Relationship between Cytotoxicity and Corneal Epithelial Cell Invasion by Clinical Isolates of *Pseudomonas aeruginosa*

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We have reported that some strains of *Pseudomonas aeruginosa* can enter corneal epithelial cells during experimental murine eye infection and when the cells are cultured in vitro. Following invasion, both the host cell and the intracellular bacteria can remain viable for up to 24 h. Others have reported that toxin-mediated damage of epithelial cells contributes to the pathogenesis of *P. aeruginosa* keratitis. To clarify the relationship between cell invasion and cytotoxicity, fourteen *P. aeruginosa* isolates were compared for their capacity to enter epithelial cells and for their ability to induce cytotoxicity. Bacterial invasion was quantified by gentamicin survival assays both in vivo and in vitro. Cytotoxicity was examined qualitatively by trypan blue exclusion assays and quantitatively by chromium release assays in vitro. A significant inverse correlation was found between the ability to induce cytotoxicity and epithelial cell invasion as measured by gentamicin survival assays. Both cytotoxic and noncytotoxic strains were identified among corneal and noncorneal isolates; all isolates that were not cytotoxic were capable of epithelial cell invasion. Efficient host cell invasion could not be demonstrated for cytotoxic strains; however, the gentamicin survival assay relies upon host cells retaining viability in order to yield useful results, and this may limit the effectiveness of this assay for testing epithelial cell invasion by cytotoxic strains. Since all of the corneal isolates that were tested were virulent in vivo, the results show that there are at least two different types of *P. aeruginosa*-induced disease, one caused by strains that are cytotoxic and the other involving bacteria that can enter epithelial cells and survive intracellularly without killing the host cell.

We have reported previously that *Pseudomonas aeruginosa* is able to invade corneal epithelial cells in a murine experimental model of corneal wound infection (4). Using an in vitro corneal cell invasion assay, we have shown that intracellular bacteria can survive within viable cells for up to 24 h (5). However, studies have suggested that some *P. aeruginosa* strains are able to kill eukaryotic cells, as was recently demonstrated for strain PA103 during interaction with Madin-Darby canine kidney (MDCK) cells (1). During gentamicin survival assays, which are commonly used to quantify bacterial invasion of host cells, intracellular bacteria are protected from killing by gentamicin, which does not penetrate efficiently into viable eukaryotic cells. If *P. aeruginosa* strains kill cells, intracellular bacteria may be released into the extracellular environment, or the host cells may allow the antibiotic to enter them. In both situations, the internalized bacteria would become susceptible to the antibiotic that normally kills only extracellular bacteria. We examined cytotoxic capability and the ability to invade cells by gentamicin survival assays for various isolates of *P. aeruginosa* recovered from human infections. The results show that there are both cytotoxic and noncytotoxic strains of *P. aeruginosa* among clinical isolates and that an inverse correlation exists between cytotoxicity and bacterial invasion of cells as quantified by the gentamicin survival assay.

MATERIALS AND METHODS

Animals. Six-week-old C57BL/6 mice were anesthetized by intraperitoneal injection with 0.2 ml of a cocktail containing 6.7 mg of ketamine hydrochloride per ml (Parke-Davis, Morris Plains, N.J.) and 1.3 mg of xylazine per ml (Haver,

Shawnee, Kans.). Following anesthesia, three full-thickness epithelial abrasions were produced on the left cornea with a 26-gauge needle. The cornea was immediately inoculated with 5 μ l of a bacterial suspension. At various times after infection, mice were sacrificed by cervical dislocation. All procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Preparation of bacteria. Ten nonmucoid human *P. aeruginosa* corneal isolates were tested: two serogroup O6 strains, 6294 and 6487; five serogroup O11 strains, 6073, 6077, 6206, 6382, and 6389; one serogroup O8 strain, 6354; and two serogroup O10 strains, 6452 and 6436. In addition, four noncorneal isolates of *P. aeruginosa* were tested: one serogroup O1 strain, PAK; one serogroup O7 strain, 1244; and two serogroup O2 strains, PAO1 and 19660. *Escherichia coli* HB101 was used as a control. Bacteria were grown overnight at 37°C on a Trypticase soy agar plate (3).

Preparation of primary cell cultures. SHEM was prepared as previously described (11), but with bovine pituitary extract (10 μ g/ml) used in place of cholera toxin. All reagents, apart from Ham's F-12 nutrient mixture (HyClone Laboratories, Logan, Utah) and Eagle minimal essential medium (Whittaker Bioproducts, Walkersville, Md.), were obtained from Sigma Chemical Co., St. Louis, Mo. Primary rabbit epithelial cell cultures were prepared as previously described (15). In brief, eyes were washed in saline and the corneas were excised and rinsed in Hanks balanced salt solution. The epithelium was removed after treatment of the anterior cornea with Dispase II (Boehringer Mannheim, Indianapolis, Ind.) in SHEM (15). Epithelial cells were grown in 15-mm-diameter tissue culture wells (Costar, Cambridge, Mass.) in the presence of SHEM. Ten- to 12-day-old cell cultures were used for all experiments.

Cytotoxicity assays. Two separate methods were used to measure cytotoxicity induced by *P. aeruginosa*. In the first method, $\sim 10^5$ corneal epithelial cells were incubated for 24 h with 12.5 μ Ci of sodium chromate (^{51}Cr) and cells were then washed to remove extracellular chromium. The bacterial inoculum was prepared by resuspension of bacteria into Ham's F-12 medium to an inoculum of 10^9 /ml. Triplicate wells of epithelial cells were incubated with 200 μ l of the bacterial suspension from each *P. aeruginosa* strain tested. One set of wells was not inoculated with bacteria and served as a control for spontaneous release of ^{51}Cr and to determine the total amount of radioactivity taken up by the cells. After 3 h of bacterial-epithelial cell interaction at 37°C, the supernatant and three 200- μ l washes of each well were collected and pooled, and the released radioactivity was measured with a gamma counter (Gamma 550; Beckman Instruments, Inc., Irvine, Calif.). The counts per minute of the radioactivity released from cells following lysis with the detergent Triton X-100 (0.5%) was divided by the total

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intracellular counts per minute initially present and multiplied by 100 to determine the percentage of chromium released from the epithelial cells.

In the second method, cytotoxicity was assessed in a semiquantitative manner by trypan blue dye exclusion assays. Unlabeled cells were incubated with bacteria as described above, and then trypan blue was added to each well. The percentage of cells that took up the stain was estimated by microscopy. A cytotoxicity score was based on the percentage of stained cells (1, <1%; 2, 1 to 5%; 3, 5 to 10%; 4, >15%). These experiments were masked such that the investigator making the estimates was not aware of either the identity of the bacterial strain used or the control wells that were not inoculated with bacteria. Each well was presented for assessment at least three times, and the average score was used in data analysis.

In vitro invasion assays. Cells were inoculated with 200 μ l of a suspension of Ham's F-12 medium containing $\sim 2 \times 10^5$ CFU of *P. aeruginosa*. Following a 1- or 3-h incubation period, the inoculum was removed and all wells were washed with 200 μ l of Ham's F-12 medium to remove nonassociated bacteria. Gentamicin (200 μ g/ml) was added for 2 h to kill extracellular bacteria, and then cells were washed once with medium to remove the antibiotic. Triton X-100 was added to lyse cells, the contents of the well were thoroughly mixed, and a viable count was performed to quantify survivors (intracellular bacteria). Other wells were used to determine that gentamicin was able to kill all of the bacteria present in a well. These wells were inoculated with bacteria and then lysed with Triton X-100 without prior antibiotic treatment. Gentamicin was then added for 2 h to ensure that all bacteria present in the well were killed by the antibiotic. As a control for each set of experiments, three empty wells were inoculated with the bacterial suspension to determine baseline adherence to plastic, and another three wells were used to confirm that bacteria that were attached to plastic were susceptible to gentamicin killing. At least three wells were used for each strain during each experiment, and experiments were repeated at least three times. The results are shown in the figures as the mean and the standard deviation of the data collected from one representative experiment.

In vivo invasion and association assays. C57BL/6 mice were anesthetized by intraperitoneal injection; corneas were scratch injured as previously described (4) and then inoculated with 10^8 CFU of *P. aeruginosa*. At least five mice were used for each strain. Three hours later, animals were killed and corneas were removed and washed in 5 ml of Ham's F-12 medium. Bacterial association with epithelial cells was determined by viable counts following homogenization of some of the corneas. To determine bacterial invasion, the remaining corneas were treated with gentamicin for 2 h and washed twice with Ham's F-12 medium prior to homogenization to release intracellular bacteria.

Transmission electron microscopy. Infected mouse eyes were removed immediately after sacrifice and prepared for transmission electron microscopy by standard methodology. In brief, whole eyes were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 3 h and then postfixed in 1% osmium tetroxide in phosphate buffer at room temperature for 2 h. Samples were dehydrated with a graded series of ethanol and embedded in resin. Thin sections of the cornea were examined with a Zeiss EM 100A electron microscope set at 60 kV.

Statistics. The Spearman rank correlation coefficient test was used to determine correlation among the various tests of invasion and cytotoxicity. The Mann-Whitney U test was used to analyze the in vivo data.

RESULTS

Cytotoxicity measured by chromium release assays. There were major differences among corneal isolates of *P. aeruginosa* with regard to their ability to induce cytotoxicity of corneal epithelial cells as measured by chromium release assays (Fig. 1). Some strains were not cytotoxic in that they released <20% of the intracellular chromium, comparable to both spontaneous release and the negative control strain, *E. coli* HB101, while strains at the other end of the spectrum were highly cytotoxic, causing the release of greater than 40% of the radioactive chromium. The noncorneal isolate 19660, used extensively in corneal infection models (6-9), was found to be highly cytotoxic by this method.

Cytotoxicity measured by trypan blue exclusion assays. The trypan blue method visually illustrated the finding of little or no cytotoxicity with some strains and massive cell destruction with others (Fig. 2). Cytotoxicity occurred as expanding circular regions of dead and dying cells (Fig. 2C and D). Trypan blue exclusion assay scores obtained for the various strains (Fig. 3) correlated well with the quantitative results of the chromium release assays ($P < 0.0001$, Spearman rank correlation coefficient test).

Gentamicin survival assays to measure bacterial invasion. After 1 h of incubation with corneal cells, all *P. aeruginosa*

FIG. 1. Chromium release assay results. After uptake of radiolabeled chromium, *P. aeruginosa* isolates or *E. coli* HB101 isolates were added to primary cultures of rabbit corneal epithelial cells, and the amount of radiolabeled chromium released from cells over the next 3 h was measured and used to quantify bacterial cytotoxic activity. All *P. aeruginosa* strains were isolated from human corneal infections except strain 19660. The bars indicate the means of the counts per minute released, and the error bars indicate the standard deviations of the data.

strains invaded at least 50-fold more efficiently than *E. coli* HB101, and the differences among the *P. aeruginosa* strains were relatively small (Fig. 4). There was no correlation between invasion results at 1 h and cytotoxicity results at 3 h ($P = 0.7$ compared with chromium release assays and $P = 0.4$ compared with trypan blue assays). By 3 h, there were large increases in the number of viable bacteria recovered from within cells for strains with low cytotoxicity (Fig. 4). For example, strains 6487 and 6294 had nearly a 50-fold increase in epithelial cell invasion at 3 h compared with the invasion measured at 1 h. In contrast, for cytotoxic strains (those releasing $\geq 25\%$ of the chromium as shown in Fig. 1 or yielding a score of ≥ 2 as shown in Fig. 3), the number of gentamicin survivors did not increase significantly according to a comparison of invasion in 1 h with invasion in 3 h. In fact, invasion levels of some cytotoxic strains actually decreased between 1 and 3 h and were lower than results obtained after 3 h with the non-invasive strain *E. coli* HB101. The difference in behavior between cytotoxic and noncytotoxic strains was so striking that at 3 h there was a significant inverse correlation between invasion results and cytotoxicity as measured by either chromium release assays ($P = 0.010$, Spearman rank correlation test) or trypan blue exclusion assays ($P = 0.009$, Spearman rank correlation test). This inverse correlation between the ability to survive within cells during gentamicin treatment and cytotoxic ability was also observed for three nonocular strains of *P. aeruginosa* tested. All three strains (PAO1, 1244, and PAK) were efficient invaders when epithelial cells were inoculated with 2×10^5 CFU of bacteria for 3 h ($[4.2 \pm 0.1] \times 10^4$ CFU, $[1.1 \pm 0.2] \times 10^4$ CFU, and $[3.8 \pm 0.3] \times 10^3$ CFU, respectively) compared with the negative control strain *E. coli* HB101 ($[4.1 \pm 0.5] \times 10^2$ CFU), and none of these *P. aeruginosa* strains were cytotoxic as determined in trypan blue exclusion assays (trypan blue exclusion assay score = 1 for all samples).

Intracellular bacterial invasion in vivo. An inverse correlation between in vitro cytotoxicity and intracellular invasion by *P. aeruginosa* strains, as determined by gentamicin survival assays, was also noted in corneas infected for 3 h in vivo (Fig. 5). However, in this instance only the most cytotoxic strains ($\geq 40\%$ chromium release as shown in Fig. 1) showed reduced

FIG. 2. Cytotoxicity as observed with the trypan blue exclusion assay. The trypan blue exclusion assay allows for the visual examination of corneal cell cytotoxicity by *P. aeruginosa* isolates. (A) Uninoculated corneal cells (magnification, ca. $\times 30$); (B) 3-h incubation with strain 6294; no cytotoxicity observed (magnification, ca. $\times 30$); (C) 3-h incubation with strain 6206 (massive cytotoxicity was observed) (magnification, ca. $\times 30$); (D) same as C (cytotoxicity occurred as expanding circular regions) (magnification, ca. $\times 80$).

FIG. 3. Trypan blue exclusion assay results. Strains of *P. aeruginosa* were evaluated for their cytotoxic activity by assigning a score between 1 and 4 based on an estimation of the percentage of cells that had taken up the dye trypan blue (1, no cytotoxicity; 4, staining observed for $\geq 15\%$ of cells). Bars represent the means of triplicate determinations, and error bars, where shown, indicate the standard deviations. (Some results have no error bars; this occurred since there was no variability in scores given in multiple observations.) Cytotoxicity results correlated well with those obtained with chromium release assays ($P = 0.0001$, Spearman rank correlation coefficient test). Two of the 10 ocular isolates (strains 6487 and 6294) exhibited no cytotoxicity in this assay.

levels of invasion by gentamicin survival assays ($P = 0.0086$ by Mann-Whitney U test, $n = 35$ determinations). There was no significant difference in in vivo intracellular survival between the two noncytotoxic strains 6487 and 6294 and the moderately cytotoxic strains (defined as $<40\%$ chromium release as shown in Fig. 1, $P > 0.05$).

The difference in in vivo intracellular survival between highly cytotoxic and less cytotoxic strains was not due to differences in their ability to colonize the cornea in vivo; there were no significant differences between any of the strains tested in the

FIG. 4. Intracellular invasion by *P. aeruginosa* strains in vitro. Viable intracellular bacteria in corneal cells were quantified by gentamicin survival assays. After 1 h of incubation of 2×10^5 bacteria with epithelial cells, all *P. aeruginosa* strains invaded more efficiently than *E. coli* HB101, and the differences among *P. aeruginosa* strains were relatively small. After 3 h of incubation with 2×10^5 bacteria, there were large increases in invasion only for strains that were not cytotoxic. Bars represent the means of triplicate determinations, and error bars indicate the standard deviations. Invasion results for some cytotoxic strains decreased and became lower than those for *E. coli* HB101. There was a significant inverse correlation between 3-h invasion results and cytotoxicity as measured by either chromium release assays (Fig. 1) ($P = 0.010$) or trypan blue exclusion assays (Fig. 2) ($P = 0.009$).

FIG. 5. Intracellular invasion by *P. aeruginosa* strains in vivo. Viable intracellular bacteria in corneal cells in intact eyes were quantified by gentamicin survival assays. For whole mouse corneas infected in vivo for 3 h following scratch injury, only the most cytotoxic strains showed reduced levels of invasion by gentamicin survival assays ($P = 0.0086$, Mann-Whitney U test; $n = 35$). There was no significant difference in invasion in vivo between the two noncytotoxic strains (6487 and 6294) and the moderately cytotoxic strains (yielding $\leq 40\%$ release of chromium, $P > 0.05$). The bars represent the medians of the data; upper and lower quartiles are recorded above the bars.

total number of bacteria recovered from corneas after 3 h of infection in vivo (the median [lower quartile, upper quartile] CFU values with the various *P. aeruginosa* strains were as follows: 6294, $1.1 [0.8 \text{ to } 1.2] \times 10^6$; 6487, $1.1 [0.9 \text{ to } 1.4] \times 10^6$; 6452, $1.1 [0.9 \text{ to } 1.7] \times 10^6$; 6382, $1.4 [1.1 \text{ to } 1.7] \times 10^6$; 6389, $1.5 [1.2 \text{ to } 2.0] \times 10^6$; 19660, $0.7 [0.7 \text{ to } 1.2] \times 10^6$; 6206, $1.0 [0.8 \text{ to } 1.9] \times 10^6$).

Electron microscopy. Numerous studies (6-9) have documented the ability of the cytotoxic *P. aeruginosa* 19660 strain to cause corneal infections in mice, but less information is available regarding the ability of noncytotoxic strains to cause corneal disease in mice. We examined the ultrastructural changes that occurred in a scratched cornea infected with *P. aeruginosa* 6294, a strain that is invasive but not cytotoxic (Fig. 6). In the region where the cornea was scratched and inoculated with bacteria, there was massive inflammation of the underlying stroma (the relatively acellular region located beneath the epithelium). Polymorphonuclear leukocytes were present in the stroma, thus confirming that the swelling was due to inflammation (these cells are not found in healthy corneas). The overlying epithelial cells were stretched out of shape (probably because of traction by the edematous stroma); otherwise, the epithelial cells appeared healthy. Corneas that were scratched but not inoculated with bacteria did not demonstrate corneal inflammation, and in contrast to inoculated corneas, the scratched area healed in several hours.

Partial characterization of cytotoxic activity. With strain 6206, neither bacteria that were heat killed nor bacterial culture supernatants (from which bacteria were removed) were able to induce corneal cell cytotoxicity (trypan blue exclusion assay score = 1 for all samples). Bacteria that were thoroughly washed before inoculation remained cytotoxic to cells in vitro as measured by the trypan blue exclusion assay (score = 4 for all samples), thus confirming that the bacteria and not toxic exoproducts produced by growth on Trypticase soy agar plates were responsible for cytotoxicity. Cytotoxicity did not occur when assays were performed at 4°C (score = 1 for all samples). These results indicated that viable whole bacteria were re-

FIG. 6. Micrograph of cornea infected with strain 6294, which is not cytotoxic to epithelial cells in vitro, demonstrates how corneal disease occurs in vivo without direct epithelial cell cytotoxicity. The corneal cells appear healthy; however, the entire epithelial layer is physically torn away from an inflamed and swollen underlying stroma. The presence of polymorphonuclear leukocytes in the stroma is indicative of inflammation, since these are normally not present in this tissue. These features suggest that disease caused by this strain is mostly inflammatory in nature. E, epithelium; S, stroma; P, polymorphonuclear leukocyte.

quired to initiate cytotoxicity in vitro. Most cytotoxic strains did not carry plasmids, which indicated that genes coding for bacterial cytotoxic factors were not plasmid borne (data not shown).

DISCUSSION

We have found a significant inverse relationship among 14 *P. aeruginosa* strains in their ability to invade corneal epithelial cells after 3 h and their ability to induce epithelial cell cytotoxicity. Strains could be classified as either cytotoxic, according to chromium release or trypan blue dye exclusion assays, or invasive, according to gentamicin survival assays. The results of in vivo experiments supported the in vitro findings: highly cytotoxic strains were found in significantly lower numbers inside cells during experimental infection in mice than were *P. aeruginosa* isolates with low-to-moderate in vitro cytotoxicity, despite the fact that there was no difference among any of the strains in their ability to colonize the cornea. Therefore, it appears that the determination as to whether a strain of *P. aeruginosa* is invasive in epithelial cells is related to its ability to kill eukaryotic cells, with strains with low cytotoxicity showing high levels of invasion and vice versa. Results of gentamicin survival assays are generally believed to reflect the number of bacteria that have invaded cells. Our results suggest that this assay may not accurately assess the ability of cytotoxic strains to invade cells, since gentamicin is excluded only by viable eukaryotic cells. Even if cytotoxic strains did initially invade cells, once the cell was killed by the bacterium either the gentamicin could enter the host cells and kill the intracellular organisms or the internalized bacteria would be released from dead cells and be killed by extracellular antibiotic, thereby giving false-negative results. This phenomenon may explain the inverse correlation between invasion and cytotoxicity when invasion was measured 3 h after bacteria were applied to corneal cells.

To document that cytotoxic strains could initially invade corneal epithelial cells, we modified the gentamicin survival assay by measuring intracellular bacteria after only 1 h of bacterial interaction with the corneal cells. The results of these 1-h assays indicated that cytotoxic strains were able to invade cells prior to death of the target cells, since at this time there were no significant differences in invasion levels among the various *P. aeruginosa* isolates, and each strain was at least 50-fold more invasive than *E. coli* HB101. In vivo, however, the effect of cytotoxic activity with regard to measuring intracellular bacterial levels was less pronounced, in that the moderately cytotoxic strains invaded in numbers similar to those of non-cytotoxic strains. These results suggest that most *P. aeruginosa* strains can enter corneal cells initially and that murine corneal cells in vivo may be more resistant to *P. aeruginosa* cytotoxicity than rabbit corneal cells in vitro.

Two studies have documented that *P. aeruginosa* invades rabbit corneal epithelial cells during infection (14, 17). Using electron microscopy, the first study described gradual engulfment of bacteria by corneal cells with no evidence of cell destruction, suggesting that the strain used had low cytotoxic activity. The second of these reports described pocket formation and embedding of the bacterium in the cell membrane, followed by cellular engulfment, but noted that engulfment was then followed by destruction of epithelial cells. This result may be indicative of what occurs in the cornea during infection with a cytotoxic strain.

A large number of elegant studies of *P. aeruginosa* corneal infection have been carried out in the laboratories of Hazlett and Berk with strain 19660 (6–9), which we found to be cyto-

toxic. These investigators showed that cell lysis (cytotoxicity) is involved in the loss of the epithelium during infection with strain 19660 (6, 8, 9). Corneas infected with virulent strains of *P. aeruginosa* often lose the epithelial layer in the affected area, leaving an exposed stroma (10). While direct epithelial cell cytotoxicity may account for this finding with some strains, our results suggest that detachment of the epithelial cell layer following infection with an invasive but poorly cytotoxic strain such as *P. aeruginosa* 6294 may be due to massive swelling of the underlying stroma. This swelling results in stretching and tearing of the epithelial layer, which clearly lacks the elasticity to remain attached to the swollen stroma (Fig. 6). The swelling likely is secondary to inflammation induced by host immune factors, which has been reported to be an important component of the damage to the cornea during infection with *P. aeruginosa* (6, 12, 18). Indeed, it appears that corneal disease due to strain 6294 may largely result from inflammatory insults, as indicated by the swollen stroma and intrastromal invasion by polymorphonuclear leukocytes (Fig. 6). The role of *P. aeruginosa* epithelial cell invasion in the development of the inflammatory aspects of corneal disease is not yet clear; however, it has been shown that bacterial entry into cells can stimulate the production of cytokines such as interleukin 8, a potent mediator of inflammation (2); meanwhile, bacteria and bacterial products are protected from factors of the immune system while they reside within host cells.

These experiments were performed with corneal epithelial cells, and most of the cytotoxic strains studied were corneal isolates. The findings are, however, likely to apply to *P. aeruginosa* infections involving other systems. Various aspects of *P. aeruginosa* cytotoxicity were recently studied for MDCK cells incubated with strain PA103 (1). Similar to our findings for corneal cell infection with cytotoxic isolates, a circular pattern of MDCK cell toxicity was observed, and cytotoxicity was not seen with the bacterial culture supernatant, with heat-killed bacteria, or during incubation at 4°C. Strain PA103 was observed inside MDCK cells, but these were generally cells that had been made permeable and were dead. Although strains that were less cytotoxic for MDCK cells were identified, they were not assessed for their ability to invade cells. This study also found that cell injury correlated with the production of the 53-kDa form of exoenzyme S or other coordinately regulated factors and that MDCK cells with defects in cell surface glycosylation were resistant to cell injury. Elastase and exotoxin A were not involved. Similar findings were noted with strain PA103 during in vivo infection of the rabbit lung (13).

The significance of our results, which indicate that there is more than one pathway to disease caused by *P. aeruginosa*, extends to both our understanding of the role of various virulence factors in pathogenesis and the development of methods for treatment. Two recent studies found different results for the role of the *exxA* gene in *P. aeruginosa* virulence. Each group of investigators used an animal model for lung infection, but they used different strains. In one study, PA103, a cytotoxic strain, was used (13). A mutation in the *exxA* gene in this strain led to a total loss in the ability to induce cytotoxicity, and the effect was to render this strain avirulent. The other investigators found that the mutation in *exxA* in another strain, PAO1, did not affect virulence (19). This could be explained by the fact that, unlike PA103, PAO1 does not use cytotoxicity as a virulence mechanism; our results showed that PAO1 is not a cytotoxic strain, which confirmed previous results with this strain with MDCK cells (1). This strain is, however, able to cause disease, both in the cornea and in the lung (16, 19). If the host immune response, rather than direct bacterial toxicity, is involved in producing tissue damage during infection with

some *P. aeruginosa* strains—as is suggested by our findings for strains such as 6294—then infections with these strains might benefit from treatments that include anti-inflammatory agents.

In conclusion, we have found that clinical isolates of *P. aeruginosa* vary in their ability to induce cytotoxicity and that this ability is inversely correlated with their ability to invade epithelial cells as measured by gentamicin survival assays. Both cytotoxic and noncytotoxic invasive strains tested in this study are able to cause corneal disease and with similar infectious doses (16), suggesting that there are two different types of *P. aeruginosa*-induced disease, one that involves direct host cell cytotoxicity and another type caused by strains that can survive within viable host cells. The differences should be considered by investigators studying the pathogenesis of *P. aeruginosa* infections.

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