Pseudomonas aeruginosa-Mediated Cytotoxicity and Invasion Correlate with Distinct Genotypes at the Loci Encoding Exoenzyme S

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Pseudomonas aeruginosa, an opportunistic pathogen, is capable of establishing both chronic and acute infections in compromised hosts. Previous studies indicated that *P. aeruginosa* displays either a cytotoxic or an invasive phenotype in corneal epithelial cells. In this study, we used polarized MDCK cells for in vitro infection studies and confirmed that *P. aeruginosa* isolates can be broadly differentiated into two groups, expressing either a cytotoxic or an invasive phenotype. In vivo infection studies were performed to determine if cytotoxic and invasive strains displayed differential pathology. Invasion was assayed in vivo by in situ infection of mouse tracheal tissue followed by electron microscopy. Both cytotoxic and invasive strains entered mouse tracheal cells in situ; however, more necrosis was associated with the cytotoxic strain. In an acute lung infection model in rats, cytotoxic strains were found to damage lung epithelium more than invasive strains during the short infection period of this assay. The expression of cytotoxicity requires a functional *exsA* allele. In the strains tested, the ability to invade epithelial cells in vitro appears to be independent of *exsA* expression. Since ExsA is a transcriptional regulator of the exoenzyme S regulon, chromosomal preparations from invasive and cytotoxic strains were screened for their complement of exoenzyme S structural genes, *exoS*, encoding the 49-kDa ADP-ribosyltransferase (ExoS), and *exoT*, encoding the 53-kDa form of the enzyme (Exo53). Invasive strains possess both *exoS* and *exoT*, while cytotoxic strains appear to have lost *exoS* and retained *exoT*. These data indicate that the expression of cytotoxicity may be linked to the expression of Exo53, deletion of *exoS* and perhaps other linked loci, or expression of other ExsA-dependent virulence determinants. In the absence of a functional cytotoxicity pathway (*exsA::Ω* strains), invasion of eukaryotic cells is detectable.

Pseudomonas aeruginosa is an opportunistic gram-negative pathogen that is associated with corneal ulcer formation and infections of immunocompromised or critically ill patients, persons with cystic fibrosis, or individuals who have suffered severe burns (2). The pluripotential virulence determinants associated with successful infection as well as the range of tissues colonized and damaged by this microorganism suggest a broad genetic capacity for colonization of compromised tissue and evasion of host immune responses. We have focused on the role of exoenzyme S production in *P. aeruginosa* pathogenesis. Exoenzyme S is a member of the ADP-ribosyltransferase family of bacterial enzymes that includes many toxins (11). In both burn and acute lung infection models, exoenzyme S production correlates with the ability of *P. aeruginosa* to disseminate from epithelial colonization sites to result in systemic infection (12, 18). Damage to epithelial barriers may be a prerequisite step in the spread of *P. aeruginosa* to different tissues (1, 12).

Several in vitro models of epithelial infection have been developed to examine the role of *P. aeruginosa* virulence factors in epithelial damage. Using polarized Madin-Darby canine kidney cells (MDCK cells), Apodaca et al. demonstrated that

certain *P. aeruginosa* strains were cytotoxic (1). Cytotoxicity was dependent on live bacteria and could not be reproduced by using subcellular fractions or heat-killed bacterial cells (1). Corneal epithelial cells have served as another clinically relevant model of *P. aeruginosa* pathogenesis (3–5). Utilizing this model, Fleiszig et al. screened *P. aeruginosa* clinical isolates for their ability to invade corneal cells or induce cytotoxicity (5). Early in invasion assays (1 h), no significant difference was detected for invasion among the various *P. aeruginosa* isolates. *P. aeruginosa* strains were 50-fold more invasive than *Escherichia coli* HB101. However, at a later time of infection (3 h), *P. aeruginosa* strains could be differentiated into two groups. One group of strains was cytotoxic, demonstrated by chromium release or trypan blue dye exclusion assays. The second group was invasive, as assessed by gentamicin survival analysis. These observations confirm that *P. aeruginosa* can display an invasive phenotype and suggest that invasion is inversely correlated with cytotoxicity (5).

In this analysis, cytotoxic or invasive *P. aeruginosa* strains were analyzed for their capacity to invade polarized epithelial cells in vitro and to cause epithelial damage in vivo. The role of exoenzyme S production was analyzed by comparing the in vitro phenotypes of parental strains and *exsA* (regulatory) mutants (8). Cytotoxic and invasive strains were characterized by Southern blot analysis for their complement of exoenzyme S structural genes, *exoS* (encoding ExoS [13]) and *exoT* (encoding Exo53 [28]). Exoenzyme S production by these strains was

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also examined. The results suggest that unlike cytotoxicity, invasion may not be directly regulated by *exsA*. Cytotoxicity is linked to the expression of Exo53, the deletion of *exoS*, or the expression of other ExsA-dependent virulence determinants. Loss of the cytotoxic phenotype due to an *exsA* mutation allows detection of the invasive phenotype, suggesting that invasion and cytotoxicity may be sequential events.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Four nonmucoid corneal *P. aeruginosa* isolates were used in the invasion analysis and acute lung infection model (5). Two of the isolates, 6073 and 6206, were characterized as cytotoxic in the corneal cell assay and were serogroup O11 (5). The second pair of isolates, 6294 and 6487, were serogroup O6 and characterized as invasive (5). One cytotoxic strain, PA103 (O11), and one invasive strain, 6294, were used in the in situ tracheal infection studies. To characterize exoenzyme S expression and genotype, a larger group of *P. aeruginosa* strains was analyzed. Strains that displayed a cytotoxic phenotype with corneal cells included PA103, 6073, 6077, 6206 (O11), 19660 (O2), 6436 (O10), 6354 (O8), 6452 (O10), 6382 (O11), and 6389 (O11); invasive strains included 6294, 6487, PAO1 (O2), and PAK (O1).

For invasion and association assays, *P. aeruginosa* isolates were cultivated on Trypticase soy plates, harvested, and adjusted to a cellular density of 10^7 /ml in buffered minimal essential medium with Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, Mo.) containing 0.6% (wt/vol) bovine serum albumin and 20 mM HEPES, pH 7.4 (5). To measure epithelial damage in vivo, *P. aeruginosa* cells were maintained and grown as previously described (1). For exoenzyme S production, isolates were cultivated as previously described (7).

Association and invasion of *P. aeruginosa* on polarized MDCK cells. MDCK type II cells were maintained and passaged as previously described (1). For invasion assays, cells were cultured on 12-mm-diameter, 0.4- μ m-pore-size Transwells (Corning Costar Corp., Cambridge, Mass.) and used for experiments after 3 days of growth. The cells were inoculated with *P. aeruginosa* strains by the addition of 150 μ l of a bacterial suspension containing approximately 10^7 CFU/ml. Following a 1- or 3-h incubation at 37°C, the inoculum was removed and the cells were washed three times with minimal essential medium to remove nonassociated bacteria. Associated bacteria were measured by the addition of 0.25% Triton X-100 to lyse MDCK cells followed by a thorough mixing of the well contents and viable counts. To measure intracellular *P. aeruginosa*, gentamicin (200 μ g/ml) was added and the mixture was incubated for 2 h to kill extracellular bacteria. The antibiotic was removed by washing once with medium, Triton X-100 was added, and the cellular lysate was diluted to determine the number of surviving intracellular bacteria. At least three wells of cells were used for each strain in each experiment, and all experiments were repeated at least three times. The MICs of gentamicin for cytotoxic and invasive strains were determined. These values ranged from 1 to 25 μ g/ml, with values for all but one strain, 6073, falling in the range of 1 to 5 μ g/ml. The ability to invade cells did not correlate with gentamicin susceptibility differences. The isolate for which the MIC of gentamicin was highest, 6073 (25 μ g/ml), was phenotypically cytotoxic and not invasive. In each invasion assay, a well without eukaryotic cells was seeded with the same inoculum and exposed to 200 μ g of gentamicin per ml. Although more bacteria associated with the plastic surface than with cells, none of the isolates survived gentamicin treatment. The results are presented in the tables as the means and standard deviations of the data collected from one representative series of experiments. Statistical analysis involved the *t* test for comparison of data collected for any two strains and analysis of variance for comparison of data collected for three or more strains.

Measurement of epithelial damage in vivo. Pathogen-free Sprague-Dawley rats (250 to 350 g) were anesthetized by an intraperitoneal (i.p.) injection of 60 mg of pentobarbital sodium (Nembutal) per kg of body weight; anesthesia was maintained with i.p. injections of half this dose every 2 h. The anesthetized animals underwent tracheostomies; PE-240 polyethylene tubes were inserted and were connected to constant-volume respirators (Harvard Apparatus, South Natick, Mass.). The animals were ventilated at rates that maintained partial CO₂ pressure between 30 and 40 mm Hg, end-expiratory positive pressures of 3 cm H₂O, and an FIO₂ of 1.00. A PE-50 polyethylene tube was inserted into the carotid artery for measurement of blood pressure and for obtaining blood samples. The animals were monitored during each experiment, and all protocols for animal use were approved by the Committee for Animal Research at the University of California at San Francisco.

The animals received a protein-bacterium solution consisting of 1 ml of 5% bovine serum albumin containing 2.5×10^7 CFU of *P. aeruginosa* and 0.5 μ Ci of ¹²⁵I-albumin. The radioactive albumin was used as the alveolar tracer. The protein-bacterium solution was instilled into the lower lobe of the right lung of each of the anesthetized animals (PE-50 tube inserted through the tracheal tube during mechanical ventilation). The animals were turned prior to the instillation and placed in the right lateral decubitus position to facilitate the instillation into the right lower lung; the animals were kept in this position for 4 h, the duration of the experiment. Lung epithelial permeability (see below) was also assessed by using a vascular tracer, ¹³¹I-albumin (0.5 μ Ci), injected via the artery.

The animals were deeply anesthetized at the end of the experiments, and the abdominal aorta was transected to exsanguinate the animals. A sternotomy was then performed, and the lungs were removed en bloc. The lungs were separated; aspiration of all remaining fluid from the right lung was then accomplished. Homogenized lung samples were measured for radioactivity, wet and dry weights, and hemoglobin concentration remaining in the supernatant. Radioactivity and pleural and lung fluid protein concentrations were measured.

All experiments involved four animals in each group. The results are presented as means and standard deviations. Groups were compared by using analysis of variance with the Student-Newmann-Keuls test. A *P* value of <0.05 was accepted as significant.

Lung epithelial damage was measured by several independent tests. The flux of the alveolar tracer (¹²⁵I-albumin) into blood occurs only when the alveolar epithelium has been disrupted (12, 15). The vascular tracer (¹³¹I-albumin) enters the alveolar spaces when epithelial and/or lung endothelial injury occurs. The ratio of the concentration of the protein in the final aspirate to the concentration of the protein in the initial solution instilled is indicative of the ability of the alveolar epithelium to actively transport sodium. Normally, the alveolar epithelium can actively transport sodium; liquid passively leaves the instillate, so the protein concentration increases. When the alveolar epithelium is damaged by bacteria or bacterial products, the epithelium is unable to transport sodium and no increase in the concentration of the instilled protein is observed (12, 15).

Bacterial invasion of the trachea. Six pathogen-free BALB/c mice, 23 to 26 g, were anesthetized with pentobarbital sodium i.p. and placed in a supine position to expose the trachea. An incision was made in each trachea, and 10 μ l of a bacterial solution or Ringer's lactate was injected. A 20-gauge luer stub adapter was placed into the trachea at the sternal notch, and the adapter was secured with a ligature. The mice were ventilated with a rodent ventilator at 122 breaths per min with a peak inspiratory pressure of 6.0 cm H₂O. The mice were kept on a warming blanket, and their temperatures were monitored rectally. Anesthesia was maintained by hourly doses of pentobarbital sodium. The mock infection or bacterial infection was allowed to proceed for 4 h, after which the mice received 150 mg of pentobarbital sodium per kg, their abdominal aortas were transected, and after exsanguination, the tracheas were removed. The tracheas were fixed in 2% glutaraldehyde in phosphate buffer, postfixed in osmium tetroxide, and embedded in Epon. Necrosis was gauged by the disruption of the epithelium, loss of epithelial cells, cell swelling, and pyknotic nuclei. Adherent bacteria were seen predominantly where the tracheal epithelium had been denuded and rarely detected where the epithelium was intact. Thick sections were scanned, and thin sections were taken only from areas of intact epithelium. Thin sections were cut at approximately 80 nm, stained with lead citrate and uranyl acetate, and examined under a Zeiss 10C electron microscope. Two mice were mock infected with Ringer's lactate, two mice were infected with the invasive *P. aeruginosa* isolate 6294, and two mice were infected with the cytotoxic isolate PA103.

Characterization of exoenzyme S production and genotypic analysis. Exoenzyme S production was qualitatively assessed by Western blot (immunoblot) analysis of culture supernatants from each *P. aeruginosa* strain grown under inducing conditions as previously described (7). The supernatants were harvested after 14 h of growth and concentrated 20-fold by the addition of a saturated solution of ammonium sulfate to a final concentration of 55%. Three microliters of the concentrated supernatant was loaded per lane of an 11% polyacrylamide-sodium dodecyl sulfate (SDS) gel. Duplicate gels were either stained with Coomassie blue R-250 or subjected to Western blot analysis using rabbit antiserum that recognizes both the 49- and the 53-kDa forms of exoenzyme S (7, 16). Affinity-purified horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim Biochemicals) and 4-chloro-1-naphthol (Sigma) were used to detect antibodies to exoenzyme S (16).

The complement of exoenzyme S genes was determined by Southern blot analysis (25). Two probes for *exoS*, which encodes the 49-kDa protein, or ExoS, were used in a series of Southern blots. One probe was a 404-bp internal *EagI* fragment (13). To confirm our findings, a second probe corresponding to the entire structural gene (an *NsiI*-*BamHI* 1,431-bp fragment) was used. The probe for *exoT*, which encodes the 53-kDa protein, or Exo53, was a 1,327-bp *EcoRV* fragment (28). Chromosomal DNA was isolated from each strain, subjected to restriction endonuclease digestion with *BamHI* (no internal sites in either gene), *EagI* (internal sites in both genes), or *EcoRV* (internal sites in both genes), and transferred to Magnagraph nylon supports, as previously described (8). The probes were labeled with [³²P]dCTP (3,000 Ci/mmol; New England Nuclear Research Products, Wilmington, Del.) by using a random-primed DNA labeling kit (Bethesda Research Laboratories, Bethesda, Md.).

RESULTS

Association with and invasion of polarized MDCK cells by cytotoxic and invasive strains of *P. aeruginosa*. In previous experiments, different corneal isolates of *P. aeruginosa* displayed either a cytotoxic or an invasive phenotype when assayed on corneal epithelial cells (5). Polarized MDCK cells have been used as a model for *P. aeruginosa*-mediated cytotoxicity (1). To determine whether *P. aeruginosa* could express

TABLE 1. Association with and invasion of MDCK cells by cytotoxic and invasive corneal isolates of *P. aeruginosa*

Strain	Association (mean no. of cells $\pm$ SD)		Invasion (mean no. of cells $\pm$ SD)		% Invasion ^a	
	1 h	3 h	1 h	3 h	1 h	3 h
Noncytotoxic						
6294	$(5.6 \pm 0.5) \times 10^4$	$(7.7 \pm 0.3) \times 10^4$	$(2.0 \pm 0.4) \times 10^3$	$(2.4 \pm 0.4) \times 10^4$	3.50	31.00
6487	$(4.9 \pm 0.4) \times 10^4$	$(8.2 \pm 0.5) \times 10^4$	$(1.1 \pm 0.1) \times 10^3$	$(7.0 \pm 0.4) \times 10^3$	2.20	8.50
Cytotoxic						
6206	$(7.1 \pm 1.1) \times 10^4$	$(4.1 \pm 0.2) \times 10^5$	$(1.3 \pm 0.4) \times 10^3$	530 $\pm$ 41	1.80	0.13
6073	$(9.4 \pm 1.9) \times 10^4$	$(8.0 \pm 0.4) \times 10^5$	$(1.1 \pm 0.1) \times 10^3$	590 $\pm$ 90	1.10	0.07

^a Defined as the percentage of associated bacteria that survives gentamicin treatment.

an invasive phenotype on polarized MDCK cells, bacterial suspensions were added to cells grown on Transwell filters, and bacterial association with and invasion of cells were measured. Two cytotoxic strains (6206 and 6073) and two invasive strains (6294 and 6487) were analyzed at two time points (Table 1). At the early time point (1 h), there was no significant difference between cytotoxic and invasive strains in their ability to associate with or invade cells. Low levels of invasion were detected for both cytotoxic and invasive isolates at 1 h. At 3 h, the percentage of associated bacteria that had invaded increased approximately 4- to 9-fold for the invasive isolates but decreased 13- to 15-fold for the cytotoxic isolates. In addition, the total number of associated bacteria for the cytotoxic isolates increased six- to eightfold, while there was little to no increase for invasive isolates. These results parallel earlier studies of corneal cells in that cytotoxic strains demonstrated a reduced ability to invade at 3 h whereas invasive strains showed the opposite pattern (5). Our results indicate that both cytotoxic and invasive *P. aeruginosa* strains were able to enter polarized MDCK cells at early time points. Expression of the cytotoxic phenotype at later times may mask early invasion events by allowing gentamicin access to the intracellular environment and killing of exposed bacteria.

Expression of the exoenzyme S regulon and MDCK cell invasion. MDCK cell cytotoxicity was previously correlated with the expression of the exoenzyme S regulon by *P. aeruginosa* (1). With this assay, mutation of *exsA*, a transcriptional activator required for exoenzyme S production, resulted in a noncytotoxic phenotype. PA103 and PA103 *exsA*:: Ω were analyzed for their capacity to invade MDCK cells (Table 2). At 1 h with an inoculum of 10^7 CFU/ml (as in Table 1), low numbers of both strains associated with MDCK cells, but invasion was not detectable. By 3 h, association by the parental cytotoxic strain, PA103, increased over 2,000-fold, yet invasion was not detectable. Association of PA103 *exsA*:: Ω increased approximately 20-fold, and invasive bacteria were detected after 3 h. When the inoculum was increased to 10^8 CFU/ml, the cytotoxic parental strain demonstrated a reduced capacity to invade relative to that of the *exsA*-deficient derivative. These

results suggested that the ability to invade may be independent of or negatively regulated by ExsA.

An *exsA* mutation does not affect the invasive phenotype. In MDCK cell studies, *P. aeruginosa* PAO1 demonstrated a relatively noncytotoxic phenotype (1). Invasion assays were subsequently performed with corneal cells, in which PAO1 expressed an invasive phenotype (5). The *exsA* mutation was introduced into strain PAO1 (8), and the effect on invasion was measured in the MDCK cell assay. As shown in Table 3, PAO1 *exsA*:: Ω appeared to have increased association and invasion relative to those of the parent strain, PAO1; however, the percent invasion remained constant. Thus, the overall invasion characteristics of a noncytotoxic strain appeared to be unaltered by an *exsA* mutation.

In vivo evaluation of cytotoxic and invasive *P. aeruginosa* strains. Cytotoxic (6206 and 6073) or invasive (6487 and 6294) *P. aeruginosa* strains were tested in an acute lung infection model developed in rabbits (12) and adapted to rats for this study. Figure 1 shows the results of calculating the ratio of the total albumin concentrations in the aspirate and in the instillate. The two invasive strains, 6487 and 6294, showed an increase in the aspirate/instillate total protein ratio, indicating an alveolar epithelium capable of active transport. In contrast, the two cytotoxic strains, 6206 and 6073, had aspirate/instillate ratios of total albumin concentrations of 1 or less. For comparison, PA103, a cytotoxic strain in this model, had a ratio (mean $\pm$ standard deviation) of 1.00 ± 0.22 ($n = 10$). In animals receiving albumin solutions not containing bacteria, albumin concentrates to the same degree as measured in the animals that received the invasive strains (1.5 ± 0.18 ; $n = 4$). These results suggest that the invasive bacteria did not impair alveolar function, while the cytotoxic strains injured the alveolar epithelium and impaired the ability of the epithelium to actively transport sodium.

The influx of the vascular radioactive protein tracer (^{131} I-albumin) entering the airspaces of the lung was also examined (data not shown). We noted a trend for larger quantities (approximately twofold) of the vascular protein tracer to enter the airspaces of the lungs of animals receiving the cytotoxic strains,

TABLE 2. Effect of an *exsA* mutation on the phenotype of *P. aeruginosa* PA103 on polarized MDCK cells

Strain	Association ^a			Invasion			% Invasion ^b		
	1 h, 10^7 CFU/ml	3 h		1 h, 10^7 CFU/ml	3 h		1 h, 10^7 CFU/ml	3 h	
		10^7 CFU/ml	10^8 CFU/ml		10^7 CFU/ml	10^8 CFU/ml		10^7 CFU/ml	10^8 CFU/ml
PA103	470 $\pm$ 60	$(9.5 \pm 0.3) \times 10^5$	$(2.1 \pm 0.4) \times 10^6$	0	0	$(1.6 \pm 0.1) \times 10^3$	0	0	0.08
PA103 <i>exsA</i> :: Ω	480 $\pm$ 90	$(9.3 \pm 0.5) \times 10^3$	$(3.8 \pm 0.3) \times 10^5$	0	980 $\pm$ 240	$(4.7 \pm 0.4) \times 10^4$	0	11.00	12.50

^a Expressed as mean numbers of cells $\pm$ standard deviations at the indicated incubation times and with the indicated inoculum sizes.

^b Defined as the percentage of associated bacteria that survives gentamicin treatment.

TABLE 3. Effect of an *exsA* mutation of *P. aeruginosa* PAO1 on MDCK cell invasion^a

Strain	Association (mean no. of cells $\pm$ SD) ^b	Invasion (mean no. of cells $\pm$ SD) ^c	% Invasion ^d
PAO1	$(4.5 \pm 0.8) \times 10^4$	$(3.3 \pm 0.2) \times 10^3$	7.3
PAO1 <i>exsA</i> :: Ω	$(8.5 \pm 0.2) \times 10^4$	$(6.2 \pm 0.6) \times 10^3$	7.3

^a An inoculum size of 10^7 CFU/ml was used, and the cultures were incubated for 3 h.

^b There is a significant increase in association when PAO1 *exsA*:: Ω is compared to PAO1 (*t* test, $P = 0.008$).

^c There is a significant increase in invasion when PAO1 *exsA*:: Ω is compared to PAO1 (*t* test, $P = 0.009$).

^d No difference was demonstrated when the percentages of associated bacteria that invade were calculated. Percent invasion is defined as the percentage of associated bacteria that survives gentamicin treatment.

6206 and 6073, than for animals receiving the invasive isolates, 6487 and 6294. The ^{125} I-albumin ratios measured for the invasive strains, 6487 and 6294, were similar to ratios measured when protein alone (without a bacterial inoculum) was instilled into the airspaces (0.31 ± 0.4 [mean $\pm$ standard deviation]).

Figure 2 depicts the percentage of the alveolar tracer (^{125} I-albumin) leaving the lung airspaces and entering the circulation after 4 h. The level of ^{125} I-albumin in the blood of animals infected with the invasive strains was equal to the value obtained when the instillate contained only labeled protein ($1.26\% \pm 1.0\%$). In contrast, the values obtained for animals infected with the cytotoxic strains were significantly elevated, suggesting that alveolar epithelial injury had occurred. For comparison, infection with strain PA103 produced a level of $13.01\% \pm 4.4\%$ ^{125} I-albumin in the blood. Together, these data suggest that cytotoxic strains damaged the alveolar epithelium during the 4-h experiment. Invasive strains did not injure the epithelium during this interval.

Bacterial invasion of the trachea. Electron microscopic analysis of the alveolar epithelium of mice and rats infected

FIG. 1. Ratio of albumin concentration measured in the final aspirate to that in the instilled solution. The invasive strains, 6487 and 6294, are associated with ratios of albumin concentration of >1 . This suggests that the bacteria did not damage the alveolar epithelium's ability to actively transport sodium. In contrast, the cytotoxic isolates, 6206 and 6073, caused a reduction in this ratio in every experiment. This result suggests that cytotoxic bacteria injured the alveolar epithelium's ability to actively transport sodium. There is a significant difference in the ratios when cytotoxic and invasive strains are compared (*, $P < 0.05$). C, results of control experiments using animals instilled with a protein solution without bacteria.

FIG. 2. Percentages of alveolar tracer, ^{125} I-albumin, that entered the circulation over the 4-h experimental period. The invasive strains, 6487 and 6294, did not appear to injure the alveolar epithelium, as the percentage of the alveolar tracer measured in the circulation in these experiments was similar to the percentage of the alveolar tracer measured in experiments where a protein solution without bacteria had been instilled. In contrast, the instillation of the cytotoxic strains, 6206 and 6073, caused a significant percentage of the alveolar tracer to enter the circulation (*, $P < 0.05$).

with invasive and cytotoxic *P. aeruginosa* isolates was performed. The narrowwidth of the alveolar epithelium, however, precluded differentiation of intracellular bacteria from bacteria that had penetrated between epithelial cells (data not shown). Results from this study and prior studies indicate that *P. aeruginosa* interacts with corneal (5) and kidney (1) epithelial tissues in similar manners. To determine if *P. aeruginosa* invaded epithelial tissue in vivo, an in situ tracheal infection was developed. Epithelial tissue of the trachea is amenable to histologic examination utilizing electron microscopic techniques that are sensitive enough to detect bacterial invasion. Mouse tracheas were exposed for 4 h to 10^7 CFU of an invasive strain (6294) or a cytotoxic strain (PA103) or to no bacteria (Ringer's lactate). Sections were made through bacterial foci of infected tracheal epithelium. Tracheas exposed to Ringer's lactate had no foci of infection. Figure 3A is an electron micrograph of the tracheal epithelium and the matrix immediately below the epithelium postinfection with strain PA103. Bacteria were localized in the matrix of the cells close to the basal region (Fig. 3A). Epithelial cells containing PA103 and adjacent cells appeared damaged (Fig. 3A and B). In some sections, tracheal epithelium was found to be sloughed off (data not shown). Figure 3C shows a section of tracheal epithelium infected with the invasive strain 6294. Less necrosis was noted in these sections and overall damage to the tracheal epithelium was less severe when strain 6294 was used for the infection. The results of these experiments indicate that cytotoxic and invasive *P. aeruginosa* isolates are found within epithelial cells soon after in vivo infection.

Expression of exoenzyme S in cytotoxic and invasive strains.

In previous studies, a series of *P. aeruginosa* corneal isolates was screened for expression of either a cytotoxic (PA103, 6073, 6077, 6206, 19660, 6436, 6354, 6452, 6382, and 6389) or an invasive (6294, 6487, PAK, and PAO1) phenotype (5). *P. aeruginosa* cells were grown under inducing conditions for exoenzyme S production, and supernatants were harvested, concentrated 20-fold, and subjected to SDS-polyacrylamide gel

FIG. 3. Electron micrographs of infected mouse tracheal tissue. (A) Thin section of mouse trachea infected with the cytotoxic strain PA103 (magnification, $\times 10,125$). Note the presence of cilia at the apical surface. Necrosis was noted in tracheal samples infected with strain PA103. The presence of a bacterium at the basal region of the cell is indicated (arrow). (B) A different area of the same trachea shown in panel A. Two PA103 bacteria (arrows) are located within the cytoplasm, free of eukaryotic membrane material (magnification, $\times 25,500$). (C) Invasive strain 6294 was applied to the trachea in the same manner as for panel A. After the 4-h incubation period, bacteria were found intracellularly (arrows). Less necrosis was associated with the application of the 6294 strain than with PA103 (magnification, $\times 25,500$).

electrophoresis and Western blot analysis. The top panel of Fig. 4 shows a Coomassie blue-stained SDS-polyacrylamide gel of concentrated extracellular proteins. The protein patterns tend to be variable; however, strains showing the highest levels of cytotoxicity appeared to express several unique bands in the 60- to 70-kDa region of the gel. A relatively large abundance of a 70-kDa extracellular protein appears in the lanes corresponding to strains PA103, 6073, 6077, 6206, 19660, and 6436. In previous studies of strain PA103, the expression of the 70-kDa protein was shown to be coordinately regulated with exoenzyme S production and to be dependent on a functional *exxA* allele (8).

The bottom panel of Fig. 4 is a Western blot of the extracellular proteins reacted with antiserum that recognizes the 53- and 49-kDa forms of exoenzyme S. The results of this analysis indicated that all strains produced the 53-kDa form of exoenzyme S. Except for strain PAK, the strains that produced a large amount of the 70-kDa protein (PA103, 6073, 6077, 6206, 19660, and 6436) also appeared to be the greatest producers of the 53-kDa form of exoenzyme S. A cytotoxic strain, 6452, and two invasive strains, 6294 and 6487, produced a small but detectable amount of the 53-kDa protein. Unexpectedly, only the invasive strains (6294, 6487, PAK, and PAO1) produced the 49-kDa form of exoenzyme S. Immunoreactive products that migrate faster than the 49-kDa band are breakdown products of exoenzyme S.

Southern blot analysis of chromosomal DNA from cytotoxic and invasive strains. Chromosomal DNA was isolated from each strain, digested with *Bam*HI, *Eag*I, or *Eco*RV, and analyzed by agarose gel electrophoresis and Southern blotting (8, 25). DNA fragments corresponding to *exoS*, encoding the 49-kDa form of exoenzyme S, and *exoT*, encoding the 53-kDa

FIG. 4. Coomassie blue-stained SDS-polyacrylamide gel analysis of concentrated extracellular proteins from *P. aeruginosa* strains grown for exoenzyme S production (top) and Western blot analysis of a duplicate gel probed with antibodies that recognize exoenzyme S (bottom). Supernatants were harvested and concentrated as described in Materials and Methods. Each lane represents an individual strain (as indicated between the panels). The 11% polyacrylamide gel (top) was stained with Coomassie blue R-250. The Western blot was reacted with antiserum that recognizes both forms of exoenzyme S and then with a peroxidase-labeled secondary antibody. Secondary antibody binding was detected by using 4-chloro-1-naphthol as a peroxidase substrate. The migration of the 53- and 49-kDa exoenzyme S proteins (left of each panel) and molecular mass markers shown in kilodaltons (right of each panel) are indicated.

FIG. 5. Southern blot analysis of *P. aeruginosa* chromosomes and hybridization with *exoS* or *exoT* probes. Chromosomal DNAs were isolated from various strains and digested with *Bam*HI. Fragments were separated on a 0.8% agarose gel, transferred to Magnagraph nylon paper, and probed with labeled restriction fragments corresponding to *exoS* (top) or *exoT* (bottom). *Hind*III-treated lambda DNA served as a series of molecular size markers. These fragments were detected by labeling phage DNA by the random priming method and subsequent hybridization. Molecular sizes in kilobases (right of each panel) and the migration of the chromosomal *Bam*HI fragment containing the *exoS* or *exoT* gene (left of each panel) are indicated.

protein, were used as probes in the top and bottom panels, respectively, of Fig. 5. Because *exoS* and *exoT* are approximately 80% identical at the nucleotide sequence level, cross-hybridization between the two chromosomal fragments containing each gene is detectable even under high-stringency conditions (28). For clarity, only the *Bam*HI digest is shown in Fig. 5. All 14 strains examined contained the DNA corresponding to *exoT* (a *Bam*HI fragment that comigrates with the 23-kb marker). However, only the invasive strains contained a copy of *exoS* (a 2.3-kb *Bam*HI fragment). Longer exposures did not reveal other bands hybridizing with *exoS* or *exoT* sequences. Southern blot analysis utilizing restriction endonucleases that digest at internal sites within each gene, *Eco*RV and *Eag*I, demonstrated the same pattern of the loss of *exoS* in the cytotoxic isolates. Our results suggest that cytotoxicity may be related to the deletion of *exoS* or other linked loci.

DISCUSSION

P. aeruginosa has been suitably described as an opportunistic pathogen expressing a wide array of virulence determinants (17). How different products work together or individually at each stage of infection and in the context of the whole bacterium is a difficult problem that we have approached using tissue culture and animal models and examining different strains (1, 3–5). These studies confirm previous observations with corneal cells that *P. aeruginosa* can follow either an invasive or a cytotoxic infection pathway (5). All strains tested with MDCK cells had variable capacities to invade at an early time point; however, at later time points, invasion was detected only with *P. aeruginosa* strains that lacked the ability to produce cytotoxic factors. The inverse relationship between cytotoxicity and invasion as proposed by Fleiszig et al. (5) was directly tested in this study by analyzing invasion in strains that lacked a key component of the MDCK cell cytotoxic pathway, ExsA

(1). ExsA is a transcriptional activator protein that is required for the synthesis and secretion of both forms of exoenzyme S as well as other extracellular proteins (8, 10, 27). The absence of cytotoxicity of *exsA* strains suggested that either exoenzyme S or other coordinately regulated or secreted proteins were responsible for MDCK cell death (1). If cytotoxicity and invasion are inversely correlated, elimination of the cytotoxicity pathway should allow detection of invasion. An *exsA* mutation introduced into an invasive strain may have no effect if invasion and cytotoxicity are independently regulated. Conversely, if ExsA negatively regulates invasion, an increase in invasion of an *exsA* strain may be detectable. The experiments were performed with PA103 (cytotoxic) and PAO1 (invasive) and their *exsA*:: Ω derivatives. The *exsA* mutation rendered PA103 invasive in the MDCK epithelial cell model and did not alter the invasion characteristics of PAO1. These results favor the hypothesis that *P. aeruginosa* invasion and cytotoxicity are independently regulated. This interpretation, however, must be tempered since the wild-type strains PAO1 and PA103 are not isogenic and differ in many other characteristics.

As in previous analyses using corneal cells, cytotoxic strains in this study appeared to be invasive for polarized MDCK cells at early time points. It is unclear whether invasion or association of bacteria with eukaryotic target cells is required for cytotoxicity to occur. One possibility is that upon entry into the host cell, genes involved in cytotoxicity are induced. By 3 h, cell death may be caused by the accumulation of bacterial products within the cell cytoplasm. Alternatively, direct association of the bacterium with the eukaryotic membrane (polarized transfer) as opposed to bacterial uptake may be required for the expression of cytotoxicity. Elegant studies demonstrating the polarized transfer of two *Yersinia* cytotoxins, YopE and YopH, into eukaryotic cells have been performed (19, 24, 26). Polarized transfer of YopE and YopH requires the participation of a type III export pathway (24, 26). Several gene products critical for exoenzyme S synthesis and secretion are homologous to *Yersinia* proteins involved in production of the Yops virulence determinants (10, 27). Recent studies indicate that exoenzyme S is exported from *P. aeruginosa* by a type III secretion mechanism (29). These data suggest that the intoxication mechanism mediating epithelial cell cytotoxicity by exoenzyme S and/or perhaps other coordinately regulated extracellular proteins may closely resemble that of the yersiniae.

In previous studies, the ability to adhere to cells was postulated to be an important step for expression of the cytotoxic phenotype, as PA103 appeared to adhere to MDCK cells better than the noncytotoxic mutant, PA103 *exsA*:: Ω (1). Results presented in this study indicate that at early time points, differences in adherence between PA103 and PA103 *exsA*:: Ω were not detectable. Differences in adherence were noted at 3 h, after cytotoxicity had occurred with the parental strain. Among *P. aeruginosa* strains isolated from corneal infections, little difference was measured for adherence at 1 h. More association with cytotoxic strains than noncytotoxic strains by 3 h was measured (5). The general trend for an increase over time in adherence by cytotoxic strains may be explained by the predilection of *P. aeruginosa* to bind to injured tissue. This property has been observed in other studies where injury to the cornea (6, 9, 22) or trachea (21, 23) is required to initiate infection. It is unclear why PAO1 demonstrates the opposite pattern of an increase in association when the cytotoxicity pathway is rendered nonfunctional (as by an *exsA* mutation). Perhaps in strain PAO1 *exsA*:: Ω there is a slight enhancement in the production of cell-damaging factors that are not coordinately regulated with the exoenzyme S regulon, leading to the observed increase in association relative to that of PAO1.

Cytotoxic and invasive *P. aeruginosa* isolates were tested for their ability to damage lung epithelium in an acute infection model in rats and were assayed for in vivo invasion of tracheal epithelial cells. Data from both models suggest that cytotoxic strains induced higher levels of epithelial damage and necrosis than invasive strains early in infection. Thus, the potential to produce cytotoxins at early time points may directly correlate with the ability to breach the epithelium and spread to other tissues, particularly the pleural fluids and bloodstream. In situ infection of tracheal epithelium indicated that cytotoxic and invasive isolates could invade cells early in infection. Bacteria appeared free in the cytoplasm without evidence of eukaryotic membrane borders. These data do not address the viability or replication of intracellular bacteria but do raise the possibility that *P. aeruginosa* may be able to evade the immune response by invading epithelial cells. In chronic *P. aeruginosa* infections such as those in cystic fibrosis patients, noncytotoxic variants may arise after in vivo selection for less virulent, mucoid strains. Noncytotoxic variants may persist because of their ability to invade cells. Invasive or noncytotoxic variants may also contribute to detrimental inflammatory responses of infected cystic fibrosis patients. Fleiszig et al. demonstrated that corneal infections with an invasive strain (6294) resulted in inflammation of the underlying stroma, which attracted polymorphonuclear leukocytes to this layer (5). Although selection for noncytotoxic variants that invade epithelial cells in cystic fibrosis patients is an attractive model, Pier et al. have shown that epithelial cells with a defective CFTR gene show defects in the uptake of *P. aeruginosa*. These authors postulate that the defect in bacterial uptake may foster extracellular microcolony formation (20), leading to chronic infection.

Exoenzyme S production has been correlated with the ability of *P. aeruginosa* to spread from epithelial colonization sites to the bloodstream (18). Since these early studies, the structural gene encoding each form of exoenzyme S has been cloned and sequenced, and the respective recombinant proteins have been produced (13, 28). The eukaryotic target specificities for the two enzymes appear to be the same (14, 28). The major detected difference between the two forms of exoenzyme S is the rate of catalysis (14). The 53-kDa protein possesses approximately 0.2% of the ADP-ribosyltransferase activity of the 49-kDa enzyme (28). Thus, if ADP-ribosyltransferase activity correlated with epithelial damage, one would expect that production of the 49-kDa form of the enzyme would be correlated with cytotoxicity. We, however, found exactly the opposite pattern in that cytotoxic strains possessed *exoT* only and appeared to have lost *exoS*. The expression pattern paralleled the genetic analysis in that deletion of *exoS* resulted in the lack of expression of the 49-kDa form of exoenzyme S. Several models may account for these observations. The deletion of *exoS* may eliminate other genetic information that represses cytotoxicity. Alternatively, ExoS may compete with other, more potent cytotoxins for the secretion or intoxication apparatus of *P. aeruginosa*, resulting in a negative correlation between the production of ExoS and cytotoxicity. On the other hand, production of ExoS may enhance invasion by subverting host cell signal transduction through the covalent modification of host receptor or regulatory proteins. Because *P. aeruginosa* may produce several extracellular proteins that are coordinately regulated by ExsA, it is difficult to pinpoint the role of either form of exoenzyme S in cytotoxicity or invasion until the appropriate mutant strains are constructed.

These studies confirm that *P. aeruginosa* invades several types of epithelial cells in vitro and in vivo. The ability to invade is inversely correlated with cytotoxicity (5). The cytotoxic pathway can be eliminated by introducing mutations in

the transcriptional activator of the exoenzyme S regulon, ExsA. Strains with defects in the cytotoxic pathway display an invasive phenotype. The control of the invasion pathway appears to be independent of ExsA expression in the strains tested in this analysis. In contrast, cytotoxicity depends on a functional *exoA* allele and appears to be correlated with either expression of *exoT* and/or other coordinately regulated genes of the exoenzyme S regulon or deletion of *exoS*.

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