INFECTION AND IMMUNITY, Jan. 1996, p. 37–43 Vol. 64, No. 1
0019-9567/96/$04.00+0

Contribution of Specific *Pseudomonas aeruginosa* Virulence Factors to Pathogenesis of Pneumonia in a Neonatal Mouse Model of Infection

H. B. TANG,[1] E. DiMANGO,[2] R. BRYAN,[1] M. GAMBELLO,[3] B. H. IGLEWSKI,[3] J. B. GOLDBERG,[4] AND A. PRINCE[1]*

Departments of Pediatrics[1] and Medicine,[2] College of Physicians and Surgeons, Columbia University, New York, and Department of Microbiology and Immunology, University of Rochester, Rochester,[3] New York, and Channing Laboratory, Department of Medicine, Harvard Medical School, Boston, Massachusetts[4]

Received 14 August 1995/Returned for modification 3 October 1995/Accepted 12 October 1995

We sought to identify which *Pseudomonas aeruginosa* products are involved in initiating respiratory tract infection. Defined mutants derived from strain PAO, i.e., PAOR1 (*lasR*), PAO-pmm (*algC*) (an LPS mutant), and AK1152 (which is Fla$^-$ and lacks functional pili), were significantly less virulent than PAO1 in a BALBc/ByJ neonatal mouse model of infection as measured by their abilities to cause acute pneumonia, bacteremia, and death. All three mutants were also less adherent to epithelial cells in an in vitro binding assay. PAOR1 and AK1152 were less able to elicit epithelial production of interleukin-8 than PAO1. LasR was found to be required for the optimal expression of neuraminidase under conditions of increased osmolarity, as might be present in certain pathological conditions. PAO-*exsA*::Ω, which lacks exoenzyme S expression, was fully virulent, causing at least as much pathology as PAO1. The expression of several *P. aeruginosa* virulence factors appears to be required to establish pulmonary infection in the neonatal mouse.

Pseudomonas aeruginosa is an opportunistic pathogen which rarely causes pulmonary disease in normal hosts but which is an important cause of acute pneumonia in immunocompromised patients, including neonates (18) and patients with human immunodeficiency virus infection (11), and of chronic pneumonia in patients with cystic fibrosis (CF). *P. aeruginosa* expresses numerous gene products which are postulated to contribute to the pathology associated with *Pseudomonas* pulmonary infection. Such virulence factors include adhesins responsible for binding to specific epithelial receptors, exoenzymes which modify eukaryotic targets, and exoproducts which interfere directly with immune clearance mechanisms. The phenotypes of *P. aeruginosa* strains associated with chronic pulmonary infection in CF patients have been well studied. These organisms produce the mucoexopolysaccharide alginate, usually lack pili and flagella (19), express rough lipopolysaccharide (LPS) (15), and produce relatively low levels of exotoxins (35, 36). The properties of these organisms that are involved in the initial stages of infection, i.e., the gene products expressed when bacteria are inhaled from an environmental source, abruptly exposed to the increased osmolarity and temperature of the upper respiratory tract, and then forced to adapt in order to elude mucociliary clearance and to establish a nidus of infection, are less well understood.

Several studies have demonstrated that *P. aeruginosa* pili facilitate attachment to respiratory epithelial cells (10, 27, 34, 37), although the organisms also possess additional, less well defined adhesins (29). Using a neonatal mouse model of pulmonary infection, we have previously demonstrated that the expression of pili, while important in facilitating respiratory tract infection, is not essential for this process to occur (32). The roles of many of the other well-characterized *P. aeruginosa* exoproducts in the development of pulmonary infection are not well studied. Most of the data regarding *P. aeruginosa* virulence are derived from animal models in which the normal immune system is compromised by delivering a cutaneous burn, traumatizing the cornea, rendering the experimental animal neutropenic (33), or delivering the bacterial inoculum in the context of agar beads implanted within the lung to mimic a chronic endobronchial infection (5). Since these models bypass the normal route of entrance of the organism into the respiratory tract, they may not be appropriate for defining the virulence factors which facilitate the acquisition of *P. aeruginosa* from environmental sources into the lungs of susceptible hosts.

We chose to study the relative virulences of several mutants of *P. aeruginosa* PAO1 by using the immunologically naive, but competent, neonatal BALBc/ByJ mouse (22, 30). In these animals phagocytic cells such as polymorphonuclear leukocytes (PMNs) and alveolar macrophages are probably most important in clearing inhaled bacteria (2, 31). To determine which of the many *P. aeruginosa* virulence factors are involved in the initial establishment of respiratory infection, mutants were tested for their ability to adhere to epithelial monolayers in vitro, to elicit an inflammatory response as measured by the stimulation of a major neutrophil chemokine, interleukin-8 (IL-8), by epithelial cells, and to produce pneumonia, bacteremia, and mortality in a neonatal mouse model of acute infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *P. aeruginosa* strains used are described in Table 1. All of the strains were mutants of the prototypic strain PAO1 and, with the exception of AK1152, were constructed by gene replacement techniques in which a wild-type gene was replaced with one containing a tetracycline resistance cartridge or an Ω fragment preventing expression. The *lasR* mutation in PAOR1 affected the production of *P. aeruginosa* autoinducer, elastase, and alkaline protease (13, 24, 28). PAO-pmm, an *algC* mutant, lacked the

* Corresponding author. Mailing address: Department of Pediatrics, Columbia University, 650 W. 168th St., New York, NY 10032. Phone: (212) 305-4193. Fax: (212) 305-2284. Electronic mail address: asp7@columbia.edu.

TABLE 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Description	Source and/or reference
Strains		
PAO1	Prototroph	16
PAOR1	Sm^r Δ/*lasR*::*tet*	B. Iglewski (13)
AK1152	Fla$^-$ Mot$^-$ mutant of PAO1	T. Montie (9)
PAO-pmm	PAO1 *algC*::*tet*	J. Goldberg (6)
PAO1-*exsA*::Ω	Ω insertion at bp 2358 within *exsA*	D. Frank (12)
Plasmids		
pSW200	Amp^r 1.8-kb stabilizing fragment	B. Iglewski (13)
pLP105	Amp^r *lasR lasI*	B. Iglewski (28)
pMJG1.7	Amp^r *lasR*	B. Iglewski (13)

phosphomannomutase gene, resulting in a defective LPS core, the lack of O side chains, and the inability to synthesize alginate (6). The mutation in *exsA* resulted in undetectable secretion of the ADP-ribosylating enzyme exoenzyme S but no changes in exotoxin A, elastase, or phospholipase excretion (12). AK1152 was a nonmotile PAO1 mutant generated by chemical mutagenesis (9). All strains were tested for piliation by susceptibility to phage F116 (25) and for the expression of functional flagella by motility in soft agar. Bacteria were grown in M9 minimal medium with aeration at 37°C to late log phase for adherence studies as previously described (27).

Adherence assay. Epithelial cells from human nasal polyps were isolated by the protease method and maintained in primary culture with supplemented DMEM-F12 medium (27). The cells were plated on collagen-coated 24-well plates and grown to confluence. The immortalized human epithelial cell lines 9HTEo− and 1HAEo− were previously characterized by D. Gruenert (14). Mouse L4 adenoma cells were obtained from the American Type Culture Collection and maintained under standard conditions.

The number of *P. aeruginosa* organisms binding to epithelial cells was determined by previously published methods (27). Briefly, bacteria grown to late log phase were metabolically labeled with [^{35}S]methionine and washed with 10 mM NaCl, and a 0.1-ml inoculum of 5×10^8 CFU/ml was added to each well of confluent monolayer and left for 60 min at 37°C. Nonadherent bacteria were rinsed off by three successive washes with phosphate-buffered saline (PBS). The monolayers and adherent *Pseudomonas* cells were solubilized with 2% sodium dodecyl sulfate and scintillation counted (Packard Tricarb liquid scintillation spectrometer). Each datum point was recorded six times, and means and standard deviations were determined. Statistical analysis was performed by analysis of variance (Microsoft Excel 5.0).

IL-8 assay. 1HAEo− cells were assayed for IL-8 production in response to incubation with bacterial strains. IL-8 serves as an important neutrophil chemokine associated with pulmonary inflammation (26), and IL-8 expression by epithelial cells is elicited by several *P. aeruginosa* gene products, such as pilin, flagellin, and the *Pseudomonas* autoinducer, a low-molecular-weight homoserine lactone derivative (8, 24). Monolayers grown to confluence in DMEM-F12 medium with 10% fetal calf serum were serum starved for 18 h prior to stimulation. Bacterial strains grown to late log phase were washed and resuspended in PBS. An aliquot of 10^7 CFU/ml was incubated for 60 min at 37°C with the epithelial cell monolayers. Subsequently, the monolayers were washed three times with PBS and reincubated with DMEM-F12 with 100 μg of gentamicin per ml for 18 h. IL-8 in the epithelial cell supernatant was assayed with an enzyme-linked immunosorbent assay kit (R&D, Minneapolis, Minn.). Prior kinetic studies showed that peak IL-8 production from 1HAEo− cells occurred at 8 to 24 h following stimulation with *P. aeruginosa* PAO1. Each datum point was determined in quadruplicate, and each experiment was performed at least two times. Statistical analysis was performed by analysis of variance (Microsoft Excel 5.0 software).

Mouse model of infection. Pregnant BALBc/ByJ mice (Jackson Laboratories, Bar Harbor, Maine) were housed in individual cages in a room free of known murine pathogens. An entire litter of 7- to 10-day-old mice were inoculated intranasally with a single strain of *P. aeruginosa* as previously described in detail (32). Bacterial strains grown to late log phase were washed twice in sterile PBS and concentrated to deliver an inoculum of approximately 5×10^8 CFU/ml in 10 μl. The animals were sacrificed with CO_2 24 h after inoculation. The right lung and spleen were homogenized in PBS and plated individually onto MacConkey-lactose agar plates, while the left lung was placed in formalin for histopathological studies. Any animals found dead at 24 h were treated similarly.

Evidence of pulmonary infection was defined as the recovery of $>10^3$ CFU of *P. aeruginosa* per 100 μl of homogenized lung from lung cultures. Histopathological analysis of the lungs was performed with the assistance of a veterinary pathologist. Pneumonia was confirmed by the presence of gram-negative rods, leukocyte infiltration, consolidation, and hemorrhage. The degree of infection was categorized as diffuse if consolidation and leukocyte infiltration involved an entire field (magnification, ×10) and more than five such fields were found in a given specimen. The disease was characterized as focal if areas of consolidation were limited in extent and appeared in the context of normal pulmonary histology at a magnification of ×10.

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. Differences in rates of pneumonia, bacteremia, and mortality at 24 h were analyzed for statistical significance by chi-square analysis.

Neuraminidase assays. Neuraminidase activity was measured by following the hydrolysis of the fluorescent substrate 4-methylumbelliferylneuraminic acid (Mu NeuNAc) (23). Stationary-phase culture supernatants harvested from organisms grown in M9 medium plus 10 mM $FeCl_2$ (to diminish the production of fluorescent pigments) were concentrated with polyethylene glycol, extensively dialyzed against phosphate buffer, and incubated with 25 μM MuNeuNAc in 50 mM sodium acetate (pH 4.9) in duplicate wells of a 96-well microtiter plate. Fluorescence was read in a TiterTek fluorimeter and standardized for the protein content of the supernatant (4). A sonicate of *Escherichia coli* expressing the *Vibrio cholerae* neuraminidase (pCVD364) served as a positive control.

RESULTS

Adherence properties of the PAO1 mutants. The ability to attach to specific host receptors is thought to be an important prerequisite to infection. The mutants and control strain were compared for the ability to attach to primary cultures of human respiratory epithelial cells derived from nasal polyps (NHNP cells), to transformed tracheal epithelial cells (9HTEo− cells), or to mouse adenoma cells (Fig. 1). In each case the parental strain, PAO1, was more adherent than any of the mutants tested. The nonmotile Fla$^-$ strain AK1152 was Pil$^-$ (resistant to the pilus-specific phage F116), although by Western blot (immunoblot) hybridization it was found to express pilin protein (data not shown). Thus, its lack of adherence in these assays may be ascribed to its lack of a functional ligand as well as to its inability to swim. The other mutants tested were clearly motile and expressed functional pili but nonetheless adhered to epithelial cells less than the parental strain.

The different epithelial cell types tested also differed in their affinities for PAO1 ligands. The murine cells expressed more receptors for PAO1 than the other cell types, with approximately 100-fold more organisms bound to the murine cells than to the primary human epithelial cells. The human nasal polyp (NHNP) cells had the lowest affinity for *P. aeruginosa* ligands. However, even the relatively low levels of binding to the human cells are biologically relevant, as the same inoculum stimulated a relatively large amount of IL-8 production (Fig. 2).

In 10-day-old mice this inoculum of PAO1 caused pneumonia characterized by the infiltration of PMNs into bronchi and dense consolidation of parenchymal tissue in 50% of the animals infected (Fig. 3 and 4). One-third of the infected animals became bacteremic, and 20% died.

Characteristics of PAOR1. PAOR1 was significantly impaired in its ability to attach to the epithelial cells, with only 26% binding to nasal polyp cells compared with a PAO1 control, 58% binding to 9HTEo− cells, and 7.9% binding to murine L4 cells. LasR-dependent exoproducts may be involved in modifying epithelial cell surface structures to facilitate attachment. LasR, along with the product of *lasI*, the *Pseudomonas* autoinducer, acts as a transcriptional activator of several known virulence factors, including elastase and alkaline protease (24). We speculated that LasR might be involved in the regulation of the expression of neuraminidase, a *P. aeruginosa* exoproduct which can cleave terminal sialic acid residues from sialylated superficial gangliosides, exposing the GalNAcβ1-4Gal receptor for *Pseudomonas* pili (4).

Neuraminidase expression was measured in supernatants harvested from stationary-phase cultures of PAO1 and

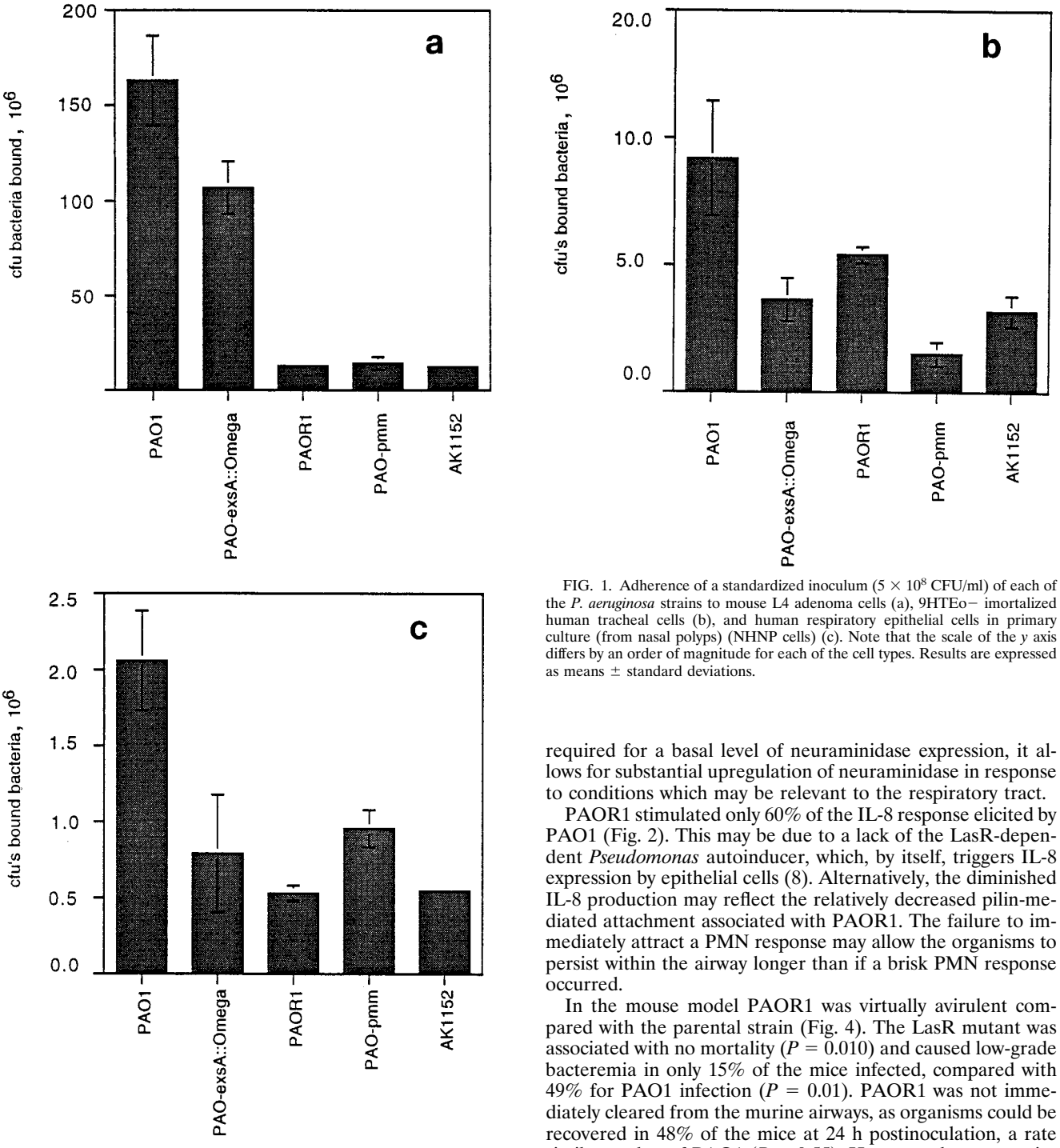

FIG. 1. Adherence of a standardized inoculum (5×10^8 CFU/ml) of each of the *P. aeruginosa* strains to mouse L4 adenoma cells (a), 9HTEo− imortalized human tracheal cells (b), and human respiratory epithelial cells in primary culture (from nasal polyps) (NHNP cells) (c). Note that the scale of the *y* axis differs by an order of magnitude for each of the cell types. Results are expressed as means ± standard deviations.

PAOR1, complemented by the cloned *lasR* and *lasI* genes (Fig. 5). Basal levels of neuraminidase expression were not significantly different in PAO and PAOR1; however, in the presence of an osmotic stress (high salt concentration), there was a 3.5-fold increase in neuraminidase production, which was *lasR* dependent. The NaCl-induced increase in neuraminidase production was noted to occur in strains expressing either a plasmid or chromosomal copy of *lasR* and *lasI* but not in PAOR1 expressing the plasmid vector alone. Although LasR is not required for a basal level of neuraminidase expression, it allows for substantial upregulation of neuraminidase in response to conditions which may be relevant to the respiratory tract.

PAOR1 stimulated only 60% of the IL-8 response elicited by PAO1 (Fig. 2). This may be due to a lack of the LasR-dependent *Pseudomonas* autoinducer, which, by itself, triggers IL-8 expression by epithelial cells (8). Alternatively, the diminished IL-8 production may reflect the relatively decreased pilin-mediated attachment associated with PAOR1. The failure to immediately attract a PMN response may allow the organisms to persist within the airway longer than if a brisk PMN response occurred.

In the mouse model PAOR1 was virtually avirulent compared with the parental strain (Fig. 4). The LasR mutant was associated with no mortality ($P = 0.010$) and caused low-grade bacteremia in only 15% of the mice infected, compared with 49% for PAO1 infection ($P = 0.01$). PAOR1 was not immediately cleared from the murine airways, as organisms could be recovered in 48% of the mice at 24 h postinoculation, a rate similar to that of PAO1 ($P = 0.55$). However, there was minimal pathology associated with this colonization, and in very few of the infected animals (15%) was there any evidence of bacterial replication within the lung. Thus, despite a functional pilus to mediate attachment and normal motility, there appears to be a requirement for LasR-dependent gene products to cause infection of the respiratory tract.

Characteristics of the LPS mutant. The *algC* mutation of PAO-pmm rendered this mutant avirulent (Fig. 4). Despite normal piliation and motility, this strain was less adherent to epithelial monolayers. Binding to human nasal polyp cells was 46%, that to 9HTEO− cells was 17%, and that to murine L4

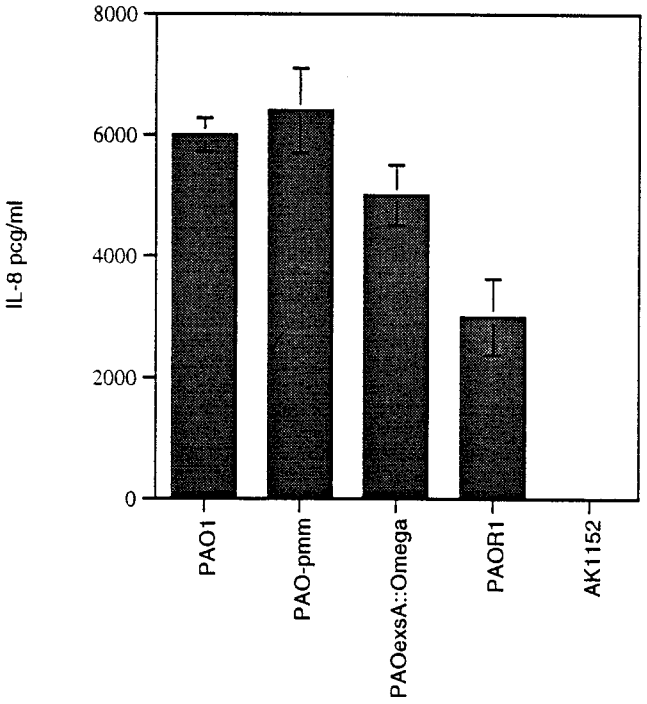

FIG. 2. Amount of IL-8 (picograms per milliliter) produced by 1HAEo− cells following exposure to a standardized inoculum of each of the *P. aeruginosa* strains. Results are expressed as means ± standard deviations.

cells was 8%, compared with that of the PAO1 control. PAO-pmm was very readily cleared from the respiratory tract; only 20% of the mice infected had any organisms recovered from either the lungs or spleen at 24 h ($P = 0.0007$), and bacteremia and mortality rates were 3 and 2%, respectively, both significantly lower than that attributed to PAO1 ($P = 0.0004$ and $P = 0.005$, respectively). In contrast, the strain was as efficient as PAO1 in eliciting IL-8 expression, demonstrating that the O side chain of LPS was not involved in the IL-8 response of epithelial cells. The histology of the lungs infected with the LPS mutant demonstrates a few small areas of focal consolidation consisting primarily of a PMN response, amid a background of predominantly normal lung architecture (Fig. 3b).

Characteristics of the Fla⁻ mutant AK1152. AK1152 was poorly adherent and unable to elicit an IL-8 response from epithelial cells in vitro. AK1152 was recovered 24 h after inoculation from one-third of the mouse lung homogenates ($P = 0.08$), and 28% of the mice were found to also have organisms in the spleen ($P = 0.01$ compared with the data obtained with PAO1). Histopathological changes associated with these infections were minimal (Fig. 3c): a small area of focal infiltration of PMNs was noted, but the majority of the tissue was normal in appearance. There were no deaths attributed to infection with AK1152.

Characteristics of PAO-*exsA*::Ω infection. Unlike the other three mutants tested, PAO-*exsA* was fully virulent (Fig. 4). This strain showed diminished adherence to the human cells in primary culture (38% of the PAO1 adherence). However, it was only minimally less adherent to the mouse adenoma cells (66% of the control value), which may be more relevant to the mouse virulence studies. This exoenzyme S mutant elicited an IL-8 response that was approximately 80% of that attributable to PAO1 (Fig. 2). In mice, the *exsA* mutant caused pneumonia in 75% of the animals inoculated. This pneumonia was characterized by relatively large areas of dense consolidation, more confluent than the focal patches of inflammation associated with infections due to the other mutants studied (Fig. 3d). Rates of bacteremia and mortality due to the exoenzyme S mutant were virtually identical to those caused by the PAO1 parent. The expression of exoenzyme S does not appear to be required for the establishment of pulmonary infection in this model system.

DISCUSSION

As with other infections which are initiated at a mucosal surface, three classes of potential virulence factors appear to be important in this case: structural components of the organism which act as ligands for specific receptors, bacterial gene products which interfere with the normal function of immune clearance mechanisms, and exoproducts which modify targets in eukaryotic cells. The data presented in this report suggest that *P. aeruginosa* must express several key virulence factors, i.e., a complete LPS molecule, LasR-dependent exoenzymes, and adhesins and flagella, to initiate pulmonary infection. The requirement for multiple virulence determinants may explain why the normal host is rarely infected by this ubiquitous organism.

The host response to a complex pathogen such as *P. aeruginosa* is multifaceted. Several independent investigators have provided evidence that PMN-mediated clearance and nonopsonic phagocytosis of *P. aeruginosa* from the airways of CF patients are impaired. The conditions in the neonatal murine lung, which, like the human lung, is relatively resistant to *P. aeruginosa* infection (22), may provide a reasonable approximation of what might occur early in CF. In the neonatal mouse, phagocytic function is less effective than it is in an adult animal (30). *P. aeruginosa* virulence factors which inhibit phagocytic function, such as the O chain of LPS, elastase-mediated cleavage of complement receptors and neutrophil ligands (3), and destruction of immunoglobulins, including immunoglobulin A, may be particularly important in such early stages of infection. The neutrophil response to *P. aeruginosa* seen in the mouse airway is similar to what is observed in bronchoalveolar lavage fluid from young infants with CF, which contains excessive numbers of PMNs and neutrophil chemoattractants such as IL-8 (7). Nonopsonic phagocytosis is also impeded in the airway because of a lack of glucose, which is important specifically in the ingestion of *P. aeruginosa* but not other opportunists (2).

Numerous studies have demonstrated a role for pili in the pathogenesis of *P. aeruginosa* infection, particularly in the CF lung (10, 27). Pili are important in mediating attachment to both epithelial receptors and phagocytic cells (19). However, the expression of pili is not sufficient for pathogenicity, and the role of pili in triggering IL-8 expression may actually be to enhance clearance of the organisms by the recruited PMNs. All of the strains studied, with the exception of the Pil⁻ Fla⁻ strain AK1152, stimulated biologically significant amounts of IL-8 in vitro. In the mouse, the stimulation of the murine IL-8 homolog may have elicited the influx of neutrophils to clear the infecting organisms from the airway. Since pili can function as a ligand for phagocytic cells, pilin-mediated adherence may initiate a protective immunologic response by the host and provoke the selection of nonpiliated mutants in a chronic infection (19).

A second requirement for the establishment of *P. aeruginosa* pneumonia appears to be the expression of the complete LPS complex. The LPS mutant PAO-pmm does not express either the LPS O side chain or the A-band LPS and synthesizes an

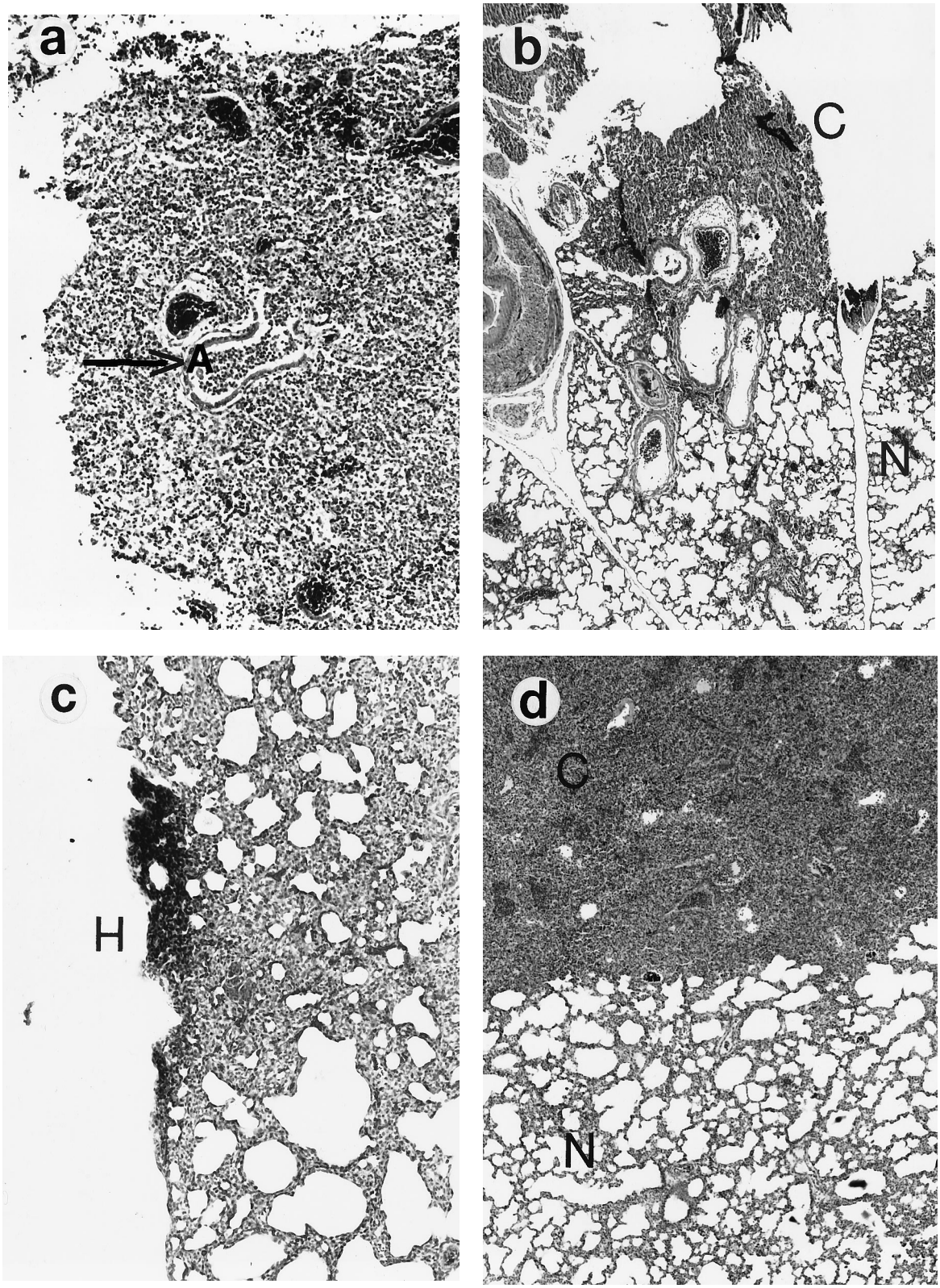

FIG. 3. Photomicrographs of hematoxylin-and-eosin-stained sections of murine lung 24 h following infection with *P. aeruginosa*. (a) PAO1 control demonstrating a confluent pneumonia with PMN infiltration of the airways (A). Magnification, ×100. (b) PAO-pmm. Focal consolidation (C) is limited in extent, with most of the parenchyma appearing normal (N). Magnification, ×40. (c) AK1152. A small amount of hemorrhage and infiltrate (H) is visible surrounded by tissue that is normal in architecture. Magnification, ×100. (d) PAO-*exsA*::Ω. An area of very dense consolidation (C) with hemorrhage into the airways is apparent next to a region of normal lung (N). Magnification, ×40.

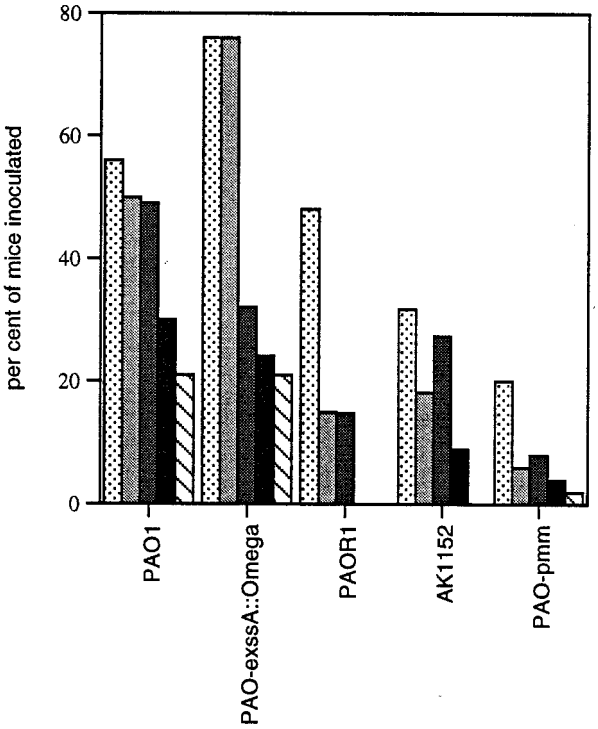

FIG. 4. Composite virulence of the *P. aeruginosa* strains in 7- to 10-day-old mice. The *y* axis shows the percentages of mice affected. Symbols: ▨, total colonization (isolation of more than 10 CFU/100 μl of minced lung tissue) ▩, pneumonia ($>10^3$ CFU/100 μl of homogenized lung tissue) and pneumonia confirmed histopathologically; ▩, total bacteremia (isolation of any organisms from the spleen); ■, $>10^3$ CFU/100 μl of homogenized spleen tissue; ▧, death. PAO1, $n = 34$; PAO-*exsA*::Ω, $n = 34$; PAOR1, $n = 27$; AK1152, $n = 22$; PAO-pmm, $n = 50$.

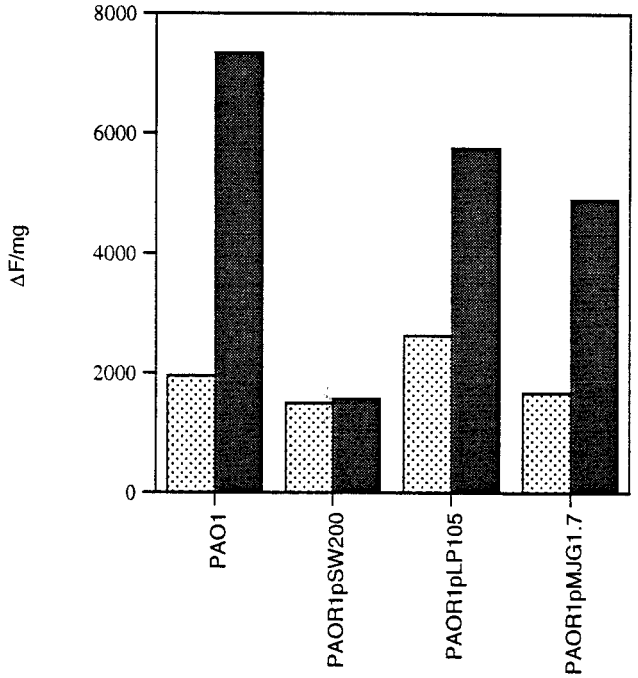

FIG. 5. Neuraminidase production by PAO1 and PAOR1 expressing cloned *lasR* and *lasI*. Neuraminidase activity (change in fluorescence [ΔF] per milligram of protein in the culture supernatant) is plotted for each strain grown in M9 minimal medium (238 mOsm; control) (▨) or in M9 medium plus 400 mM NaCl (■). PAOR1pSW200, PAOR1 ($LasR^-$) plus the plasmid vector pSW200; PAOR1pLP105, $LasR^+$ $LasI^+$; PAOR1pMJG1.7, $LasR^+$.

incomplete LPS core (6). This LPS mutant was the least virulent of the strains tested, demonstrating that LPS is critical even in the initial stages of infection. *P. aeruginosa* strains lacking O antigen are serum sensitive and can be lysed in the presence of complement (15). In the absence of the complete LPS molecule, these organisms appear to be readily cleared by the host. Since these are presumably nonimmune mice, nonopsonic phagocytosis by macrophages is likely to be a major mechanism of bacterial clearance (2, 31). The piliated LPS mutants can interact with epithelial receptors and trigger an IL-8 response; few organisms ever reach the lung, and the vast majority are gone within 24 h. It is of note that the instillation of a large dose of wild-type *Pseudomonas* LPS (as is present in AK1152) appears to be sufficient to cause some pathology. Although AK1152 did not adhere or trigger an IL-8 response, this strain did cause more pneumonia and bacteremia in the mice than did the LPS mutant, suggesting that it was more difficult to clear AK1152 from the bloodstream than to clear the strain which did not express a complete LPS complex.

The relative avirulence of PAOR1 implies that secreted exoproducts are also critical in the establishment of *P. aeruginosa* pneumonia. The *lasR* mutant has functional pili, is fully motile, and has no known alteration in LPS synthesis, yet it is virtually avirulent. The LasR mutants are not immediately cleared from the airways, as the number of mice which became colonized was similar to the number colonized by the wild-type strain. However, the *lasR* mutant failed to replicate or invade to any significant degree and caused no mortality.

As a central regulatory gene, *lasR* may be involved in the expression of several, possibly as yet undefined, virulence factors which have multiple effects. LasR was not required for neuraminidase expression, yet it appeared that under conditions found in vivo, with higher osmolarity of even the normal respiratory tract, the increased expression of neuraminidase and other LasR-dependent exoproducts such as elastase might interfere with immune clearance mechanisms (13). Phagocytosis is likely to be more efficient in the absence of elastase, which can cleave immunoglobulins and neutrophil ligands.

The *exsA*::Ω mutant served as an important, and unanticipated, control in that it was as virulent as the parental strain PAO1 in virtually all aspects of pathogenesis assayed. Although this exoenzyme S mutant was less adherent to the human cells, it efficiently induced an IL-8 response in human cells and adhered better than any of the other mutants to the mouse L4 adenoma cells. In vitro studies have demonstrated that exoenzyme S is highly toxic to MDCK cells, a renal epithelial cell line (1), and can cause substantial alveolar injury. When the same *exsA*::Ω mutant was directly instilled into the alveolae of paralyzed intubated rabbits, it caused minimal damage compared with the parental strain, which was highly toxic, suggesting that the alveolar pathology could be attributed to exoenzyme S (17). Exoenzyme S also causes T-cell proliferation, a response which may be more important in a more chronic pulmonary infection (21). Thus, even within the same organ system, a specific virulence factor may be implicated in different stages of infection. Exoenzyme S may be more important when it is expressed by organisms within an established nidus of infection. It does not appear to be a virulence factor which facilitates colonization of the respiratory tract. Species differences may also account for differences in the susceptibility to exoenzyme S toxicity. The mouse lung may be less susceptible to exoenzyme S damage than the rabbit lung under the experimental conditions used.

As with other mucosal pathogens, it appears that several *Pseudomonas* exoproducts must be coordinately expressed to enable the organism to establish an infection in the respiratory tract (20). The gene products expressed during the acquisition of the pathogen from the environment may be quite distinct from those expressed by mutants which have adapted to the local milieu and are selected by the pressures of the normal immune response. Strategies to prevent the acquisition of *P. aeruginosa* from environmental sources may target any of several *Pseudomonas* products to significantly impair virulence. The development of vaccines or passive immunotherapy directed against multiple virulence factors may be a realistic approach to prevent *P. aeruginosa* pneumonia in the relatively limited populations of patients who are susceptible.

ACKNOWLEDGMENTS

We thank Steven Brunert for his help in processing and interpreting the histopathology, and we thank Dara Frank and Tom Montie for generously providing strains and Al Lemme for photomicroscopy.

This work was supported by the Cystic Fibrosis Foundation (H.B.T.), the New York Lung Association (E.D.) and National Institutes of Health grant DK36963 (A.P.).

REFERENCES

1. **Apodaca, G., M. Bomsel, R. Lindstedt, J. Engel, D. Frank, K. E. Mostov, and J. Wiener-Kronish.** 1995. Characterization of *Pseudomonas aeruginosa*-induced MDCK cell injury: glycosylation-defective host cells are resistant to bacterial killing. Infect. Immun. **63:**1541–1551.
2. **Barghouthi, S., K. D. Everett, and D. P. Speert.** 1995. Nonopsonic phagocytosis of *Pseudomonas aeruginosa* requires facilitated transport of D-glucose by macrophages. J. Immunol. **154:**3420–3428.
3. **Berger, M., R. U. Sorensen, M. F. Tosi, D. G. Dearborn, and G. Doring.** 1989. Complement receptor expression on neutrophils at the inflammatory site, the *Pseudomonas*-infected lung in cystic fibrosis. J. Clin. Invest. **84:**1302–1313.
4. **Cacalano, G., M. Kays, L. Saiman, and A. Prince.** 1992. Production of the *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. J. Clin. Invest. **89:**1866–1874.
5. **Cash, H. A., D. C. Strauss, and J. A. Bass.** 1982. *Pseudomonas aeruginosa* exoproducts as pulmonary virulence factors. Can. J. Microbiol. **29:**448–456.
6. **Coyne, M. J., K. S. Russell, C. L. Coyle, and J. B. Goldberg.** 1994. The *Pseudomonas aeruginosa algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. J. Bacteriol. **176:**3500–3507.
7. **Dean, T. P., Y. Dai, J. K. Shute, M. K. Church, and J. O. Warner.** 1993. Interleukin-8 concentrations are elevated in bronchoalveolar lavage, sputum, and sera of children with cystic fibrosis. Pediatr. Res. **34:**159–161.
8. **DiMango, E. D., H. Zar, R. Bryan, and A. S. Prince.** 1995. Diverse *P. aeruginosa* gene products stimulate respiratory epithelial cells to express interleukin-8 production. J. Clin. Invest. **96:**2204–2210.
9. **Drake, D., and T. C. Montie.** 1988. Flagella, motility and invasive virulence of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **134:**43–52.
10. **Farinha, M. A., B. D. Conway, L. M. G. Glasier, N. W. Ellert, R. T. Irvin, R. Sherburne, and W. Paranchych.** 1994. Alteration of the pilin adhesin of *Pseudomonas aeruginosa* PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte cells and decreased virulence in mice. Infect. Immun. **62:**4118–4123.
11. **Fichtenbaum, C. J., K. F. Woeltfe, and W. G. Powderly.** 1994. Serious *Pseudomonas aeruginosa* infections in patients infected with human immunodeficiency virus: a case control study. Clin. Infect. Dis. **19:**417–422.
12. **Frank, D. W., G. Nair, and H. P. Schweizer.** 1994. Construction and characterization of chromosomal insertional mutations of the *Pseudomonas aeruginosa* exoenzyme S trans-regulatory locus. Infect. Immun. **62:**554–563.
13. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000–3009.
14. **Gruenert, D. C., C. B. Basbaum, M. J. Welsh, W. E. Finkbeiner, and J. A. Nadel.** 1988. Characterization of human tracheal epithelial cells transformed by an origin defective simian virus 40. Proc. Natl. Acad. Sci. USA **85:**5951–5955.
15. **Hancock, R. E. W., L. M. Mutharia, L. Chan, R. P. Darveau, D. P. Speert, and G. B. Pier.** 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum sensitive, nontypable strains deficient in lipopolysaccharide side chains. Infect. Immun. **42:**170–177.
16. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
17. **Kudoh, I., J. P. Wiener-Kronish, S. Hashimoto, J.-F. Pittet, and D. Frank.** 1994. Exoproduct secretions of *Pseudomonas aeruginosa* strains influence severity of alveolar epithelial injury. Am. J. Physiol. **267:**L551–L556.
18. **Leigh, L., B. J. Stoll, M. Rahman, and J. McGowan.** 1995. *Pseudomonas aeruginosa* infection in very low birth weight infants: a case-control study. Pediatr. Infect. Dis. J. **14:**367–371.
19. **Mahenthiralingam, E., M. E. Campbell, and D. P. Speert.** 1994. Non-motility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. Infect. Immun. **62:**596–605.
20. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
21. **Mody, C. H., D. E. Buser, R. M. Syme, and D. E. Woods.** 1995. *Pseudomonas aeruginosa* exoenzyme S induces proliferation of human T lymphocytes. Infect. Immun. **63:**1800–1805.
22. **Morissette, C., E. Skamene, and F. Gervais.** 1995. Endobronchial inflammation following *Pseudomonas aeruginosa* infection in resistant and susceptible strains of mice. Infect. Immun. **63:**1718–1724.
23. **Myers, R. W., R. T. Lee, Y. C. Lee, G. H. Thomas, L. W. Reynolds, and Y. Uchida.** 1980. The synthesis of 4-methylumbelliferyl α-ketoside of N-acetylneuraminic acid and its use in a fluorometric assay for neuraminidase. Anal. Biochem. **101:**166–174.
24. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127–1130.
25. **Pemberton, J. M.** 1973. F116: a DNA bacteriophage specific for the pili of *Pseudomonas aeruginosa* strain PAO. Virology **55:**558–560.
26. **Richman-Eisenstat, J. B. Y., P. G. Jorens, C. A. Hebert, I. Ueki, and J. A. Nadel.** 1993. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway disease. Am. J. Physiol. **264:**L413–L418.
27. **Saiman, L., and A. Prince.** 1993. *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J. Clin. Invest. **92:**1875–1880.
28. **Seed, P. C., L. Passador, and B. H. Iglewski.** 1995. Activation of the *Pseudomonas aeruginosa lasI* gene by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. J. Bacteriol. **177:**654–659.
29. **Simpson, D. A., R. Ramphal, and S. Lory.** 1992. Genetic analysis of *Pseudomonas aeruginosa* adherence: distinct genetic loci control attachment to epithelial cells and mucins. Infect. Immun. **60:**3771–3779.
30. **Sordelli, D. O., M. Djafari, V. E. Garcia, P. A. Fontan, and G. Doring.** 1992. Age-dependent pulmonary clearance of *Pseudomonas aeruginosa* in a mouse model: diminished migration of poylmorphonuclear leucocytes to *N*-formylmethionyl-leucyl-phenylalanine. Infect. Immun. **60:**1724–1727.
31. **Speert, D. E., B. A. Loh, D. A. Cabral, and I. E. Salit.** 1986. Nonopsonic phagocytosis of nonmucoid *Pseudomonas aeruginosa* by human neutrophils and monocyte-derived macrophages is correlated with bacterial piliation and hydrophobicity. Infect. Immun. **53:**207–212.
32. **Tang, H., M. Kays, and A. Prince.** 1995. Role of *Pseudomonas aeruginosa* pili in acute pulmonary infection. Infect. Immun. **63:**1278–1285.
33. **von Specht, B.-U., B. Knapp, G. Muth, M. Broker, K.-D. Hungerer, K.-D. Diehl, K. Massarat, A. Seeman, and H. Domdey.** 1995. Protection of immunocompromised mice against lethal infection with *Pseudomonas aeruginosa* by active or passive immunization with recombinant *P. aeruginosa* outer membrane protein F and outer membrane I fusion proteins. Infect. Immun. **63:**1855–1862.
34. **Woods, D. E., J. A. Bass, W. G. Johanson, Jr., and D. C. Straus.** 1980. Role of adherence in the pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. Infect. Immun. **30:**694–699.
35. **Woods, D. E., M. S. Schaffer, H. R. Rabin, G. D. Campbell, and P. A. Sokol.** 1986. Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. J. Clin. Microbiol. **24:**260–264.
36. **Woods, D. E., P. A. Sokol, L. E. Bryan, D. G. Storey, S. J. Mattingly, H. J. Vogel, and H. Ceri.** 1991. In vivo regulation of virulence in *Pseudomonas aeruginosa* associated with genetic rearrangement. J. Infect. Dis. **163:**143–149.
37. **Woods, D. E., D. C. Straus, W. G. Johanson, Jr., V. K. Berry, and J. A. Bass.** 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. **29:**1146–1151.

Editor: B. I. Eisenstein