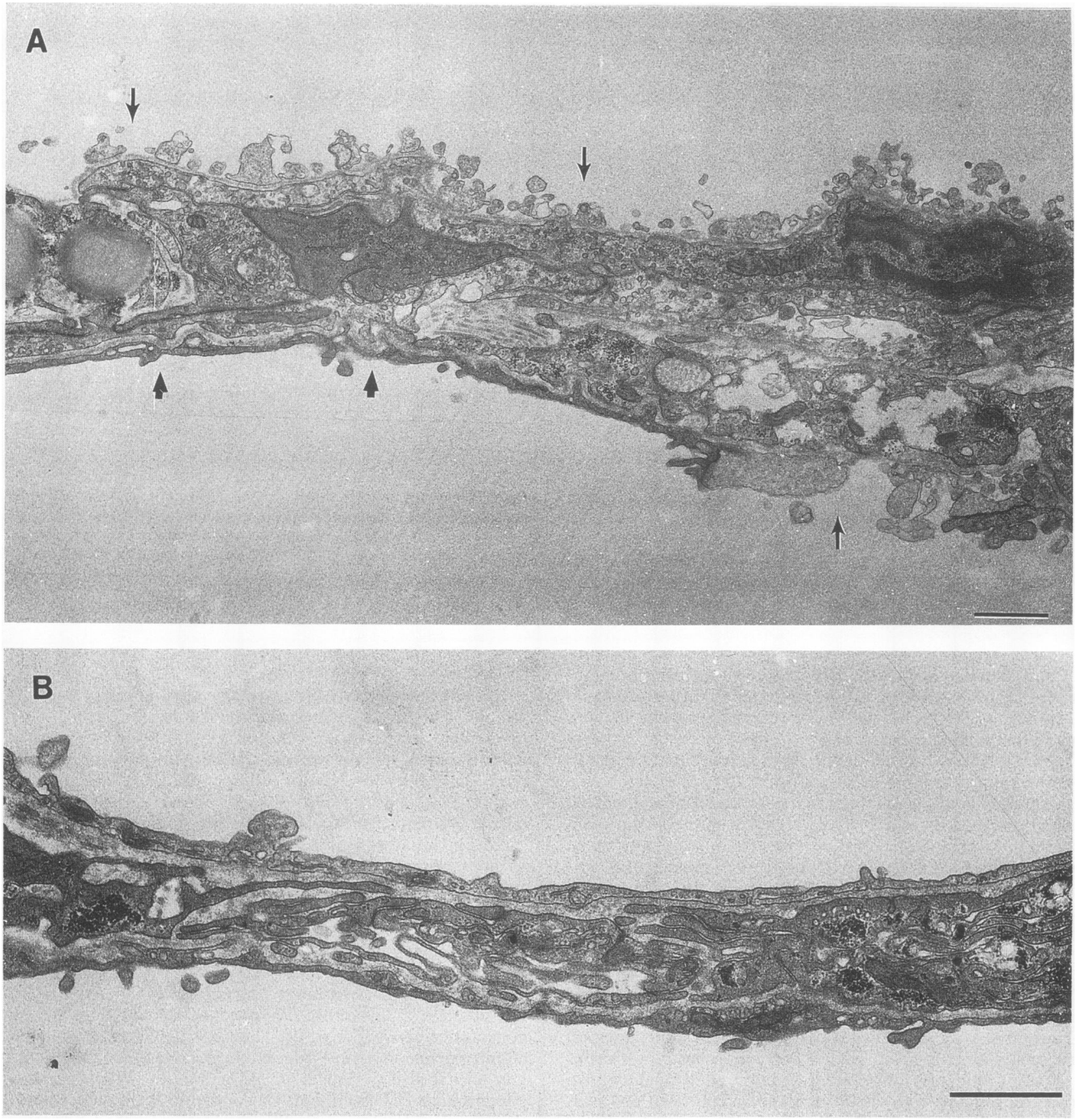

FIG. 4. Morphological evidence of pneumolysin injury to the alveolar capillary membrane. (A) Representative rat lung after the instillation of pneumolysin (100 HU/ml) into the airspace before fixation. Injury to type 1 epithelial cells was manifested by edema and severe disruption of cellular membranes (arrows). Although epithelial cell injury was diffusely seen in most alveoli, it was strikingly focal within individual alveoli, with totally disrupted epithelial cells bordered by apparently unaffected cells (arrowheads). Magnification, ×12,000. (B) Rat lung after instillation of inactivated pneumolysin. No evidence of cellular injury was detected. Magnification, ×18,000. Bars, 1 μm.

caused extensive damage to the alveolar epithelium of isolated perfused rat lungs (Fig. 4). Pneumolysin appeared to primarily injure type I alveolar epithelial cells, as manifested by edema, the appearance of cytoplasmic blebs, and the disruption of cellular membranes. Epithelial cell injury was evident in nearly every alveolus but was strikingly focal within individual alveoli, with totally disrupted epithelial cells connected by morphologically intact intercellular tight junctions to apparently unaffected cells. Although injury to other lung cells was much less pronounced, the alveolar interstitium was noted to be markedly thickened and edematous (Fig. 4). Thus, these physiological and morphological

studies of isolated perfused rat lungs demonstrate that purified native pneumolysin disrupts the alveolar epithelium, predominantly injuring type I epithelial cells.

DISCUSSION

Pneumococcal infection persists in the antibiotic era as a common and serious disease. Despite the use of appropriate antibiotics, mortality from bacteremic infections remains high, especially during the first week of infection. Polyvalent pneumococcal vaccines have been developed to prevent this early mortality in high-risk populations, but the efficacy of this approach in very young, debilitated, and immunocompromised individuals is uncertain. The failure of conventional antibiotic therapy, the unproven protection by immunization of high-risk patients, and the emergence of multiply antibiotic-resistant *S. pneumoniae* strains emphasize the need for a better understanding of the pathogenesis of pneumococcal pneumonia and bacteremia.

Pneumolysin, an oxygen-labile, intracellular 53-kDa cytolysin that is released upon autolysis, appears to be a major virulence factor in pneumococcal disease. Pneumolysin elicits an acute humoral response in humans (16, 20, 21), often with immune complex formation (23). In mice, immunization with purified pneumolysin increases survival after subsequent challenge with virulent pneumococci (28), and pneumolysin-deficient *S. pneumoniae* mutants show reduced virulence that can be restored after reconstitution of pneumolysin production (3, 4). Furthermore, these studies suggest that pneumolysin plays a role in early mortality from *S. pneumoniae* infection, as immunization with pneumolysin or infection with pneumolysin-deficient mutants postponed but did not prevent death. Pneumolysin also injures pulmonary artery endothelial cells (32) and may be an important mediator in the pathogenesis of alveolar hemorrhage during the early stages of pneumococcal pneumonia.

Pneumolysin injures human nasal and tracheobronchial ciliated epithelia (33), but its effect on the pulmonary alveolar epithelium has not been described until now. The alveolar epithelium is important as the limiting membrane for alveolar capillary water and solute transport (5, 34). By analogy with other types of surface epithelium, it may also provide a primary barrier to tissue penetration by alveolar microorganisms. Finally, alveolar epithelial cells are presumably exposed to the highest concentrations of toxins produced by intra-alveolar *S. pneumoniae*.

For these reasons, we investigated the effects of *S. pneumoniae* toxins on pulmonary alveolar epithelial cells. Incubation with type 14 *S. pneumoniae* autolysates caused substantial ^{51}Cr release from isolated RAEC. Biochemical and immunological studies identified pneumolysin as a major *S. pneumoniae* BAS cytotoxin for cultured RAEC. Furthermore, pneumolysin caused markedly increased alveolar epithelial cell permeability in isolated perfused rat lungs, apparently by predominantly injuring type I epithelial cells. The amount of pneumolysin producing appreciable epithelial cell injury (10 to 100 HU/ml) represents the lysis of approximately 10^6 to 10^7 CFU of pneumococci per ml. Although the exact alveolar concentrations of bacteria in pneumococcal pneumonia are unknown, these values are within the range that might be expected in vivo.

In addition to its direct cytotoxicity, pneumolysin may produce further lung tissue injury through its ability to induce inflammation. Purified pneumolysin has been shown to cause ocular inflammation in animals (19) and is known to activate the classic complement pathway (29). Also, purified recombinant pneumolysin instilled into rat lungs causes an inflammatory pneumonitis that appears histologically identical to that produced by injection of type 3 *S. pneumoniae* (13). Of note, the instillation of modified pneumolysins produced by site-directed mutagenesis revealed that the toxin-induced histological changes correlated with the hemolytic activity of the toxin rather than with the ability of the toxin to activate complement (13).

In summary, pneumolysin is a major soluble *S. pneumoniae* cytotoxin for the pulmonary alveolar epithelium. Direct pneumolysin injury to the alveolar epithelium, as well as to the pulmonary endothelium, may produce alveolar flooding and hemorrhage during the earliest stages of pneumococcal pneumonia. The resulting serous exudate may in turn promote the rapid multiplication of *S. pneumoniae* within the alveoli. Furthermore, by injuring the alveolar epithelium and pulmonary endothelium, pneumolysin may play a central role in facilitating the penetration of alveolar organisms into the bloodstream during pneumococcal bacteremia.

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