Persistent Infection with *Pseudomonas aeruginosa* in Ventilator-associated Pneumonia

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***Rationale*: *Pseudomonas aeruginosa* is one of the leading causes of gram-negative ventilator-associated pneumonia (VAP) associated with a mortality rate of 34 to 68%. Recent evidence suggests that *P. aeruginosa* in patients with VAP may persist in the alveolar space despite adequate antimicrobial therapy. We hypothesized that failure to eradicate *P. aeruginosa* from the lung is linked to type III secretory system (TTSS) isolates.**

***Objectives*: To determine the mechanism by which infection with *P. aeruginosa* in patients with VAP may evade the host immune response.**

***Methods*: Thirty-four patients with *P. aeruginosa* VAP underwent noninvasive bronchoalveolar lavage (BAL) at the onset of VAP and on Day 8 after initiation of antibiotic therapy. Isolated pathogens were analyzed for secretion of type III cytotoxins. Neutrophil apoptosis in BAL fluid was quantified by assessment of nuclear morphology on Giemsa-stained cytocentrifuge preparations. Neutrophil elastase was assessed by immunoenzymatic assay.**

***Measurements and Main Results*: Twenty-five out of the 34 patients with VAP secreted at least one of type III proteins. There was a significant difference in apoptotic rate of neutrophils at VAP onset between those strains that secreted cytotoxins and those that did not. Neutrophil elastase levels were positively correlated with the rate of apoptosis ($r = 0.43$, $P < 0.01$). Despite adequate antimicrobial therapy, 13 out of 25 TTSS$^+$ isolates were recovered at Day 8 post-VAP, whereas eradication was achieved in all patients who had undetectable levels of type III secretion proteins.**

***Conclusions:* The increased apoptosis in neutrophils by the TTSS$^+$ isolates may explain the delay in eradication of *Pseudomonas* strains in patients with VAP. Short-course antimicrobial therapy may not be adequate in clearing the infection with a TTSS secretory phenotype.**

Keywords: ventilator-associated pneumonia; *Pseudomonas aeruginosa*; antimicrobial therapy; outcome

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Ventilator-associated pneumonia due to *Pseudomonas aeruginosa* has been associated with high rates of relapse despite adequate antimicrobial therapy.

What This Study Adds to the Field

Failure to eradicate *P. aeruginosa* in ventilator-associated pneumonia is linked to a type III secretory system, which is implicated in apoptosis of alveolar neutrophils.

Pseudomonas aeruginosa is a leading cause of nosocomial infections and is responsible for 10% of all hospital-acquired infections (1, 2). It is the most common antibiotic-resistant pathogen causing VAP (3), and the most common cause of fatal episodes of VAP (4). Unlike many other causes of VAP, *Pseudomonas* is consistently associated with a measurable attributable mortality (5, 6). Infections caused by *P. aeruginosa* are often severe and life threatening and are difficult to treat because of the limited susceptibility to antimicrobial agents and the high frequency of an emergence of antibiotic resistance during therapy (7), thus resulting in severe adverse outcomes.

Among its large arsenal of virulence factors, *P. aeruginosa* encodes a type III secretion system (TTSS) (8), which has attracted significant attention in recent years. Upon cell contact, the needlelike type III secretion machinery is deployed, allowing bacteria to inject toxins directly into the cytoplasm of the host cell (9). To date, four TTSS effector molecules have been described. Exoenzyme U (ExoU) is a necrotizing toxin with phospholipase activity (10) that leads to rapid lysis of mammalian cells (11–13). ExoY is an adenylate cyclase that increases intracellular levels of cAMP (14). The other two known effectors are ExoS and ExoT, highly homologous to each other, having a carboxy-terminal ADP-ribosyltransferase (ADPRT) domain and an amino-terminal GTPase-activating (GAP) domain (15, 16). The amino-terminal GAP activity acts on Rho family GTPases, whereas the carboxy-terminal ADPRT activity is directed toward Ras and other host cell proteins (17–19). As a result of these enzymatic activities, intoxication with ExoS is associated with several observable phenotypes, including cytotoxicity and inhibition of bacterial internalization by both phagocytic and nonphagocytic mammalian cells (12, 20).

Recently, we have demonstrated that *P. aeruginosa* could be isolated from the alveolar space 8 days from onset of VAP despite adequate antimicrobial therapy (21). Because the presence of a functional type III secretion system has been associated with a poor outcome in patients with VAP (22), we hypothesized that persistent alveolar infection with *Pseudomonas* VAP is correlated with the expression of TTSS phenotype. The objective of this investigation was to determine the mechanism by which infection with *Pseudomonas* infection in patients with VAP may evade the host immune response. Therefore, the aims of this study were to analyze the interaction between *P. aeruginosa* isolates and alveolar neutrophils *in vivo* and to evaluate the cytotoxic contribution of the type III secretion system on polymorphonuclear leukocyte (neutrophil) viability *in vitro*.

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METHODS

Study Population

The study protocol was reviewed and approved by the institutional review board of the State University of New York at Buffalo. Written, informed consent was obtained from all subjects or their legal representatives. Only patients with first episode of *P. aeruginosa* VAP defined as bacterial growth of 10^4 colony forming units (cfu)/ml or more from bronchoalveolar lavage fluid (BALF) were enrolled. Exclusion criteria included polymicrobial infection and discordant antimicrobial therapy.

Data Collection

Clinical data recorded on study enrollment included age, sex, reasons for mechanical ventilation, duration of mechanical ventilation before study onset, prior antibiotic therapy, temperature, leukocyte count, ratio of Pa_{O_2}/Fi_{O_2}, time to first antibiotic dose from VAP onset, the Acute Physiology and Chronic Health Evaluation II score (23), and the Multiple Organ Dysfunction Score (MODS) (24).

Collection and Processing of Respiratory Specimens

BAL was obtained at time of suspected VAP onset before antimicrobial therapy and was repeated on Day 8 after completion of antibiotic therapy. One-half the collected samples were sent for microbiology processing, and the rest was filtered through two layers of sterile gauze and centrifuged at 500 × *g* for 10 minutes at 4°C to separate the supernatants from the cell pellet. BAL fluid total protein levels were measured by a modified Lowry assay (25).

To assess the clonal distribution of *P. aeruginosa* isolates, DNA typing was conducted on all samples obtained at VAP onset using repetitive-element–based polymerase chain reaction (26). Sample relationships were designated as follows: indistinguishable, no band differences; similar, one band difference; and different, two or more band differences.

Analysis of Type III Secretory Protein Phenotype

P. aeruginosa isolates were cultured under TTSS-inducing conditions in minimal media supplemented with nitrotriacetic acid and lacking calcium medium (27). Cultures were incubated with shaking overnight at 37°C before dilution to optical density, 600 (OD_{600}) of 0.1 in fresh minimal media supplemented with nitrotriacetic acid and lacking calcium medium and cultured for a further 5 hours. Bacterial cells were harvested by centrifugation and the supernatant removed. Cell-free supernatant from each sample was concentrated using Centricon tubes (10 kD; MWCO, Millipore, MA). The concentration of protein in all preparations was determined by the Biorad Dc protein quantification kit (Bio-Rad Laboratories, Hercules, CA). Standardized protein concentrations (20 μg) were loaded onto 12.5% Tris polyacrylamide gels (Bio-Rad Laboratories) and run under denaturing conditions. Polyacrylamide gels were transferred to polyvinylidene fluoride membrane and immunoblotted with anti-PcrV, anti-ExoS or anti-ExoU as previously described (28).

Cytotoxicity Assay

PMNs were collected from whole blood obtained by venipuncture from healthy volunteers and purified by density gradient centrifugation. PMNs were washed twice and resuspended to 10^7/ml in modified HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid)-buffered saline. The viability of PMNs, which was determined by trypan blue staining, was more than 95%.

Each of the clinical isolates was cultured overnight in Luria-Bertani medium. Bacterial cells were pelleted and washed three times in sterile phospate-buffered saline (PBS), diluted to an OD_{600} of 0.1, and regrown in Luria-Bertani medium for a further 1.5 hours. After this, cultures were harvested, washed with lactated Ringer's solution and finally resuspended in 100 μl Ringer's:PBS solution (2:1 ratio by volume). The viability of PMNs in the presence of bacterial isolates was assessed by coincubation of samples containing 5×10^6 cfu/ml of *P. aeruginosa* and 5×10^6 PMNs/ml. Cytotoxicity was assayed 2 hours after infection by lactate dehydrogenase release using the Cyto Tox96 kit (Promega, San Luis Obispo, CA) according to the manufacturer's instructions.

Neutrophil Apoptosis

Neutrophil apoptosis was assessed by light microscopy (×200) analysis of cytospin cells stained with Wright's Giemsa method and by identification of nuclear changes (condensation of chromatin and simplification of nuclear structure) characteristic of apoptosis (29, 30). Two blinded investigators assessed the percentage of neutrophil apoptosis on cytospin preparations by analyzing 500 cells per slide each. The analysis was performed on two different slides from the same patient. Data were reported as the percentage of apoptotic cells. To validate the light microscopic method of assessment of neutrophil apoptosis, we used a second independent method based on annexin V binding with quantification by flow cytometry (31). The extent of neutrophil apoptosis was compared with the percentage of neutrophil apoptosis determined by nuclear morphology and light microscopy (linear regression slope, 0.79; $P = 0.03$, n = 6).

Neutrophil elastase in BAL fluid was measured in duplicate by a commercial immunoenzymatic assay kit (PMN Elastase EIA; Alpco Diagnostics, Windham, NH).

Statistical Analysis

Continuous variables were compared using unpaired Student's *t* test or the Mann-Whitney *U* test if the variables were not normally distributed. Categorical variables were compared using χ^2 test with Yates correction or Fisher's exact test when necessary. Correlations were analyzed with Spearman's rank correlation. Parametric data are presented as mean ± SD and nonparametric data as medians with 95% confidence intervals or ranges. A *P* value of less than 0.05 was determined as significant. Calculations were performed using SPPS 12.0 (SPSS, Inc., Chicago, IL).

RESULTS

Study Population

Thirty-four patients with *P. aeruginosa* VAP were enrolled between May 2003 and July 2006. Exacerbation of chronic obstructive pulmonary disease (21%) and severe community-

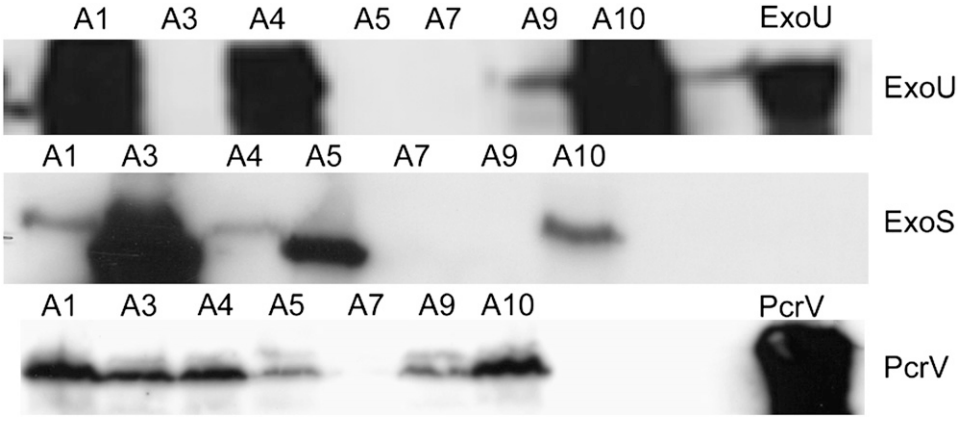

Figure 1. Immunoblot analysis of exoenzyme U (ExoU), ExoS, and PcrV secretion from a subset of the *Pseudomonas aeruginosa* clinical isolates examined.

acquired pneumonia (35%) accounted for the majority of the admission diagnoses followed by cerebrovascular accidents (24%) and abdominal pathology (9%). All participants received a total of 7 days of antibiotics therapy. The average time to first antibiotic dose from onset of VAP was 6.6 ± 3.2 hours (range, 3–16 h). The regimens consisted of a combination of vancomycin plus cefipime (n = 7), vancomycin plus piperacillin/tazobactam (n = 19), or vancomycin plus imipenem/cilastatin (n = 8). None of our patients has received an aminoglycoside as a single agent for the therapy of VAP. All isolates were analyzed in blinded fashion to the presence of a type III secretion system. Twenty-five patients had *Pseudomonas* strains that were capable of secreting detectable concentrations of at least one of the type III proteins assayed (PcrV, ExoU, and ExoS) (Figure 1). PcrV was secreted by the majority of strains (23/25) that exhibited either ExoU or ExoS cytotoxin secretion. ExoU secretion phenotype was detected in 12 isolates and ExoS in 11. Two isolates secreted PcrV alone without detectable ExoS or ExoU secretion. Of the 25 type III secretory *Pseudomonas* isolates, there were two distinct pairs considered as indistinguishable and three strains considered as similar. The 18 remainder isolates were distinct (Figure 2). Of interest, susceptibility profile showed fluoroquinolone resistance in 14 out of the 25 isolates, 11 of which (79%) were characterized as ExoU, PcrV secreters. Resistance to imipinem-cilastatin was observed in 6 out of the 25 isolates followed by cefipime (n = 3) and piperacillin/tazobactam (n = 2).

Characteristics of Type III Secretory Phenotypes

Table 1 summarizes the clinical characteristics of patients with *Pseudomonas*-positive type III secretory phenotypes and those with *Pseudomonas*-negative type III secretory phenotypes. There were no significant differences between the two groups in terms of age, sex, burden of comorbidities, severity of hypoxemia, use of prior antibiotics, time to first dose of antibiotics, or severity of illness. Only the duration of mechanical ventilation before VAP onset was significantly longer in those patients who harbored isolates capable of type III cytotoxin secretion.

Upon examining the characteristics of the BAL fluid, the mean quantitative bacterial colony counts and the percentage of neutrophils were comparable between both groups (Table 2). However, the degree of neutrophilic apoptosis was increased significantly in patients with *Pseudomonas*-positive type III secretory phenotypes compared with *Pseudomonas*-negative type III secretory phenotypes. There was also a significant difference in the levels of neutrophils elastase levels between the two groups. Moreover, the degree of neutrophilic apoptosis was positively correlated with BAL elastase levels ($r = 0.49$, $P = 0.003$; Figure 3).

Cytotoxicity of Type III Secretory Phenotypes

All type III secretory phenotype strains exhibited significant *in vitro* cytotoxicity against neutrophils (Figure 4). The highest toxicity was detected in isolates that cosecreted ExoU and PcrV, followed in decreasing order by strains that secreted both ExoS and PcrV, and PcrV alone. Of the phenotype-negative isolates, cytotoxicity was present albeit minimal compared with $TTSS^+$ strains ($P < 0.001$).

Clinical Implications of Type III Secretory Phenotypes

A repeat BAL at Day 8 of VAP onset revealed the persistence of *P. aeruginosa* colonies in the alveolar space in 13 (52%) out of the 25 patients with type III secretory phenotypes. The bacterial burden ranged from 2×10^1 to 6×10^2 cfu/ml. In comparison, none of the patients with negative type III secretory phenotype had *Pseudomonas* isolates recovered from BAL culture. Analysis of the secretory pattern of these isolates revealed that nine had the ExoU/PcrV and four had the ExoS/PcrV phenotypes. Furthermore, BAL assays revealed higher levels of neutrophil elastase in the alveolar space, which corresponded to an advanced degree of *in vitro* neutrophil cytotoxicity in those patients with persistent *Pseudomonas* isolates compared with their counterparts who had cleared the bacterium (Figure 5).

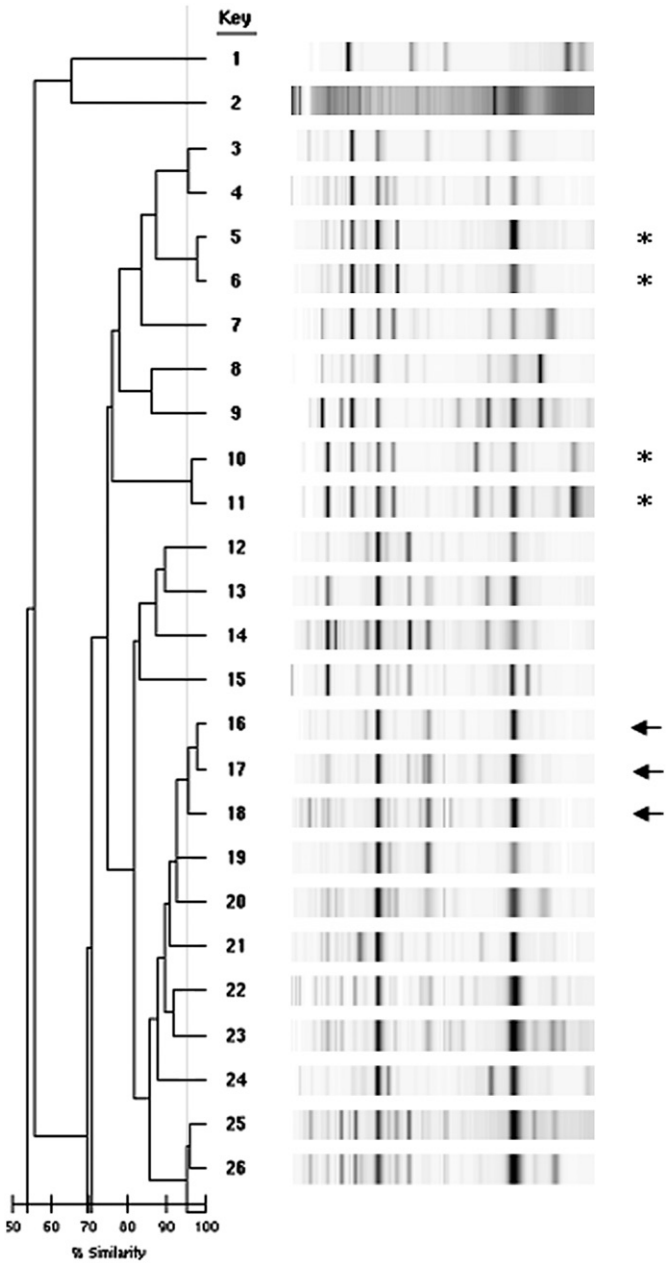

Figure 2. DNA genotyping of *Pseudomonas aeruginosa* isolates by repetitive-element–based polymerase chain reaction assay. Row no. 26 is a replica of no. 25, which was used as a quality control. Sample relationships were designated as follows: *indistinguishable, no band differences; *arrow*, similar, one band difference, and different, two or more band differences.

Outcome

After VAP treatment, relapse occurred in 8 of the 13 patients who had persistent *Pseudomonas* in the alveolar space at end of

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY POPULATION AT THE TIME OF VENTILATOR-ASSOCIATED PNEUMONIA ONSET

	Type III Secretory Phenotype (+) ($n = 25$)	Type III Secretory Phenotype (−) ($n = 9$)	*P* Value
Age, yr, mean ± SD	64.2 ± 12.8	63.7 ± 11.5	0.90
Sex, no. male/female	18/7	5/4	0.42
Charlson index, median (range)	3 (1–6)	3 (1–6)	0.48
Prior antibiotics	14 (56%)	3 (33%)	0.44
Pa_{O_2}/FI_{O_2}, mean ± SD	229.1 ± 98.6	234.1 ± 73.0	0.88
Bilateral infiltrate	16 (64%)	4 (45%)	0.43
Days of MV before VAP, mean ± SD	15.6 ± 4.7	12.2 ± 2.7	0.02
Bacteremia	3 (12%)	0	0.55
APACHE II score, mean ± SD	25.7 ± 5.6	21.6 ± 4.9	0.07
MODS, median (range)	3 (2–8)	3 (1–5)	0.21
Time to first dose of antibiotics from VAP onset, h, mean ± SD	6.7 ± 3.2	6.3 ± 3.0	0.77

Definition of abbreviations: APACHE II = Acute Physiology and Chronic Health Evaluation II; MODS = Multiple Organ Dysfunction Score; MV = mechanical ventilation; VAP = ventilator-associated pneumonia.

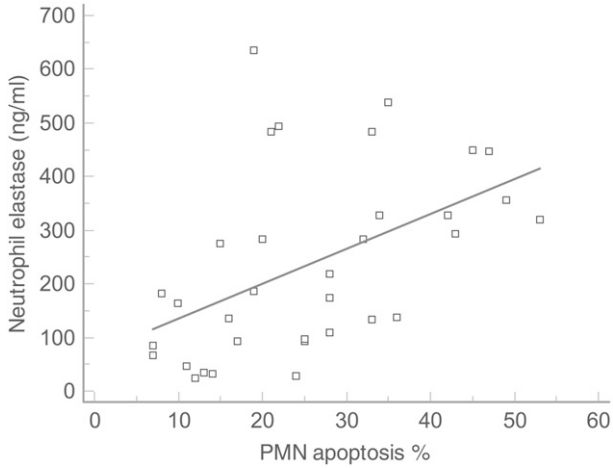

Figure 3. Scatterplot between the rate of polymorphonuclear neutrophil (PMN) apoptosis and bronchoalveolar neutrophil elastase ($r = 0.49$, $P = 0.003$).

therapy. The relapse was attributed to the same *Pseudomonas* strain in 7 of the 8 cases. Overall crude mortality was 59% (95% confidence interval [CI], 41 to 75%). Multiorgan failure was the immediate cause of death in 80% of cases. The survival rate for patients with nonsecretory type III phenotype was 66% (6 of 9) (95% CI, 30 to 93%) compared with 32% (8 of 25) for those with type III secretory phenotype isolates (95% CI, 15 to 54%). Patients exposed to prior antibiotics had no worse outcome that those who did not.

DISCUSSION

The results of the present study show that (*1*) VAP due to *P. aeruginosa* type III secretory phenotypes was associated with increased neutrophilic apoptosis *in vivo* and increased release of neutrophil elastase, (*2*) *in vitro* neutrophil cytotoxicity correlated significantly with the *Pseudomonas* ExoU/PcrV and ExoS/PcrV phenotypes, and (*3*) positive type III secretory phenotypes were associated with persistent alveolar infection and higher rate of relapse despite 7 days of adequate antimicrobial therapy.

Our analysis demonstrated that 71% of *Pseudomonas* isolates from patients with VAP were capable of secreting type III effector proteins. In contrast to patients with cystic fibrosis in whom the rate of type III secreting isolates ranged between 12 and 27.5% (32, 33), our observations agree with those of other investigators who noted that 77 to 91% of patients with *Pseudomonas* VAP harbor *Pseudomonas* strains that secrete TTSS proteins (22, 34). Although we have not examined the TTSS genetic constructs of these isolates, the phenotypes of the TTSS secretory isolates were predominantly either ExoU/PcrV or ExoS/PcrV. None of our isolates expressed both effector proteins. This mutually exclusive relationship between these two proteins has been documented previously (22, 34), the significance of which remains unclear.

Several *in vitro* and *ex vivo* investigations have studied the interaction between *P. aeruginosa* and eukaryotic cells using different infection models (35–37). These studies have made it possible to elucidate the mechanisms by which the type III secretion system enhances bacterial pathogenicity despite the host immune response. After a bacterial infection, successful eradication of bacterial infections depends on increased neutrophil survival (38). Once the invading pathogen has been neutralized, neutrophil apoptosis is initiated as an integral part of the normal resolution of the inflammatory response in the lung. Our study suggests that neutrophil apoptosis might be induced prematurely by *P. aeruginosa* isolates that secrete type III secretion proteins. In contrast, early neutrophil apoptosis did not occur in the presence of *Pseudomonas* strains that did not secrete type III proteins. Killing neutrophils may thus represent an important strategy for pathogen survival. Indeed, previous reports have shown cytotoxic $TTSS^+$ *Pseudomonas* strains and isogenic noncytotoxic strains were equally well ingested by neutrophils and macrophages, but only the cytotoxic TTSS strains were able to escape the bactericidal activity by inducing oncosis of the host cells (39). *In vitro* experiments point to the fact that both ExoS and ExoU are primary virulence factors in acute *P. aeruginosa* infection (13, 14, 36, 37). The enzymatic activity of ExoS induces cytoskeletal alterations that have numerous deleterious effects on

TABLE 2. CHARACTERISTICS OF BRONCHOALVEOLAR LAVAGE OF STUDY POPULATION

	Type III Secretory Phenotype (+) ($n = 25$)	Type III Secretory Phenotype (−) ($n = 9$)	*P* Value
BAL colony count, $\times 10^5$ cfu/ml	11.8 ± 4.9	12.6 ± 5.8	0.78
BAL, % PMNs	85.7 ± 7.4	83.9 ± 7.5	0.53
Neutrophil elastase, ng/ml	293.2 ± 161.8	78.8 ± 54.6	<0.001
Neutrophil apoptosis, %	29.1 ± 12.4	16.0 ± 9.4	0.004

Definition of abbreviations: BAL = bronchoalveolar lavage; PMNs = neutrophils.

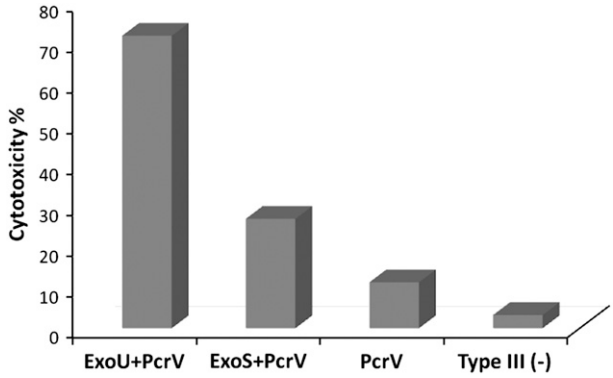

Figure 4. Cytotoxicity of *Pseudomonas aeruginosa* isolates toward human neutrophils. ExoS = exoenzyme S; ExoU = exoenzyme U.

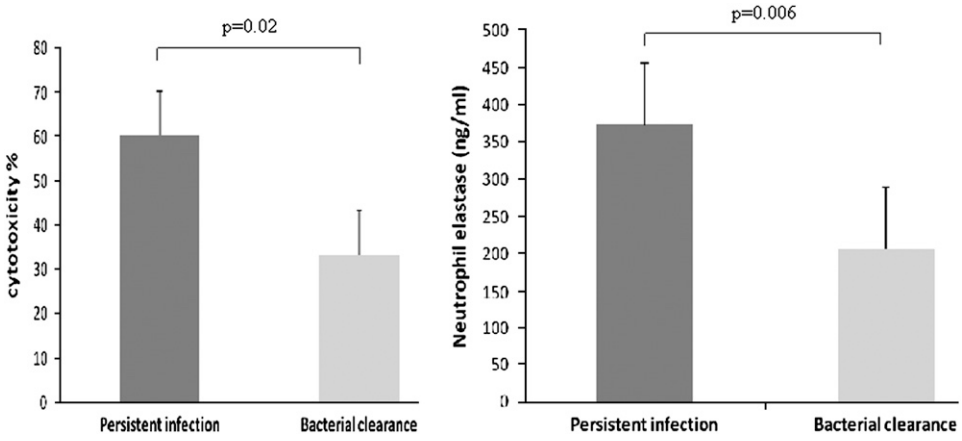

Figure 5. Comparison of *in vitro* polymorphonuclear neutrophil (PMN) cytotoxicity between patients with persistent alveolar *Pseudomonas* infection and those with bacterial clearance (*left*). Comparison of bronchoalveolar lavage neutrophil elastase between patients with persistent alveolar *Pseudomonas* infection and those with bacterial clearance (*right*).

host cells, including altered DNA synthesis and decreased cellular adherence and viability (40, 41). ExoU, through its potent phospholipase A2 activity, induces cell death across several cell lines including neutrophils, epithelial cells, and macrophages (39, 42). ExoU also has recently been shown to inhibit caspase-1–driven proinflammatory cytokine production, thereby circumventing innate immune responses (43). We should indicate that there are likely other virulence products that help *P. aeruginosa* persist in the lungs of our patients. *P. aeruginosa* pigment, pyocyanin, has been implicated in apoptosis of human peripheral blood neutrophils via generation of reactive oxygen species and lowering of cAMP (44). Whether these two virulence systems, TTSS and pyocyanin, act in concert or operate independently of each other deserves further investigation.

Our results indicate that there were higher concentrations of neutrophil elastase in BAL fluid recovered from patients with $TTSS^+$ *P. aeruginosa* isolates than in the BAL fluid from patients with $TTSS^-$ isolates. The correlation between the degree of apoptosis and BAL neutrophil elastase suggests an uncontrolled release of toxic mediators by the neutrophils in the alveolar milieu in an attempt to control the invading pathogens. Despite the increased concentrations of elastase, the $TTSS^+$ strains were the strains that persisted on Day 8 post-therapy. These results suggest that the type III secretion proteins may successfully decrease host immune responses (43). Similar results were documented by the observation that isolates of *P. aeruginosa* were intact and viable even at extremely high neutrophil elastase concentrations (45).

Failure to sterilize the alveolar space despite adequate antimicrobial therapy has been reported previously (6, 21). In a retrospective study of VAP due to *P. aeruginosa*, Crouch Brewer and colleagues (6) reported persistent *Pseudomonas* infection after repeated BAL in 31% of all enrollees. After excluding patients who had received discordant antimicrobial therapy, half of those patients had BAL cultures still growing *P. aeruginosa*. Our results extend these findings by identifying a trait that would likely predict failure to cure *Pseudomonas* pneumonia in patients requiring mechanical ventilation. The latest American Thoracic Society guidelines for the management of VAP recommended shortening the duration of therapy for 7 days for patients with VAP provided that the etiologic agent was not *P. aeruginosa* (46). This recommendation of shorter duration of therapy might hold true for patients with $TTSS^-$ isolates of *Pseudomonas* but not for the $TTSS^+$ strains. In the absence of an approved diagnostic test for type III secretory proteins, therapy for *Pseudomonas* VAP should be extended for more than 7 days, although the duration and the results of such therapy need to be examined further in light of our findings.

The mortality rate of 59% from *P. aeruginosa* is comparable to that reported 10 to 20 years ago (6, 47) despite improved intensive care unit care and the availability of more potent antimicrobial therapy. Even in the most recent studies of patients with *P. aeruginosa* pneumonia, $TTSS^+$ strains were associated with poor clinical outcomes (22, 28). This unacceptably high mortality rate of *Pseudomonas* VAP warrants changes to the current approach to therapy. Innovative antibiotic regimens and longer duration should be considered, but adjuvant therapy might prove to be more efficacious in shifting the balance between the virulence of the infecting pathogens and the immune response of the host toward eradication of these microbes. Among these interventions, passive immunization against components of the type III secretory system could tilt this balance. In a mouse model of *P. aeruginosa* pneumonia, intravenous administration of polyclonal antibodies against PcrV (a protein involved in translocation of type III–secreted toxin) resulted in survival of all animals (48). Complementary studies in a rabbit model of *P. aeruginosa*–induced septic shock associated with lung injury showed that treatment with anti–PcrV IgG significantly reduced lung injury, bacteremia, and plasma tumor necrosis factor-α levels compared with animals treated with control IgG, as well as improved hemodynamic parameters (49). Other treatments would include directed vaccination against one or more of the TTSS components. Although this intervention would not eliminate *P. aeruginosa* VAP, it might decrease morbidity and/or mortality associated with this disease.

The current study is limited by the small number of patients recruited from a single institution, potentially limiting its generalizability. However, the poor prognosis of patients with *Pseudomonas* type III secretory phenotype observed in our study parallels that reported in other clinical investigations (5, 6). We have focused on two phenotypic expressions of the TTSS, ExoU/PcrV and ExoS/PcrV, which are considered the most cytotoxic of the effector proteins. The contribution of the other effector proteins (ExoT or ExoY), whether expressed alone or in conjunction with ExoU or ExoS, remains unknown. We should indicate that we have based our definition of relapse on phenotypic profile of *Pseudomonas* isolates. Although pulsed field gel electrophoresis remains the "gold standard" to assess *Pseudomonas* typing, Rello and colleagues (50) have shown previously using pulsed field gel electrophoresis that most recurrent episodes of *P. aeruginosa* pneumonia in ventilated patients occur due to persistence of strains present in a prior infection. Therefore, we consider our findings of recurrent pneumonia to be a relapse rather than reinfection.

In conclusion, *Pseudomonas* strains may differ markedly in their ability to cause severe infections. The secretion of type III secretion proteins appears to be an effective apoptosis-inducing agent for *P. aeruginosa*, allowing the bacteria to persist in the lungs of susceptible hosts. The current practice of identifying bac-

terial genus or species might be insufficient to characterize the disease potential, the duration of treatment, and the prognostic implications of this bacterium.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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