CONCISE COMMUNICATION

Penetration of Clinical Isolates of *Pseudomonas aeruginosa* through MDCK Epithelial Cell Monolayers

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*Pseudomonas aeruginosa* causes both invasive (bacteremic) and chronic noninvasive infections. A simple in vitro system to screen *P. aeruginosa* clinical isolates for their capacity to penetrate MDCK cell monolayers has been developed. By means of this system, *P. aeruginosa* clinical isolates, including 32 blood and 45 respiratory isolates, were examined. When monolayers were infected with $3.5 \times 10^7$ cfu of bacteria, significantly more blood (93.7%) than respiratory (54.4%) isolates ($P < .001$) were detected in the basolateral medium after 3 h. Penetration ability was usually independent of cytotoxicity. Only 8 (4 blood and 4 respiratory) isolates were cytotoxic, possessed *exoU*, and passed through the monolayer after epithelial cell death, associated with a marked drop in transepithelial electrical resistance. Conversely, noncytotoxic isolates with high penetration ability but without severe epithelial damage were invasive. This system is well suited for screening clinical isolates and their mutants for specific genes conferring the invasiveness phenotype.

*Pseudomonas aeruginosa* is an important pathogen among patients with compromised immunity, including those with cystic fibrosis (CF), neutropenia, thermal burns, and AIDS [1]. Among patients with CF or diffuse panbronchiolitis, *P. aeruginosa* is the most predominant pathogen, causes chronic infection, and is difficult to eliminate, but usually it does not invade the bloodstream. This organism, however, is invasive in other patient groups and causes bacteremia associated with high mortality, particularly in those with neutropenia.

Bacteria have to penetrate epithelial barriers to invade the bloodstream. In addition to the host’s status, virulence factors of the pathogen could be involved in different types of infections induced by *P. aeruginosa*. Indeed, human blood isolates of *P. aeruginosa* cause lethal endogenous bacteremia in mice with neutropenia induced by cyclophosphamide, but human respiratory isolates do not [2]. Blood isolates penetrate human intestinal Caco-2 epithelial cell monolayers to a greater degree than do respiratory isolates [3]. These phenotypic findings suggest that blood isolates carry virulence determinants that confer the invasive phenotype. Despite evidence of putative virulence determinants in *P. aeruginosa*, none has been characterized yet.

Recently, it was reported that *P. aeruginosa* strains can be differentiated into 2 groups: strains with a cytotoxic phenotype, such as PA103, and strains with an invasive but noncytotoxic phenotype, such as PAO1 [4]. Whereas the invasiveness of *P. aeruginosa* has been evaluated in MDCK cells by gentamicin survival assay [4], penetration of *P. aeruginosa* isolates through MDCK cell monolayers has not been investigated. Recently, it was also reported that *P. aeruginosa* PA103 carries *exoU*, which encodes a 70-kDa cytotoxic protein (ExoU), whereas PAO1 lacks it [5]. However, a correlation between its presence and the clinical sources of different *P. aeruginosa* strains has not been investigated.

To address these points, we developed an MDCK cell monolayer penetration assay [6] as an in vitro screening system for virulence of *P. aeruginosa*. When grown on permeable supports, polarized MDCK cells establish a monolayer with tight junctions, akin to Caco-2 cells [7, 8]. By use of this system, we evaluated a total of 77 clinical isolates of *P. aeruginosa* for their capacity to penetrate through MDCK cell monolayers and examined their motility, piliation, serum sensitivity, cytotoxicity for MDCK cells, and possession of *exoU*. 

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Materials and Methods

Bacterial strains. A total of 77 clinical isolates of P. aeruginosa, including 32 blood and 45 respiratory isolates, were evaluated. All blood isolates and 5 respiratory isolates were obtained at Nagasaki University Hospital, Nagasaki, Japan. The other respiratory isolates were the initial isolates obtained from patients with CF at British Columbia’s Children’s Hospital and Shaughnessy Hospital, Vancouver, Canada. The motility, piliation, and serum sensitivity of P. aeruginosa strains were evaluated as described elsewhere [9]. P. aeruginosa PAO1 and PA103 were provided by B. H. Iglewski (University of Rochester School of Medicine and Dentistry, Rochester, NY). Salmonella typhimurium SL1344 was used as a positive control for the MDCK cell monolayer penetration assay. Noninvasive rabbit enterotoxigenic Escherichia coli strain RDEC-1, which is positive for oxidation of lactose, was used as a negative control and as an internal control of monolayer integrity. P. aeruginosa strains P1 and M2 were used as serum-sensitive and -resistant controls, respectively [10].

Monolayer penetration assay. MDCK cells in MEM with 10% fetal bovine serum were seeded at 1.5 × 10^5 cells/well in Transwell filter units (Costar, Cambridge, MA) containing 0.33-cm² porous filter membranes (3.0-µm pores). After 4 days of incubation at 37°C in 5% CO₂, monolayers were infected with bacteria by adding 5 µL (~3.5 × 10⁵ cfu) of freshly grown bacteria cultured in Luria broth overnight at 37°C with 150-rpm shaking. Because MDCK cells grow to ~3.5 × 10⁵ DMCK cells/filter after 4 days of culture [6], the MOI was ~100:1 bacteria/cell. Bacteria in the basolateral medium were counted by plating appropriate dilutions at several time intervals. In some experiments, equal numbers of E. coli RDEC-1 were added with P. aeruginosa isolates at the same time to check for monolayer integrity, because the noninvasive E. coli strain does not penetrate the monolayer unless the tight junctions are disrupted by Ca²⁺-free medium [6, 8]. The basolateral medium was plated on MacConkey agar to distinguish lactose-positive E. coli colonies from others. Transmonolayer electrical resistance (TER) was measured with a Millicell-ESR apparatus (Millipore, Bedford, MA) at sequential timed intervals and was calculated as reported elsewhere [6, 8].

Cytotoxicity assay. To quantify cytotoxicity of P. aeruginosa isolates on the MDCK cell monolayers, the concentration of lactate dehydrogenase (LDH) released from the MDCK cells into the medium in the filter units was measured by use of a cytotoxicity assay kit (Cytotox 96; Promega, Madison, WI).

Detection of exoU. Both polymerase chain reaction (PCR) and dot blot hybridization analysis were used to detect exoU from P. aeruginosa isolates. Purification of DNA from the isolates, PCR, and dot blot hybridization were done as described elsewhere [11]. The primers used for PCR detection of exoU were designed from the sequence published elsewhere [5] for this study (accession no. U97065), as follows: sense, 5'-TAGAACGCCTATTGCGC-3'; antisense, 5'-CTCGAGCTGACCATTT-3'. A DNA probe for dot blot hybridization was prepared by amplification of a 572-bp fragment of exoU from P. aeruginosa PA103 by PCR and was simultaneously labeled with digoxigenin-11-uridine-5-triphosphate (Boehringer Mannheim, Laval, Canada), as described elsewhere [11].

Statistics. The Mann-Whitney U test was used for comparison of frequencies of phenotypes and genotype.

Results

Penetration of representative isolates through MDCK cell monolayers. P. aeruginosa PAO1 was detected in the basolateral medium by 3 h after inoculation, as was S. typhimurium SL1344, whereas P. aeruginosa strain PA103 was not detected until 6 h (figure 1A). E. coli RDEC-1 was not detected by 12 h and did not appear in the basolateral medium until at least 24 h after inoculation. There was no difference in growth rate between P. aeruginosa PAO1 and PA103 (data not shown).

Penetration of P. aeruginosa clinical isolates and phenotypes. P. aeruginosa isolates were divided into 4 groups on the basis of clinical site of isolation and their capacity to penetrate through MDCK cell monolayers (table 1). Of 32 blood isolates of P. aeruginosa, 30 (93.7%) were detected in the basolateral medium by 3 h, as were P. aeruginosa PAO1 and S. typhimurium SL1344. A total of 24 (54.4%) of 45 respiratory isolates of P. aeruginosa were detected in the basolateral medium by 3 h; this percentage was significantly lower than that among blood isolates (P < .001). Significantly greater numbers of serum-sensitive and nonmotile phenotypes were found among respiratory isolates than blood isolates (P < .001 and P < .05, respectively). There was no difference in PO4 phage sensitivity between blood and respiratory isolates (P = .559).

Cytotoxicity of P. aeruginosa clinical isolates and possession of exoU. LDH was released into the apical medium from the MDCK monolayer inoculated with PA103 by 3 h. When the monolayer was infected with PAO1, LDH was not detected in substantial levels until 10 h. LDH was not detected in the basolateral medium until 6 h and 10 h after infection with PA103 and PAO1, respectively. When cytotoxic strains were defined as those causing statistically significant levels of LDH release from MDCK cell monolayers in apical medium by 6 h, compared with spontaneous LDH release, 8 (10.4%) of 77 clinical P. aeruginosa isolates were cytotoxic (table 1). There was no significant difference in cytotoxicity between blood and respiratory isolates (4 blood and 4 respiratory; P = .609). The expected 572-bp fragment of exoU was detected by PCR from these 8 isolates only. All of these isolates were also positive for exoU by dot blot hybridization.

Coinfection of P. aeruginosa isolates and noninvasive E. coli RDEC-1. When either P. aeruginosa PAO1 or B16 was added to the apical medium simultaneously with E. coli RDEC-1, P. aeruginosa alone was detected in the basolateral medium at 3 h, whereas E. coli RDEC-1 did not appear until 10 h after inoculation (figure 1B and 1D). Similar results were obtained when the monolayer was infected with either P. aeruginosa B15 or B51 together with E. coli RDEC-1 (data not shown). In contrast, when cytotoxic P. aeruginosa strains PA103 or S50 (figure 1C and 1E) and B22 (data not shown) were each in-
Figure 1. A. Penetration of *Pseudomonas aeruginosa* PAO1 and PA103, *Salmonella typhimurium* SL1344, and *Escherichia coli* RDEC-1 through MDCK cell monolayers. Bacteria were inoculated at \(3.5 \times 10^3\) cfu/well to apical surfaces of MDCK cell monolayers. Assay was done in triplicate, and results are expressed as mean ± SD. Noninvasive *E. coli* RDEC-1 could not be recovered from basolateral medium up to 12 h after inoculation. B-E. Coinfection of *P. aeruginosa* isolates and *E. coli* RDEC-1 and their penetration through MDCK cell monolayer. Equal number of *E. coli* RDEC-1 was added to the apical chamber simultaneously with *P. aeruginosa*. Basolateral medium was plated on MacConkey agar to distinguish lactose-positive *E. coli* colonies from *P. aeruginosa* colonies.

Inoculated together with *E. coli* RDEC-1, RDEC-1 was detected at the same early time as the *P. aeruginosa* isolates.

*Changes in TER after *P. aeruginosa* infection.* TER of monolayers infected with *P. aeruginosa* PAO1 or noncytotoxic clinical isolates decreased in a time-dependent manner, similar to results with *S. typhimurium* SL1344. Cytotoxic isolates appeared in the basolateral medium later than did noncytotoxic strains but showed a markedly faster drop in TER than did these isolates. Between 4 and 10 h after infection, TER of the MDCK cell monolayers infected with PA103 and TER of cytotoxic clinical isolates were significantly lower (\(P < .05\)) than that of monolayers infected with PAO1 and noncytotoxic isolates (e.g., mean ± SD, 231 ± 109 vs. 574 ± 34 \(\Omega\)cm² at 4 h).

**Discussion**

It has been reported that the penetration of *Salmonella* through MDCK cell monolayers is similar to that through
Caco-2 cell monolayers [7]. In the current study, we applied the MDCK cell monolayer system for evaluation of several clinical isolates of *P. aeruginosa*. The data obtained in this system correlated well with those from Caco-2 cell monolayer systems reported elsewhere [3] (data not shown). Most clinical blood isolates penetrated MDCK cell monolayers by 3 h (table 1), suggesting that they possess virulence determinants capable of inducing bacteremia. Because most persistent isolates from patients with CF are mucoid, serum sensitive, and nonmotile [10, 12], we chose initial colonizing isolates from different patients with CF in an effort to match them phenotypically with blood isolates. It appears that serum resistance and motility may be necessary, but not sufficient, for bacterial penetration through the monolayer, because several motile serum-resistant respiratory isolates failed to pass through the monolayer. The significance of pilation in the penetration of isolates through monolayers was not clearly demonstrated in this study (table 1).

It was reported recently that ExoU, secreted by the type III protein secretion system and regulated by the transcriptional activator ExsA, is produced by PA103 and other cytotoxic clinical isolates of *P. aeruginosa* and that its expression correlates with the cytotoxic phenotype [5]. Indeed, PA103 with mutation in its *exoU* or *exsA* gene changes its phenotype to noncytotoxic [13] and fails to injure the epithelium in an acute lung infection model in mice [5]. We have found 8 cytotoxic isolates carrying *exoU*. Our results in coinfection and TER studies clearly revealed that these cytotoxic isolates passed through monolayers following monolayer disruption and epithelial cell death due to intoxication, whereas noncytotoxic isolates did not markedly affect monolayer integrity. These data suggest that noncytotoxic isolates with high capacity to penetrate MDCK cell monolayers are invasive by transmigrating through epithelial cells.

CF transmembrane conductance regulator (CFTR) has been reported to be able to serve as an epithelial cell receptor for internalization of *P. aeruginosa* in the lung [14]. Moreover, entry of *exoU*-negative noncytotoxic *P. aeruginosa* into corneal cells was inhibited with monoclonal antibodies and peptides specific for CFTR amino acids, suggesting that CFTR-mediated internalization of *P. aeruginosa* by corneal cells is critical to the pathogenesis of eye infection [15].

There is a possibility that other unique determinants, in addition to CFTR, are involved in penetration of *P. aeruginosa* through MDCK cells. This study introduced a new approach to characterizing putative determinants of invasiveness in *P. aeruginosa*. The MDCK cell monolayer system described herein is well suited for screening multiple clinical isolates and mutants with alterations in specific genes.

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**References**


