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# Role of *Pseudomonas aeruginosa* Pili in Acute Pulmonary Infection

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**The role of piliation in the development and course of acute pulmonary infection was examined using infant BALB/cByJ mice inoculated by intranasal instillation of isogenic Pil$^+$ and Pil$^-$ mutants of *Pseudomonas aeruginosa* PA1244, PAK, and PAO1. The piliated strains caused more cases of pneumonia, bacteremia, and mortality than the nonpiliated strains (chi-square analysis, $\alpha = 0.001$). The piliated strains were more often associated with severe diffuse pneumonias, while the nonpiliated organisms resulted in less severe, focal pneumonias, although these differences did not achieve statistical significance. Purified pilin protein used to inoculate the mice resulted in local inflammatory changes. The nonpiliated strain PA1244-NP was as virulent as the piliated strain PAO1, suggesting that expression of other virulence factors are also important in the development of acute pneumonia. This infant mouse model of pulmonary infection appears to be a useful system for the analysis of *P. aeruginosa* virulence factors involved in the pathogenesis of pneumonia.**

*Pseudomonas aeruginosa* is a common opportunistic pathogen associated with a variety of infections including hospital-acquired pneumonia and the chronic lung disease characteristic of cystic fibrosis (CF). The presumed initial step in the pathogenesis of these respiratory infections is the aspiration of environmental strains of bacteria. The organisms are thought to attach to the upper respiratory tract initially and then gain access to the lung parenchyma due to the failure of normal mucociliary clearance mechanisms.

Woods et al. and Doig et al. first demonstrated that piliated strains of *P. aeruginosa* had greater adherence to buccal epithelial cells (21) and that the loss of mucosal fibronectin exposed increased numbers of binding sites for gram-negative organisms, particularly *P. aeruginosa* (4). More recent studies have established that the carboxy terminus of pilin interacts with epithelial receptors (8) and that a GalNAcβ1-4Gal moiety available in asialylated glycolipids acts as a receptor for *P. aeruginosa* pilin (14). This receptor appears to be present in increased amounts on the surface of cells from patients with CF (12) and may contribute to their predisposition to *Pseudomonas* infection. Although *P. aeruginosa* expresses nonpilus adhesins, their role in pathogenesis is less well defined (15).

The expression of the many *P. aeruginosa* virulence factors is highly regulated, often by environmental conditions. It is likely that specific virulence factors are expressed during the initial stages of infection in response to the abrupt change in the milieu when environmental strains of bacteria come in contact with a mucosal surface. These gene products may not be expressed during the chronic stage of infection which eventually characterizes *P. aeruginosa* infections in CF patients. A number of animal models have been developed specifically to look at the unusual interactions of chronic *P. aeruginosa* infection and the CF lung, including those described by Cash et al. (1), Woods et al. (20), and Pier et al. (10). However, fewer data are available characterizing the factors of *P. aeruginosa* associated with initial infection of the respiratory tract.

To study the virulence genes involved early in the development of lung infection, we adapted a neonatal mouse model of acute pulmonary infection originally used to study *Bordetella pertussis* virulence (19). Adult mice in this model system are quite resistant to even high concentrations of *P. aeruginosa*; however, neonates are susceptible and thus provide an animal model in which to identify specific virulence factors associated with acute infection. Although a mouse with CF would be a more appropriate model in which to study the acute process of *P. aeruginosa* pulmonary infection, limitations in the life span and absence of pulmonary findings equivalent to those in humans with the disease restrict the current use of these animals (16).

In the studies presented we confirmed the role of the pilus as an important, but not essential, virulence factor involved in the pathogenesis of acute pulmonary infection and demonstrated that pilin elicits an inflammatory response in the murine lung. Strain-dependent differences in the expression of other virulence factors are demonstrated, suggesting that this model system may be useful for defining genes important in the acquisition of *P. aeruginosa* pneumonia.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The piliated strains and isogenic Pil$^-$ mutants are described in Table 1. PAO-NP was constructed by a gene replacement technique, using the same vector as described by Saiman et al. (11). All strains were tested for pili by phage susceptibility prior to animal infection. Western blot (immunoblot) analyses of phage-resistant organisms using antipilin antibody on cell lysate preparations was done to document the absence of pili in phage-resistant strains.

All strains were grown in M9 minimal medium (9) at 37°C overnight until late-log-phase growth as measured by optical density at 590 nm ($OD_{590} = 0.480$). The organisms were washed twice with sterile phosphate-buffered saline (PBS) (pH 7.4). Aliquots of 0.5 ml were centrifuged (Sorvall, SS34 rotor, 5,000 rpm), and the pellet was resuspended into 10 μl of PBS for inoculation. Samples were also serially diluted and plated to ensure constant concentrations of $1 \times 10^8$ to $3 \times 10^8$ CFU/ml.

**Purification of pilin.** Pilin protein was isolated by the method of Frost and Paranchych (6). Briefly, *Pseudomonas* strain PAO-DB2, a hyperpiliated mutant of PAO1, was grown up overnight on large sheets of M9 agar. Cells were scraped off and placed into a 1× solution of standard saline citrate (SSC) (0.15 M NaCl and 0.15 M sodium citrate adjusted to pH 7 with NaOH)–15% sucrose mixture and filtered through a fine-mesh sieve to remove agar. This solution was stirred overnight at 4°C, and pilin was sheared off in a Waring blender pulsed on lowest speed. The mixture was then centrifuged to remove debris (Sorvall, SS34 rotor, 8,000 rpm for 20 min). The supernatant was dialyzed against sterile distilled water at 4°C with frequent changes of dialysate for 16 h. The pilin protein was

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TABLE 1. Characteristics of *P. aeruginosa* strains

| Strain | Phenotype | Source (reference) |
|---|---|---|
| PA1244 | WT,[a] $Pil^+$ $F116^r$ $PO_4^s$ | J. Sadoff (2) |
| PA1244-NP | $Pil^-$ $F116^r$ $PO_4^r$ | S. Lory (8) |
| PAK | WT, $Pil^+$ $F116^s$ $PO_4^s$ | S. Lory (8) |
| PAK-NP | $Pil^-$ $F116^r$ $PO_4^r$ | L. Saiman (13) |
| PAO1 | WT, $Pil^+$ $F116^s$ $PO_4^r$ | M. Vasil (7) |
| PAO-NP | $Pil^-$ $F116^r$ $PO_4^r$ | This study |

[a] WT, wild type.

precipitated with 50% ammonium sulfate, and the resulting pellet was dissolved in 1× SSC. This mixture was again precipitated with 20% ammonium sulfate, resuspended in 1× SSC–15% sucrose solution, loaded over a discontinuous sucrose gradient, and spun for 20 h at 4°C (Beckman ultracentrifuge L7-65, SW27 rotor). The pilin band was removed, dialyzed in distilled water, and purified further on a CsCl gradient. The protein obtained was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and visualized with Coomassie blue. Western blotting using antibody to lipopolysaccharide (LPS) was negative, confirming lack of LPS contamination.

**Mouse model of infection.** Guaranteed-pregnant BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Maine) were housed in individual cages in a room free of known murine pathogens. An entire litter of 7-day-old mice were inoculated with a single strain of *P. aeruginosa*. Each animal was weighed and then inoculated with 2-μl aliquots of a bacterial suspension placed directly into the nares. This procedure was repeated until the animal received the entire 10 μl of bacterial suspension. The entire process required approximately 1 min per animal. The mice were returned to the mother following the inoculation procedure. At 24 h postinoculation, the animals were sacrificed with $CO_2$ gas and weighed. The chest was opened using sterile technique; the right lung was weighed and homogenized in 200 μl of sterile PBS until the consistency was smooth, and a 100-μl portion was plated onto MacConkey-lactose agar plates. The spleen was similarly prepared and cultured. The left lung and selected spleens were placed in 10% buffered formalin for histopathological studies. Animals found dead at 24 h were also treated in a similar manner.

For studies using purified pilin as inoculum, 10 μl of pilin suspended in sterile PBS to a final concentration of either 7.3 or 0.73 μg/ml was used to inoculate the mice. Control litters of animals were given 10-μl portions of bovine serum albumin (BSA) in PBS at the same concentrations.

**Characterization of bacteria recovered.** Bacterial colonies recovered from MacConkey-lactose plates were tested for $Tc^r$ markers (PAO-NP, PAK-NP, and PA1244-NP) and for the expression of pili by using phage susceptibility. There was no loss of antibiotic markers or variation in piliation noted among recovered *P. aeruginosa.*

**Histopathological studies.** Tissues fixed in formalin were embedded in paraffin, and sections were stained with hematoxylin and eosin, in addition to Gram staining. Since there was little variation in the weights of the lungs, evidence of pulmonary infection was defined as the recovery of $>1 \times 10^3$ CFU/100 μl of homogenized lung of *P. aeruginosa* from lung cultures. The presence of pneumonia was determined by the presence of gram-negative rods, leukocyte infiltration, consolidation from fibrin deposition, and hemorrhage into bronchi and alveoli. The degree of infection was categorized as diffuse if consolidation and leukocyte infiltration involved an entire 10× field and more than five such fields were found in a given specimen. Disease was characterized as focal if areas of consolidation were limited in extension and appeared in the context of normal pulmonary histology under 10× magnification.

The growth of *P. aeruginosa* from splenic cultures indicated the presence of bacteremia. Spleens were examined for evidence of lymphoid depletion and the presence of gram-negative rods indicative of acute *Pseudomonas* sepsis.

**Statistics.** All differences in results between piliated and nonpiliated strains were analyzed for statistical significance using chi-square analysis. The level of significance was determined in terms of alpha value, denoted by α, which gives the probability of incorrectly rejecting the null hypothesis when it is actually true.

## RESULTS

**Kinetics of *P. aeruginosa* infection in the BALB/cByJ infant mouse.** The neonatal mouse appears to be a reasonable model to study the acquisition of and host response to different strains of *P. aeruginosa*. An inoculum of $10^8$ CFU of PAK reliably reached the lungs of the animals, and all (five of five tested) had evidence of pulmonary infection by 4 h postinoculation as documented by culture (Fig. 1). The corresponding histopathological examinations of the lungs revealed bacteria within the bronchioles of the terminal airways, but not within parenchyma (Fig. 2a). By 24 h postinoculation bacterial invasion of the epithelial cells had occurred, as evidenced by the presence of gram-negative rods within lung parenchyma (Fig. 2b and c). Although difficult to demonstrate with plain photography, actual invasion of epithelial cells with one or more gram-negative rods was seen, indicating that the route of entry into lung parenchyma was by intracellular invasion. Bacteria were also found within macrophages. In addition, intrapulmonary bacterial replication also occurred, as seen by the 10-fold increase (from $10^6$ to $10^7$ CFU/100 μl of tissue) in the median number of organisms recovered (Fig. 1).

The recovery of $>10^3$ CFU of *P. aeruginosa* from lung cultures was associated with alterations in normal pulmonary histology (Fig. 3a). Diffuse leukocytic infiltrate and fibrin deposition resulted in widespread consolidation, and widespread hemorrhage was also apparent (Fig. 3b). There were numerous polymorphonuclear leukocytes within bronchioles characteristic of suppurative bronchopneumonia as well as abundant macrophages located within pulmonary parenchyma that had phagocytosed gram-negative rods (Fig. 3c). All mortalities occurred within the first 24 h, and all animals that died had growth of *P. aeruginosa* from lung and splenic cultures, indicating overwhelming sepsis as the cause of death. Of the animals that survived, most were able to clear the infection by approximately 48 h, as the number bacteria recovered began to decrease at this time point, and by 96 h the lungs were sterile (Fig. 1c). Animals that survived the first 24 h after inoculation continued to gain weight at an average rate of 0.5 g/day, similar to the sham-infected controls, indicating clearance of infection and well being. These animals had pneumonias that were local in extent and characterized by the presence of normal lung histology along with focal consolidation within a given field viewed under 10× magnification. This was in contrast to the severe pneumonias with diffuse areas of hemorrhage, fibrin deposition, and consolidation found in animals that died. Animals succumbing to infection failed to gain weight and had higher numbers of recovered organisms.

Positive spleen cultures occurred only for animals with greater than $>10^3$ CFU of *P. aeruginosa* recovered from lung cultures, indicating the source of infection to be the pulmonary tract. The median number of bacteria recovered from splenic cultures was 1,000-fold less than the median number recovered from lung cultures (Fig. 1). Most animals had sterile splenic cultures within 72 to 96 h, suggesting clearance of bacteremia. Animals with sterile spleens at 24 h had normal splenic architecture with well-defined areas of white pulp (Fig. 4a). In contrast, animals which died or which yielded $>10^6$ CFU of *P. aeruginosa* had severe lymphoid depletion with loss of white pulp (Fig. 4b).

Depending upon which *Pseudomonas* strain was used to inoculate the 7- to 10-day-old animals, mortality ranged from 0 to 60% and evidence of pneumonia was found in 5 to 85%. Adult mice tolerated an inoculum of up to $10^{12}$ CFU/ml without developing pneumonia or bacteremia, whereas infant mice less than 4 days of age had 100% mortality when inoculated with $10^8$ CFU of strain PAK or PA1244 per ml. There were no deaths attributed to the inoculation procedure. Lung and splenic cultures from mouse pups inoculated with PBS alone were sterile at all time points, demonstrating that acquisition of *P. aeruginosa* did not occur from the environment or from transmission from infected littermates (data not shown).

**Effects of pili on rates of disease.** At 24 h postinoculation, each of the three piliated strains studied was more virulent than the corresponding $Pil^-$ isogenic mutant (Fig. 5). PAK and PA1244 caused more pneumonia than the nonpiliated mutants

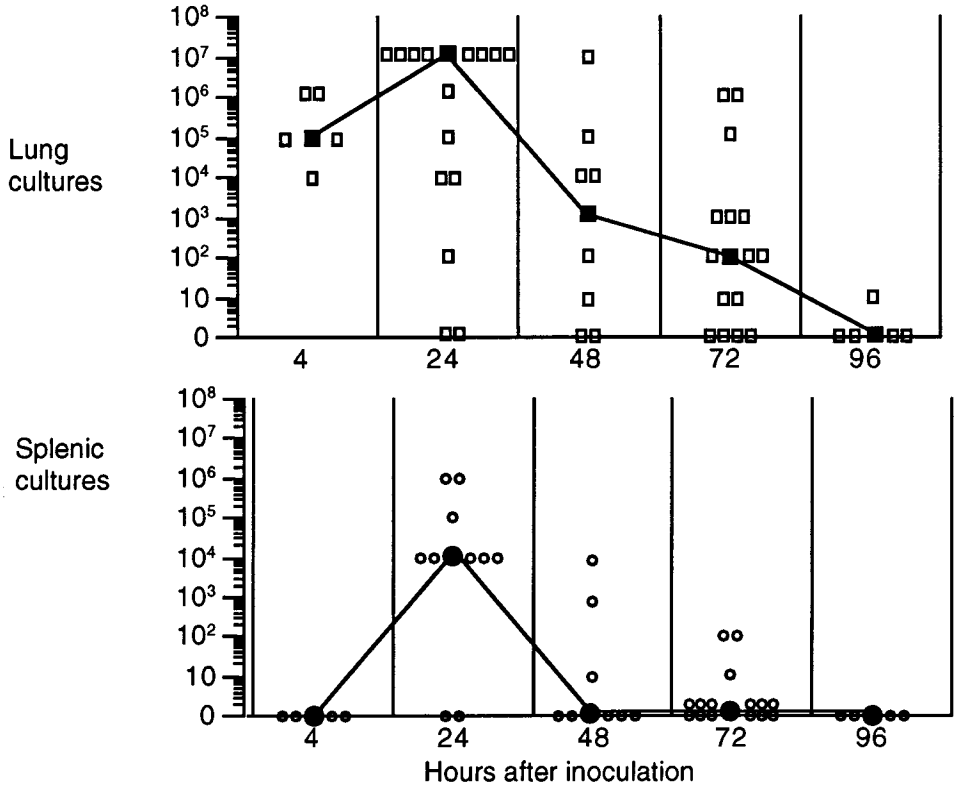



FIG. 1. Time course of *P. aeruginosa* pneumonia and bacteremia in neonatal mice. All values are listed as CFU of *P. aeruginosa* recovered per 100 μl of homogenized tissue from animals dead or sacrificed at a given time point. □, lung cultures from individual animals; ■, median CFU of all lung cultures; ○, splenic cultures from individual animals; ●, median CFU of all splenic cultures.

($\alpha = 0.10$ and 0.05, respectively). The pilus appeared to be a particularly important virulence factor for strain PAO1, as PAO-NP produced significantly less pneumonia (only 1 of 26 mice, $\alpha = 0.001$).

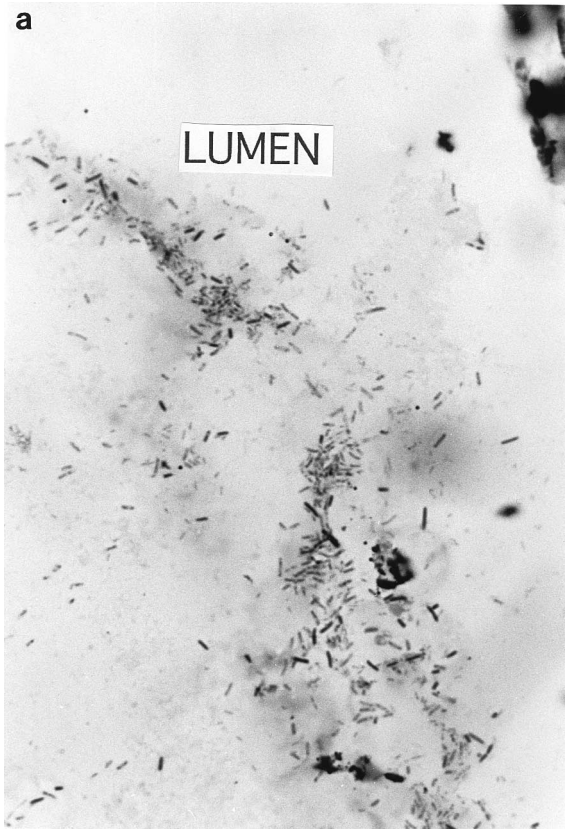
The ability to express pili was associated with disseminated infection (Fig. 2). The piliated strains were isolated from the spleen more often than the nonpiliated mutants, and the effect was most pronounced with strains PA1244 and PAO1 ($\alpha = 0.05$ and 0.001, respectively). Infection with PAK resulted in a 43% rate of bacteremia, which was higher than that associated with PAK-NP (20%) but did not reach statistical significance ($\alpha = 0.1$).

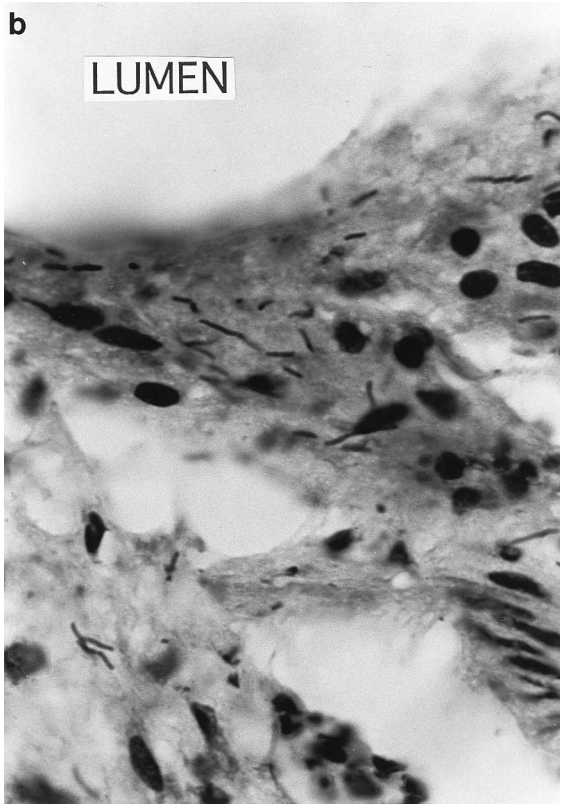
Piliation affected the rates of mortality of infected animals. All three piliated strains caused higher rates of death than the isogenic $Pil^-$ mutants. The most virulent strain appeared to be PA1244, which caused death in 60% of the animals inoculated, while PA1244-NP resulted in only 21% of the animals dying ($\alpha = 0.01$). PAO1 resulted in the most pronounced difference in that 21% of inoculated animals died, but none of those infected with PAO-NP succumbed. Although 33% of animals given PAK died, this value was higher but not significantly so than the 17% death rate for animals inoculated with PAK-NP.

**Effects of pili on pathogenesis of pneumonia.** The presence of pili appeared to affect the severity of pneumonia produced. The differences in the numbers of severe and focal pneumonias caused by each individual $Pil^+$ and $Pil^-$ strain were not large enough to achieve statistical significance (Fig. 6). However, with each individual strain, the piliated wild-type organisms resulted in a larger number of severe pneumonias, while the isogenic mutant caused a larger number of focal pneumonias. The majority of pneumonias (69%) caused by PA1244 were characterized as severe, while the majority of disease (60%) caused by PA1244-NP was found to be focal. Of all the pneumonias caused by PAO1, 40% were categorized as severe. In contrast, inoculation with PAO1-NP resulted in only one case of pneumonia in the 26 animals infected. In most cases (41 of 43) of severe pneumonia examined, there was evidence of *P. aeruginosa* adherence to respiratory epithelial cells, invasion of pulmonary parenchyma, and occasionally engulfment by monocytes (Fig. 2b and c). Bacterial invasion of pulmonary parenchyma was observed significantly less often in the focal pneumonias (5 of 53) ($\alpha = 0.001$), suggesting that the pilus may be important in establishment of pulmonary parenchymal involvement.

The contribution of pili to the pathogenesis of pulmonary infection was confirmed by using purified pilin to inoculate the mice as compared with an equivalent dose of BSA. The histology of the lungs of six of seven mice treated with 7.3 μg of purified pilin per ml demonstrated acute inflammation accompanied by invasion with polymorphonuclear leukocytes, while mice inoculated with a similar concentration of BSA had no demonstrable pathology (Fig. 7). This acute inflammatory response to pilin appeared to be dose related since mice given a 10-fold less concentrated solution had no changes noted.

Although piliated strains of *P. aeruginosa* were, in general, more virulent, there were marked differences in the virulence of the three strains used. PA1244 was the most virulent strain in this model system as it caused the highest rates of pneumonia, the majority of which were classified as severe (16 of 23), bacteremia, and mortality. Even PA1244-NP caused as much pneumonia (approximately 50%) and mortality (21%) as PAO1. PAO-NP was the least virulent strain, associated with



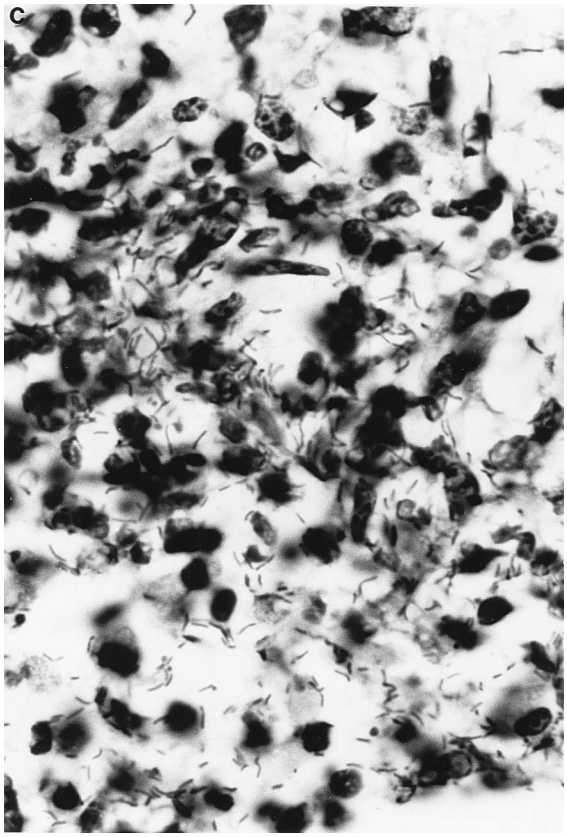




FIG. 2. Time course of *P. aeruginosa* pulmonary infection. Gram staining of lung tissue from animals (a) Gram-negative rods in bronchioles 4 h after inoculation; (b) gram-negative rods adhering to and invading epithelial surface 24 h after inoculation; (c) gram-negative rod invasion and replication in lung parenchyma 24 h after inoculation. Original magnification, ×1,500.

the lowest rates of disease and death. Inoculation with PAK or PAK-NP, resulted in similar rates of severe pneumonia, 38 and 26%, respectively, again suggesting that the expression of strain-dependent virulence factors other than pili are also important in the pathogenesis of pneumonia and its sequelae.

## DISCUSSION

The ability to synthesize functional pili appears to be important in the pathogenesis of *P. aeruginosa* infection of the mouse lung. The piliated strains used in these studies were more often associated with diffuse severe pneumonia and seemed to be more invasive, as indicated by their presence within lung parenchyma and their ability to cause bacteremia. These findings are consistent with previous studies using the burned mouse model of infection (13) and with in vitro data from several laboratories demonstrating the role of pili in mediating attachment to asialyated glycolipid receptors on epithelial cells (12). Preliminary studies done in this laboratory also show increased adherence of piliated strains to murine respiratory epithelial cells in culture (18). The histopathological studies suggest that the presence of pili alone is a major inflammatory stimulus, as pili trigger a response typical of that caused by intact piliated organisms. Recent studies have demonstrated that bronchial epithelial cells express interleukin-8, a major neutrophil chemokine, in response to *P. aeruginosa* pili (3), which may be the mechanism responsible for the inflammation elicited. Thus, the severity of the pneumonia attributed to the piliated strains may be due not only to the increased bacterial attachment associ-

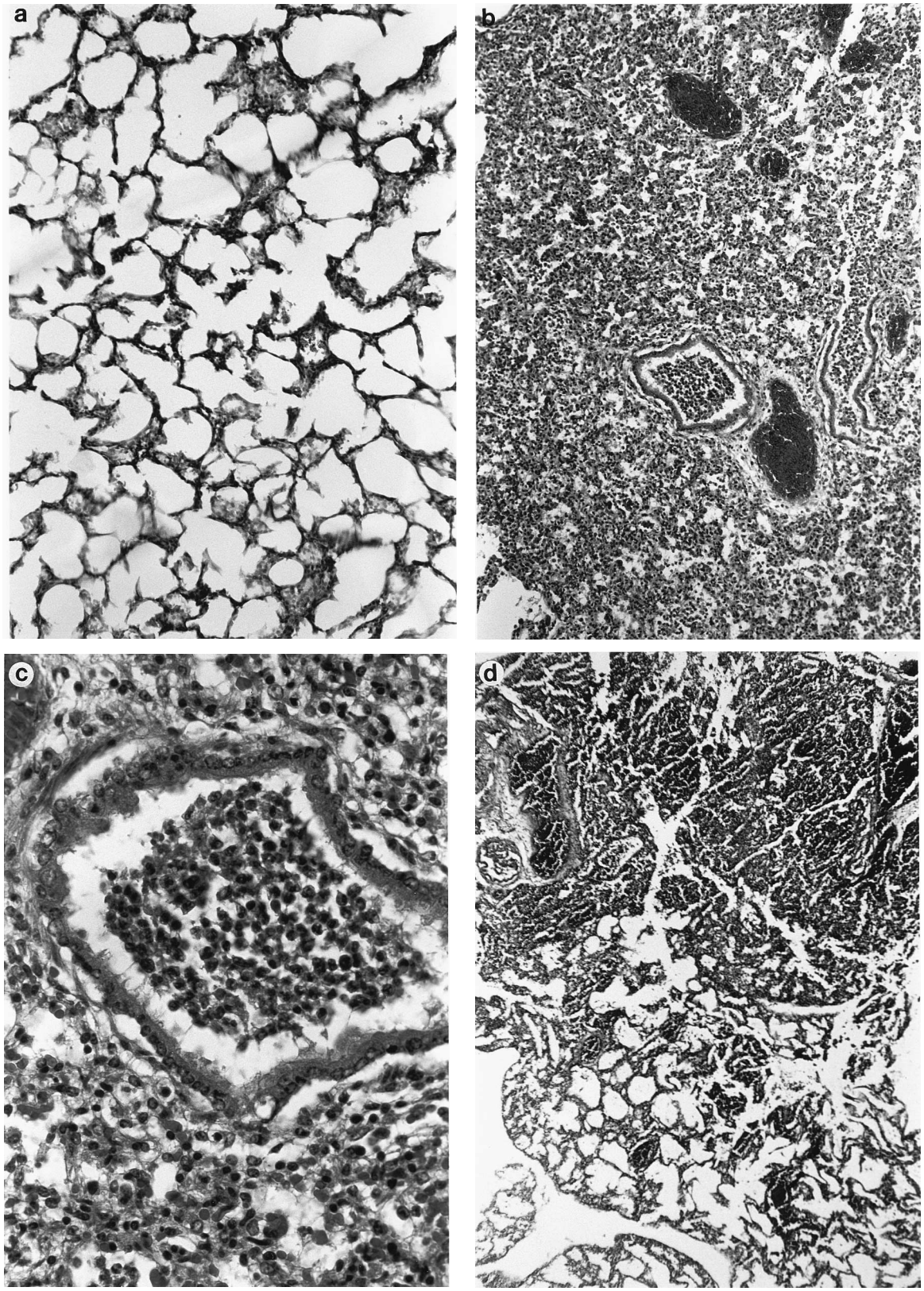



FIG. 3. Histological examination of lungs of mice infected with *P. aeruginosa*. (a) Normal lung following inoculation with PBS. Note normal alveoli and stroma free of inflammatory cells. Original magnification, ×150. (b) Severe diffuse pneumonia with consolidation, fibrin deposition, neutrophilic infiltration, and hemorrhage resulting from inoculation with strain PA1244. Original magnification, ×150. (c) Close-up of severe diffuse pneumonia, demonstrating abundance of polymorphonuclear cells in bronchioles and within stroma. Original magnification, ×600. (d) Focal pneumonia from inoculation with nonpiliated strain PAK-NP, demonstrating area of normal lung and limited area of consolidation and hemorrhage. Original magnification, ×60.

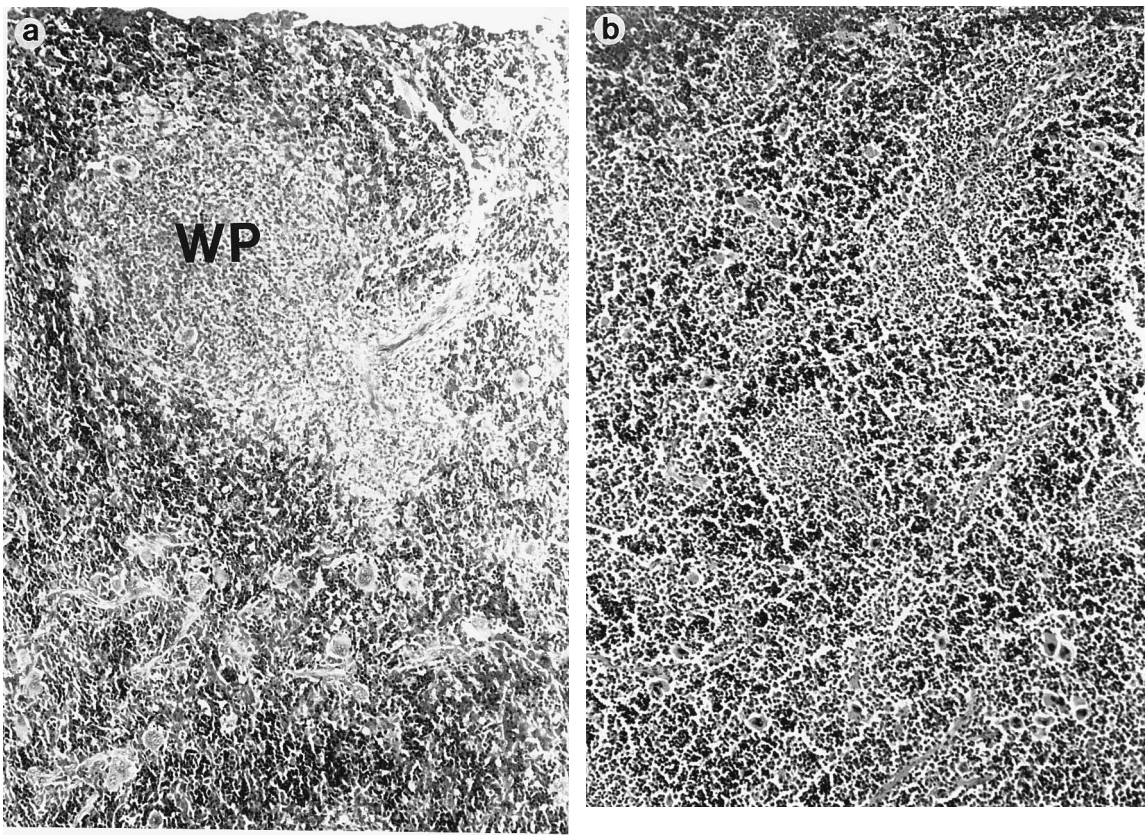



FIG. 4. Histological examination of spleens of mice infected with *P. aeruginosa*. (a) Normal splenic architecture following inoculation with PBS. Note presence of white pulp (WP). Original magnification, ×150. (b) Severe lymphoid depletion following inoculation and bacteremia with PA1244. Note absence of white pulp. Original magnification, ×150.

ated with the piliated strains but also to the host inflammatory reaction to pilin, a response not expected in infections caused by the nonpiliated strains. Although the nonpiliated strains were frequently recovered from the lungs 24 h postinoculation, the majority (67%) of the mice did not have evidence of parenchymal invasion or the severe florid pneumonias which characterized the disease caused by the piliated strains.

As all of the nonpiliated strains were constructed by the

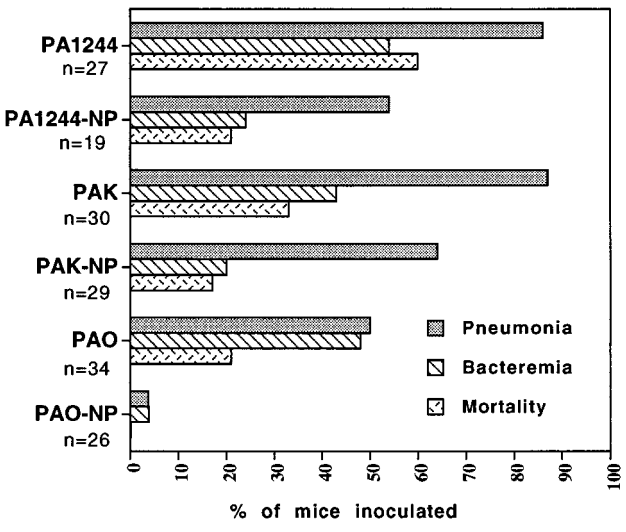



FIG. 5. Percentage of animals inoculated with indicated strain which developed pneumonia, bacteremia, or died. n equals the total number of animals used for inoculation with each strain.

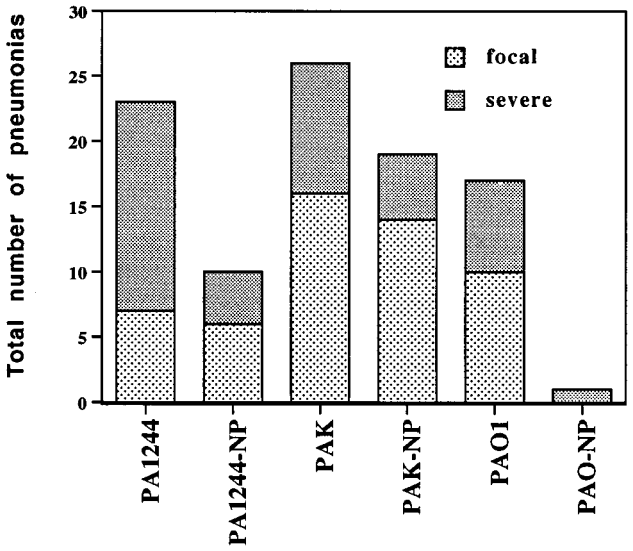



FIG. 6. Total number of focal and severe pneumonias caused by each strain. The number of animals inoculated for each strain is the same as in Fig. 5.

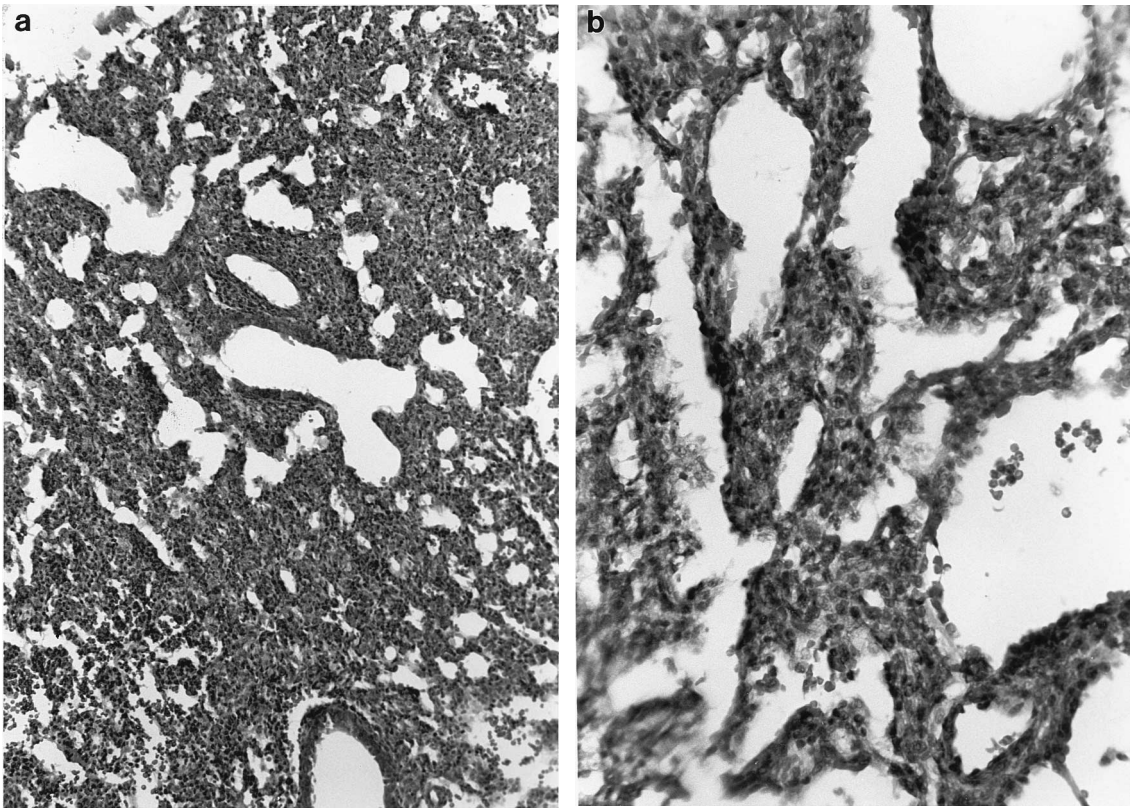



FIG. 7. (a) Histological examination of lungs of mice inoculated with pilin protein (7.3 μg/ml), with evidence of consolidation. Original magnification, ×150. (b) Close-up of tissue demonstrating polymorphonuclear infiltration. Original magnification, ×375.

insertion of the same $Tc^r$ cartridge within the *pilS* coding region, it is possible that this is responsible for the observed phenotypic differences. However, when the same $Tc^r$ marker is used to interrupt exoenzyme S expression, the mutant and parental strains are indistinguishable in this neonatal mouse system, suggesting that the antibiotic resistance marker, by itself, is not the source of the observed effects (18).

The differences in infection rate among the three strains studied suggests that the effect of piliation varied from strain to strain. The loss of piliation appeared to have the most pronounced effect on PAO1, since PAO-NP caused no deaths and <5% pneumonia and bacteremia. Indeed, pilin purified from other strains such as PAK caused less interleukin-8 production from cultured epithelial cells than PAO1 pilin such as that used in this study (3). Such evidence suggests that PAO1 pilin is especially immunogenic as compared with pilin from other strains.

Not unexpectedly, virulence properties other than pili also vary considerably among the different strains. Both PAK and PA1244 infected the mouse lungs to a greater extent than PAO1 (87 versus 56%), and PA1244 was three times more virulent than either PAK or PAO1. In addition, pulmonary infection by both PAK-NP and PA1244-NP was greater than 50%. Thus, even though infection was only focal in the majority of the animals infected with the nonpiliated strains, it seems likely that nonpilus adhesins in these strains also have a role in colonization and early infection. It has been proposed that pili function as the ligand for nonopsonic phagocytosis (17). Thus, bacteremia caused by nonpiliated strains may be due to the inability of the immunologically naive neonatal mice to efficiently phagocytose these organisms. Although the in vivo expression of specific *P. aeruginosa* gene products was not specifically documented, strains which were unable to express pili under any conditions were nonetheless quite virulent. The rates of pneumonia and resultant mortality associated with PA1244-NP infection were equivalent to those caused by the piliated strain PAO1, suggesting that either PA1244-NP expresses another ligand not produced by PAO1 or that it produces other, more potent virulence factors which compensate for its diminished attachment. Strategies to prevent *P. aeruginosa* infection must account for multiple mechanisms of pathogenesis which may be utilized by such opportunistic organisms.

*P. aeruginosa* pulmonary infection in CF has been intensively studied, but primarily at the more advanced stages of disease in which the organisms appear to be limited to the bronchial lumen, enmeshed in mucin, and surrounded by inflammatory cells. A useful feature of this infant mouse model of acute pneumonia is the ability to do histopathological studies at the acute stages of infection. At least in the infant mouse, it appears that the organisms are able to invade and perhaps replicate within epithelial cells. Although CF patients are not clinically septicemic during the initial stages of their disease, a transient infection of bronchial epithelial cells may be a part of the response to the organism. In vitro studies using transformed pulmonary cell lines have also demonstrated that *P. aeruginosa* can replicate within these cells, and invasion is clearly an aspect of *P. aeruginosa* infection of corneal epithelial cells (5). Thus, it is possible that an early stage of *P. aeruginosa* pulmonary infection includes epithelial cell infection.

This infant mouse model of *P. aeruginosa* pneumonia allows for the in vivo evaluation of bacterial and host factors important in the acute stages of pulmonary infection. The use of this

model also provides a means to test preventative and therapeutic strategies against the acquisition of these organisms.

## ACKNOWLEDGMENTS

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