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Pseudomonas aeruginosa Invasion of and Multiplication within Corneal Epithelial Cells In Vitro

SUZANNE M. J. FLEISZIG,* TANWEER S. ZAIDI, AND GERALD B. PIER

Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

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***Pseudomonas aeruginosa* is usually considered an extracellular pathogen. Using assays to determine intracellular survival in the presence of gentamicin, we have previously demonstrated that *P. aeruginosa* is able to invade corneal cells during infectious keratitis in mice. In vitro, *P. aeruginosa* was found to enter the following cells: human corneal cells removed by irrigation; epithelial cells in the cornea of rats, mice, and rabbits; and primary corneal epithelial cells cultured from rat and rabbit eyes. The level of invasion was related to the level of adherent or associated bacteria. In general, invasion was more efficient with cultured epithelial cells than with cells tested in situ. Invasion did not occur when assays were performed at 4°C. Cytochalasin D but not colchicine inhibited bacterial invasion, suggesting that bacterial entry was an endocytic process dependent on actin microfilaments but not microtubules. Bacteria that invaded cultured corneal epithelial cells were found to multiply within cells. The ability of *P. aeruginosa* to invade and multiply within corneal epithelial cells may contribute to the virulence of this organism during infectious keratitis, since intracellular bacteria can evade host immune effectors and antibiotics commonly used to treat infection.**

Pseudomonas aeruginosa corneal infection is rapidly progressive and difficult to treat and can lead to vision impairment. The majority of *P. aeruginosa* infections occur among contact lens wearers, a section of the population which is expanding in both developed and developing countries (1).

P. aeruginosa generally behaves as an extracellular pathogen. However, we recently reported that *P. aeruginosa* invades corneal epithelial cells during in vivo infection of mice (8). Using a model that involved a scratch injury to the cornea to allow infection to occur following bacterial inoculation and performing gentamicin survival assays on the excised infected tissue, we demonstrated that as many as 22% of bacteria that were associated with the cornea during infection were able to survive gentamicin treatment. By electron microscopy, we observed some bacteria within cells and others that were between cells surrounded by intact intercellular junctions. *P. aeruginosa* invasion of corneal cells has also been shown by electron microscopy to occur in the rabbit cornea during contact lens-associated infection (11) and during infection following corneal injury (18).

The ability to invade host cells is a virulence property of certain bacteria (4, 17), some of which are able to replicate inside the host cell, either within vacuoles or in the cytoplasm (4). Bacterial invasion follows adherence to the cell surface, generally involves active metabolism of both host and bacterial cells, and contributes to the infectious process by allowing the bacteria to evade host defenses and induce changes in host cell function (3). Intracellular *P. aeruginosa* may evade gentamicin and other antibiotics used clinically whenever these agents are unable to penetrate the host cell membrane (4). Although *P. aeruginosa* has been shown to invade various other cell types in culture (2, 13, 19), corneal cells remain the only cells for which *P. aeruginosa* invasion has been demonstrated during infection in vivo (8).

* Corresponding author. Present address: School of Optometry, 688 Minor Hall, University of California at Berkeley, Berkeley, CA 94720-2020. Phone: (510) 643 0990. Fax: (510) 643 5109.

In order to study *P. aeruginosa* invasion of corneal cells, we developed several in vitro models. We demonstrate here corneal cell invasion with whole animal eyes in organ culture, primary corneal epithelial cell cultures, and human superficial corneal epithelial cells removed by irrigation of the ocular surface. In addition, the results indicated that uptake of bacteria involved active function of host cells and that, after invasion, *P. aeruginosa* multiplied rapidly within cells in vitro.

MATERIALS AND METHODS

Preparation of modified SHEM medium. SHEM medium was prepared as previously described (10) but with bovine pituitary extract (10 μg/ml) used in place of cholera toxin. All reagents except Ham's F-12 nutrient mixture (HyClone Laboratories, Logan, Utah) and Eagle's minimal essential medium (Whittaker Bioproducts, Walkersville, Md.) were obtained from Sigma Chemical Co., St. Louis, Mo.

Preparation of bacteria. Bacteria were grown overnight at 37°C on a tryptic soy agar plate covered with a 12,000- to 14,000-molecular-weight-pore-size dialysis membrane (5). The inoculum was prepared by resuspension of bacteria from the membrane into Hanks' balanced salt solution (HBSS) or Ham's F-12 until the appropriate optical density was achieved. Unless otherwise specified, a corneal isolate of *P. aeruginosa* (strain 6294, serogroup O6) was used for all experiments. Other isolates used included *P. aeruginosa* PAO1 (serogroup O5) and *Escherichia coli* HB101.

Animals. For in vitro experiments, Wistar rats, New Zealand rabbits, and C57BL/6 mice were used. Animals were killed with an overdose of pentobarbital sodium (120 mg/kg), and both eyes were removed. For in vivo experiments, 6-week-old C57BL/6 mice were anesthetized by intraperitoneal injection with 0.2 ml of a cocktail containing 6.7 mg of ketamine hydrochloride (Parke Davis, Morris Plains, N.J.) and 1.3 mg of xylazine (Haver, Shawnee, Kans.) per ml as described before (8). At various times after infection, mice were killed by cervical dislocation. All procedures were done in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Interaction of bacteria with injured whole cornea in vitro. Eyes were removed from rats, and the corneal surface of each was injured by scratching (three scratches with a 26-gauge needle to produce full-thickness epithelial abrasions). Each eye was placed in a petri dish onto a sterile cotton pad soaked in HBSS, and the corneal surface was inoculated twice with 10^8 CFU of *P. aeruginosa*. The two aliquots were applied at 15-min intervals in 8-μl drops of HBSS. Following a 30-min incubation at 37°C, nonadherent bacteria were removed by sequentially rinsing the eyes in six separate washes of 5 ml of phosphate-buffered saline (PBS). Each eye was then placed into a well of a tissue culture plate and incubated at 37°C for a further 3.5 h. The eyes were then washed again six times to remove nonassociated bacteria, and the cornea was excised and rinsed in two

washes of 5 ml and one wash of 50 ml of PBS. Half of the corneas were incubated in 60 μl of HBSS containing 200 μg of gentamicin (Whittaker Bioproducts, Walkersville, Md.) per ml for 2 h at 37°C to kill extracellular bacteria (4) before being rinsed in two washes of 5 ml of PBS to remove the antibiotic. The corneas were then homogenized in 1 ml of tryptic soy broth with 0.5% Triton X-100 (Sigma Chemical Co.) added to lyse corneal cells and thus release any intracellular bacteria into solution. After a 15-min exposure to the detergent, a count of viable cells in the homogenate was performed in duplicate to determine the number of bacteria that had survived the antibiotic (intracellular organisms). Matching pairs of corneas were used to calculate the number of bacteria that had associated with the cornea (both extracellular and intracellular organisms) during the 4-h incubation. These corneas were homogenized and bacteria were counted without prior gentamicin treatment. Gentamicin was then added to the homogenate to ensure that bacteria previously associated with the cornea were not inherently resistant to gentamicin treatment. In control experiments, Triton X-100 was found not to alter the susceptibility of bacteria to killing by gentamicin (data not shown). The homogenate was washed by centrifugation at 12,000 rpm for 3 min to remove gentamicin before survivors were enumerated. In control experiments, it was established that 100% of the viable bacteria could be recovered after this washing procedure.

For each experiment, at least three eyes were used in each group, and experiments were repeated with rabbit and mouse eyes to confirm that any effects noted were not specific to rat cornea. All experiments were repeated at least twice. These mouse and rabbit corneas were inoculated with two aliquots of 5 and 30 μl of bacterial suspension, respectively.

Interaction of bacteria with uninjured whole cornea in vitro. The intact corneal surface of 16 rat eyes was incubated with bacteria for 2 h at 37°C. Nonadherent bacteria were dispersed from the eye by one quick wash in a large volume of PBS (50 ml). The cornea was removed from each eye and subjected to an extensive wash (submersion 50 times over a period of 2 min) in 1 ml of tissue culture medium to remove superficial exfoliating epithelial cells from the surface of the cornea. The number of bacteria that had invaded cells in the exfoliated cell suspension was compared with the number of internalized bacteria that remained on the cornea by gentamicin survival assays as described above.

Interaction of bacteria with exfoliated human corneal epithelial cells. Superficial corneal epithelial cells were collected from live human subjects with a corneal irrigation chamber, as previously described (5). The preparation of each cell sample involved the repetitive washing of both corneas. The cells in the 18 ml of the total eye wash that was collected from each eye were concentrated by centrifugation into 3 ml, and 10^6 bacteria were added for 4 h. This material was then divided into three aliquots; one was used to quantify association (total of adherent and intracellular bacteria), another was used to determine bacterial survival after exposure to gentamicin (intracellular bacteria), and the third was used to ensure that the antibiotic would neutralize all bacteria once they were extracellular by lysing cells with 0.5% Triton X-100 prior to treatment with 200 μg gentamicin per ml, as previously described (8). To ensure that bacteria were not protected from killing by gentamicin because of the ocular mucus in the sample (5), a control experiment was performed in which bacteria were incubated with 10 mg of porcine stomach mucin (Sigma Chemical Co.) per ml and then exposed to 200 μg of gentamicin per ml for 2 h. Mucin did not protect the bacteria from killing by the antibiotic (data not shown).

Interaction of bacteria with cultured epithelial cells. Primary rat and rabbit epithelial cell cultures were prepared as previously described (12). Briefly, eyes were washed in saline, and the corneas were excised and rinsed in HBSS. The epithelium was removed after treatment with Dispase II (Boehringer Mannheim, Indianapolis, Ind.) in SHEM medium (12). Epithelial cells were grown in 15-mm-diameter tissue culture wells (Costar, Cambridge, Mass.) in the presence of SHEM medium. Ten- to 12-day-old cell cultures were inoculated with 200 μl of a suspension of Ham's F-12 containing various concentrations of *P. aeruginosa*. Following an incubation period of either 30 min or 2 h to allow bacteria to adhere to the cells, the inoculum was removed and replaced with fresh sterile medium for the remainder of a 4-h total incubation period. All wells were then washed three times with 1 ml of Ham's F-12 to remove nonassociated bacteria. Gentamicin (200 μg/ml) was added for 2 h to one group of wells. These were washed once with medium to remove the antibiotic. Triton X-100 was added to lyse the cells, the contents of the well were thoroughly mixed, and a viable-cell count was performed to quantify survivors (intracellular organisms). Other wells were used to determine the level of bacterial association with cells; these were washed three times with 1 ml of medium before the cells were lysed with Triton X-100 without antibiotic treatment. A viable-cell count was performed to quantify bacterial association with cells. Gentamicin was added at this point (after cell lysis) for 2 h to ensure that the bacteria were all 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 the bacteria that were attached to the plastic were susceptible to gentamicin killing. At least three wells were used for each group.

Inhibitors. Cultured primary rabbit epithelial cells were used to examine bacterial association and invasion into corneal cells in the presence of various inhibitors of host cell function. All inhibitors were purchased from Sigma Chemical Co. Each inhibitor was added to cells 30 min before the assay, and each was replaced at any stage during the assay when the medium was removed. Cell cultures were inoculated with 10^5 bacteria for 2 h, and then the inoculum was removed and replaced with fresh sterile medium for an additional 2 h. Inhibitors tested included the actin-disrupting reagent cytochalasin D (10, 30, and 90 μg/ml), colchicine to inhibit microtubular cytoskeletal activity (10, 50, and 100 μg/ml), and the specific protein kinase inhibitors genistein (50, 150, and 640 μg/ml) and tyrphostin (200, 600, and 1,200 μg/ml). The viability of both the bacteria and the epithelial cells in the presence of each enzyme was monitored throughout these experiments by using viable-cell counts to assess bacterial survival and trypan blue dye exclusion to determine epithelial cell survival.

In order to examine the effect of inhibitors in an in vivo setting, the left corneas of C57BL/6 mice were scratch injured with a 26-gauge needle (three full-thickness epithelial layer abrasions) and inoculated with 10^8 bacteria (8) in the presence or absence of 90 μg of cytochalasin D per ml. Corneas were removed after 2 h, and the number of intracellular organisms was quantified by gentamicin survival assays.

Intracellular multiplication assay. Experiments were performed to test whether *P. aeruginosa* was able to replicate within cultured rabbit corneal epithelial cells. Cells were exposed to 200 μl of a bacterial suspension containing 2×10^5 organisms per ml for 1 h. After the cultures were washed, gentamicin was added to all wells for 2 h to kill the extracellular bacteria. One group of wells was used to quantify bacterial invasion (as described above) to provide a baseline for the number of intracellular organisms present at time zero. SHEM medium containing 100 μg of gentamicin per ml was added to the other wells for a further 4 or 24 h of incubation. At the end of this second incubation step, the cells were washed and treated with an additional 200 μg of gentamicin per ml for 2 h. Epithelial cells that had exfoliated from the plastic well surface during the 4-h or 24-h assay were collected from all of the wash solutions. The number of intracellular organisms in cells attached to the well and the number inside exfoliated cells were quantified separately. Following gentamicin treatment, the exfoliated cells in all wash solutions that were collected from a particular well were pooled, washed after centrifugation for 30 s to remove the antibiotic, and resuspended in SHEM medium prior to cell lysis with Triton X-100. Control experiments confirmed that 100 μg of gentamicin could kill extracellular bacteria that would otherwise grow in the SHEM medium during the assay for intracellular replication (data not shown).

Statistics. Statistical analysis of the results was done by analysis of variance (ANOVA), the Mann-Whitney *U* or unpaired *t* test for unpaired data, and the Wilcoxon or paired *t* test for paired data. All analyses were carried out with the Stat-View program (Abacus Concepts Inc., Berkeley, Calif.) on a Macintosh computer.

RESULTS

Interaction of bacteria with injured whole cornea in vitro. Following corneal injury, *P. aeruginosa* was found to adhere efficiently to the rat cornea when incubated with whole eyes in vitro for 4 h at 37°C (Table 1). A significant proportion of these organisms were also found to survive gentamicin treatment (Table 1). Similar results were obtained with mouse and rabbit eyes (Table 1). There were no survivors of gentamicin treatment when the corneas were homogenized and cells were lysed with Triton X-100 prior to addition of the antibiotic. In control experiments, it was found that 2×10^6 CFU of *P. aeruginosa* 6294 per ml were 100% susceptible to killing by 50 μg of gentamicin per ml, and up to 10^7 CFU/ml were killed by 200 μg of the antibiotic per ml of medium. It was also confirmed that 0.5% Triton X-100 did not affect bacterial viability or susceptibility to gentamicin, nor did it affect the ability to pellet bacteria by centrifugation. Another well-characterized *P. aeruginosa* strain (PAO1) was also found to invade cells on the injured rat cornea (association, $1.0 [\pm 0.3] \times 10^5$; invasion, $4.0 [\pm 0.9] \times 10^3$). *E. coli* HB101 did not invade corneal cells. Levels of invasion were not increased by inoculating eyes and performing the invasion assay in the presence of SHEM medium rather than HBSS.

Interaction of bacteria with uninjured whole cornea in vitro. Although few bacteria were found to adhere to the uninjured rat cornea, a significant proportion of these organisms were inside cells, as demonstrated by gentamicin survival assays (Table 1). Intracellular organisms were also isolated from within cells that had exfoliated into the wash solution. The number of bacteria taken up by these exfoliated cells was twofold greater (median, 225; lower quartile, 100; upper quartile, 335) than the number of bacteria inside cells that had remained on the cor-

TABLE 1. *P. aeruginosa* 6294 association with and invasion of corneal cells[a]

Treatment and model	Mean CFU ± SEM		
	Association[b]	Invasion[c]	% Invading[d]
Scratch injury			
Rat cornea	$2.9 (\pm 0.7) \times 10^5$	$5.7 (\pm 3.2) \times 10^3$	1.97
Mouse cornea	$3.6 (\pm 0.1) \times 10^5$	$5.6 (\pm 3.5) \times 10^3$	1.58
Rabbit cornea	$9.7 (\pm 0.2) \times 10^5$	$4.2 (\pm 0.1) \times 10^3$	0.43
None			
Rat whole cornea	$1.9 (\pm 0.2) \times 10^3$	$1.1 (\pm 0.2 \times 10^2$	5.6
Human corneal cells	$1.2 (\pm 0.1) \times 10^{6e}$	$1.9 (\pm 0.4) \times 10^4$	1.54
Cultured rat corneal epithelial cells	$4.3 (\pm 0.5) \times 10^4$	$1.2 (\pm 1.8) \times 10^4$	27.26
Cultured rabbit corneal epithelial cells	$5.6 (\pm 0.2) \times 10^5$	$3.5 (\pm 0.7) \times 10^4$	6.16

[a] All experiments were performed with a 4-h incubation with bacteria except for uninjured whole rat cornea (2 h). Scratch injury was produced with a 26-gauge needle (see Materials and Methods). Human corneal cells were removed by irrigation of the corneal surface.
[b] Total adherent and invading bacteria, determined by viable-cell counts.
[c] Number of invading bacteria was determined by survival after a 2-h treatment with 200 μg of gentamicin per ml.
[d] Percentage of associated bacteria that invaded.
[e] Includes nonadherent bacteria in sample (in addition to adherent and invading bacteria).

nea (median, 120; lower quartile, 30; upper quartile, 160) ($P = 0.03$, Wilcoxon test).

Interaction of bacteria with exfoliated human corneal epithelial cells. *P. aeruginosa* invaded human corneal epithelial cells removed from the eye by irrigation (Table 1). Gentamicin neutralized all bacteria in control samples that were treated with 0.5% Triton X-100 to lyse cells before antibiotic treatment.

Interaction of bacteria with cultured corneal epithelial cells. *P. aeruginosa* 6294 invaded both rat and rabbit cultured epithelial cells (Table 1). Strain PAO1 was also able to invade these cells (Fig. 1). In contrast, *E. coli* HB101 did not invade cultured cells (Fig. 1). There were statistically significant differences in levels of invasion for each of these three bacterial types ($P = 0.0001$, ANOVA), with invasion being nearly twofold greater with PAO1 than with 6294 ($P = 0.0009$, Mann-Whitney *U* test). Gentamicin neutralized all bacteria in samples containing lysed cells.

When bacteria were washed off after only 30 min, there was very little bacterial association with cells, and there was no evidence of bacterial invasion even after an additional 3.5 h of incubation (data not shown). Efficient bacterial association with and invasion of cells occurred only when the inoculum was left to interact with cells for at least 2 h. A small number of bacteria were found to adhere to empty wells (975 ± 202), but all were killed by gentamicin.

Effect of temperature on bacterial entry. Both bacterial association with rat cornea and bacterial invasion of cells on the cornea were found to be temperature dependent. Experiments in which eyes were incubated with bacteria at 4°C demonstrated that association levels dropped to only 5.5% of the levels observed at 37°C, and no invasion was detected (Fig. 2). The lack of cell invasion by bacteria was not explained by the reduction in bacterial association at 4°C, since we could carry out experiments at 37°C with a reduced bacterial inoculum (5 and 1% of the full inoculum of 2×10^8 CFU/eye) that would yield levels of bacterial association similar to those that were achieved at 4°C. Under these conditions at 37°C, invasion did occur (Fig. 2). These results suggested that invasion of corneal cells by *P. aeruginosa* may involve active processes.

Metabolic inhibitors. Colchicine did not alter bacterial association with ($P = 0.09$, Mann-Whitney *U* test) or invasion of ($P = 0.2$, Mann-Whitney *U* test) cells. Since some of the other inhibitors affected bacterial association with cells, levels of bacterial invasion are presented as the percentage of the bacteria that associated with the cells under the same conditions (Table 2). Cytochalasin D produced a dramatic dose-dependent decrease in bacterial invasion of corneal cells ($P = 0.01$,

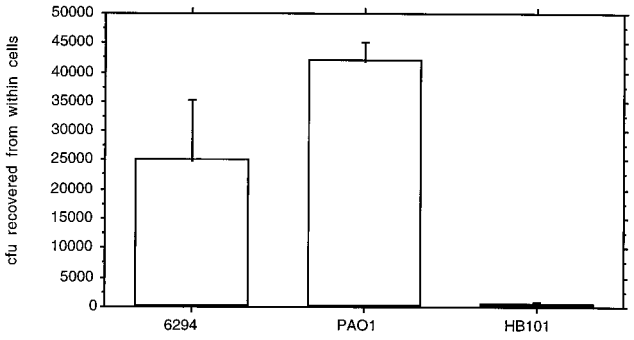

FIG. 1. Bacterial invasion of cultured rabbit corneal epithelial cells. Two *P. aeruginosa* strains and *E. coli* HB101 were used. There were significant differences between all three strains in levels of bacterial invasion ($P = 0.0001$, ANOVA). Data represent the mean + standard deviation (SD).

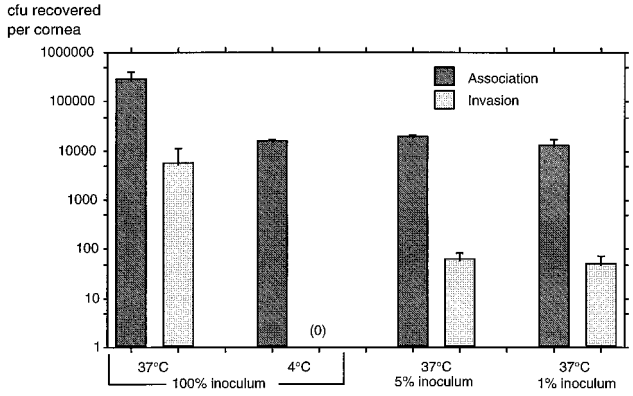

FIG. 2. Effect of temperature on *P. aeruginosa* association with and invasion of scratched injured corneal cells on rat corneas in situ. With 100% of the inoculum (2×10^8 CFU), there was a significant difference in levels of invasion between experiments performed at 37 and at 4°C ($P = 0.01$, unpaired *t* test). Bacterial association was also reduced at 4°C ($P = 0.02$, unpaired *t* test). Invasion did occur at 37°C when low levels of bacterial association were produced by reducing the inoculum to 10^7 CFU (5% inoculum) and 2×10^6 CFU (1% inoculum). Data represent the mean + SD.

TABLE 2. Alterations in *P. aeruginosa* invasion of cultured rabbit corneal epithelial cells with inhibitors of host cell function[a]

Inhibitor	Concn (μg/ml)	% Invading[b]
Cytochalasin D	0	9.87
	10	1.08
	30	0.33
	90	0.08
Genistein	0	2.14
	50	5.68
	150	1.00
	650	0.27

[a] A total of 10^5 bacteria were inoculated into each well.
[b] Percentage of associated bacteria invading in the presence of inhibitor.

ANOVA) (Table 2). The tyrosine protein kinase inhibitors tyrphostin and genistein both inhibited association ($P = 0.009$ and $P = 0.04$, ANOVA, respectively; not shown), in a dose-dependent manner for tyrphostin ($P = 0.006$, ANOVA) but not for genistein. Both inhibitors appeared to dramatically reduce invasion compared with control samples that were not treated. However, when reductions in association were also considered, only moderate to high concentrations of genistein reduced the percentage of associated bacteria that invaded (Table 2, $P = 0.01$, ANOVA). With lower concentrations of this drug and for all concentrations of tyrphostin, the percentage of associated bacteria that invaded increased. None of the four inhibitors tested affected the viability of bacteria or epithelial cells at the concentrations used.

Cytochalasin D (90 μg/ml) was also able to inhibit *P. aeruginosa* invasion of corneal cells when tested in mice infected in vivo. Two hours after inoculation with bacteria, $1.7 (\pm 0.2) \times 10^4$ CFU were recovered from within corneal cells of control animals. Only $2.4 (\pm 0.3) \times 10^3$ CFU were recovered from cells of eyes that were treated with cytochalasin D ($P = 0.0001$, unpaired *t* test).

Intracellular multiplication assay. *P. aeruginosa* was found to multiply inside primary rabbit cultured corneal epithelial cells (Fig. 3). The number of intracellular bacteria increased dramatically after 4 h, but by 24 h, the concentration was not significantly different from the initial concentration measured ($P = 0.22$, Mann-Whitney *U* test). After 72 h of incubation, the number of bacteria inside cells was insignificant. Cells that remained attached to the wells were confluent and healthy at 4, 24, and 72 h as determined by trypan blue dye exclusion. At 4 h, about 14% of intracellular bacteria were in cells that had detached from the tissue culture well, and by 24 h, 22% were inside detached cells. Of the few intracellular bacteria isolated at 72 h, 42% were from detached cells. The difference in the number of bacteria within detached cells at different time points was statistically significant ($P = 0.03$, ANOVA). Replication occurred at a slower rate (approximately fourfold slower) when samples were incubated in Ham's F-12 without supplemental CO_2 than when samples were incubated in SHEM medium with 5% CO_2 (data not shown).

DISCUSSION

We previously demonstrated that *P. aeruginosa* enters corneal epithelial cells during infection in vivo (8). Using gentamicin survival assays, we show in this report that *P. aeruginosa* can invade and survive within viable corneal epithelial cells in several in vitro models, including whole rat, rabbit, and mouse corneas after scratch injury; human superficial corneal epithelial cells removed by irrigation; and primary cultures of both rat and rabbit corneal epithelial cells. Gentamicin was effective in killing 100% of bacteria in all control samples treated with Triton X-100 to lyse cells before gentamicin was added, demonstrating that the results were not due to inherent resistance of bacteria to the antibiotic.

At 4°C, invasion of *P. aeruginosa* into whole rat cornea in vitro did not occur, and this was accompanied by a 10-fold decrease in association compared with that in eyes incubated with the same inoculum at 37°C (Fig. 2). The reduction in association can be predicted from the first law of thermodynamics, which states that for every 10°C reduction in temperature, the rate of all chemical interactions is halved. Another plausible explanation is that the receptor(s) is a glycolipid or lipoprotein whose function is inhibited at lower temperatures. A similar decrease in adherence of *P. aeruginosa* to A549 cells at 4°C has been noted (2). The decrease in association with corneal cells, however, did not explain why invasion did not occur at 4°C, since invasion was observed when similar levels of association were produced by lowering the inocula at 37°C. These findings suggest that invasion is a temperature-dependent process.

Cytochalasin D inhibited invasion, while colchicine had no effect, suggesting that host cell actin microfilaments may be required for *P. aeruginosa* invasion of corneal cells but microtubules are not involved. These findings are consistent with the eukaryotic cytoskeletal inhibition effects seen with other organisms (4, 15, 16). However, we found that it took higher concentrations of cytochalasin D to inhibit *P. aeruginosa* invasion of corneal epithelial cells than to inhibit invasion of endothelial cells (13).

There were decreases in the overall number of bacteria that associated with cells and in the overall number of bacteria that invaded cells when they were treated with the tyrosine protein kinase inhibitor genistein or tyrphostin. The effect on association was more marked than the effect on invasion with tyrphostin, so that the percentage of associated bacteria that invaded was actually increased at all concentrations. In contrast, higher concentrations of genistein reduced the percentage of associated bacteria that invaded. Genistein and tyrphostin inhibit tyrosine protein kinases by different mechanisms. Genistein inhibits binding of ATP to protein kinase, while tyrphostin competes with the tyrosine residue for binding to kinases. It

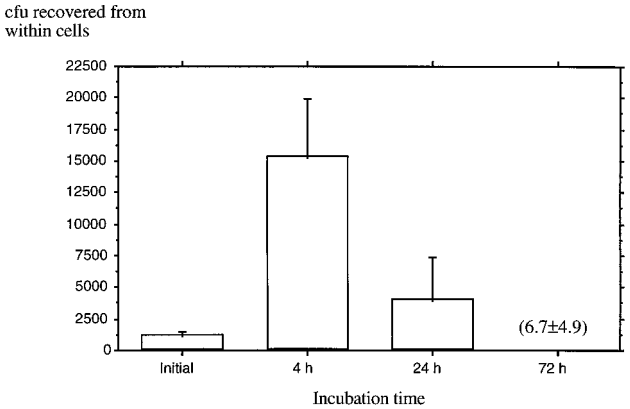

FIG. 3. Multiplication of *P. aeruginosa* 6294 within cultured rabbit primary corneal epithelial cells. The baseline level of bacterial invasion was measured after 1 h of bacterial interaction with cells (initial). Cells were then incubated in the presence of gentamicin for various time periods. There were significant changes in the number of bacteria recovered from within cells during the 72-h observation period ($P = 0.001$, ANOVA). Data represent the mean + SD.

has been noted that both genistein and tyrphostin inhibit invasion into HeLa cells by *Yersinia* species and *E. coli* strains containing *inv* (a gene that codes for *Yersinia* invasin) (14). In contrast, invasion by *Salmonella typhimurium*, which does not use invasin for entry into cells, is not inhibited by either of these drugs (14). Our results indicate that protein kinase activity may also be involved in *P. aeruginosa* entry into corneal cells and that the role of this activity is complex.

Why bacterial association with corneal cells was reduced by both protein kinase inhibitors was not clear. Perhaps specific host cell receptors involving protein kinase play a role in adherence to cells. Otherwise, it may be that these substances simply altered nonspecific physicochemical interactions, such as hydrophobicity or surface charge, that are known to be involved in the adherence of bacteria to surfaces (9).

P. aeruginosa was able to replicate within cultured rabbit corneal epithelial cells. The rate of replication was almost 10-fold in 4 h. By 24 h, the number of bacteria inside cells was reduced to initial levels. This reduction probably reflected the death of a fraction of the epithelial cells containing bacteria, either by normal cell turnover or because of bacterial invasion. In either case, this could result in the release of organisms into the extracellular environment and subsequent killing by the high concentration of gentamicin present in the surrounding medium. Otherwise, the decrease in viable intracellular bacteria may reflect bacterial exocytosis into the extracellular environment or bacterial killing by the cells themselves, which would allow gentamicin to enter the cells and kill the intracellular bacteria. Studies of endothelial cells infected with *P. aeruginosa* have demonstrated that those cells can kill a percentage of internalized bacteria and that internalized bacteria can cause cell injury (13). This late-stage decrease in intracellular bacteria may explain why we were not able to demonstrate replication in cells removed from mouse eyes infected in vivo (8). In that study, cells were removed from corneas that had already been exposed to bacteria for 24 h, and thus cells containing bacteria may have been infected for many hours prior to the beginning of the replication assay.

P. aeruginosa was able to invade superficial exfoliating corneal epithelial cells removed from humans. Since healthy uninjured cornea is almost never susceptible to infection by *P. aeruginosa*, bacterial entry into these surface cells is not likely to be a pathogenic mechanism. Indeed, uptake of bacteria into these cells could serve as a host defense against infection by entrapping bacteria and providing a mechanism for removal from the eye, since surface cells are continuously sloughed from the cornea. The results of the invasion assays performed with uninjured whole rat cornea support this hypothesis. After washing of the corneal surface, 60% of the internalized bacteria were removed from the eye within exfoliating cells.

Contact lens wear predisposes to *P. aeruginosa* infection, and thus contact lens wear must compromise corneal defenses against infection in certain situations. The longer the corneal surface is exposed to bacteria, the greater the probability that infection will occur when these defenses are compromised. If a contact lens is worn over the cornea, cells containing live bacteria could become trapped against the ocular surface, and this may then facilitate the development of infection.

We have shown here that it is possible to study *P. aeruginosa* invasion of corneal epithelial cells in a variety of in vitro systems. The fact that *P. aeruginosa* invades cells in vitro demonstrates that other factors found in vivo are not necessary for invasion to occur. Although *P. aeruginosa* adhered to and invaded corneal cells in all cases, there were large differences in the number of bacteria that interacted with corneal tissue between the various models tested. The data also demonstrate that a smaller percentage of associated bacteria invade in vitro than in our in vivo results (8). For example, 7.25% of associated bacteria survived gentamicin treatment in mouse corneas infected in vivo (8), compared with 1.58% of bacteria in corneas infected in vitro 4 h after inoculation. There are many differences between cells in vivo and the cells used in each of the in vitro models that could be responsible for differences in bacterial interactions. Corneal epithelial cells in vivo are polarized, organized in anatomically distinct layers, and in contact with the host immune system. The superficial surface of the cornea is covered by a cell surface glycocalyx as well as a thick mucus layer covered by the tear film. We have found that these surface components are able to modulate bacterial adherence to underlying corneal epithelial cells (6, 7), and they are thus also likely to affect bacterial invasion. When the whole eye is removed from an animal, several of these factors are no longer present, and when the corneal epithelial cells are removed from whole eyes, additional factors are excluded or lost. When cells are grown in culture, they may be further affected, particularly with respect to cell polarity. Bacteria attached in only small numbers to uninjured whole cornea, yet attachment to injured whole cornea or single corneal cells (removed from the cornea or grown in culture) was very efficient (Table 1). These findings suggest that tissue injury promotes bacterial association but that cell injury is not a prerequisite for this to occur. The mechanism by which corneal injury promotes bacterial interactions may involve the removal of surface modulating factors or the exposure of otherwise protected deeper layer cells or basolateral cell surfaces.

While cognizance of the differences between in vitro and in vivo settings is always critical in any biological study, the in vitro systems described here for analyzing *P. aeruginosa* invasion of corneal epithelial cells will likely provide important clues regarding the factors that influence bacterial interactions with corneal cells in vivo. Knowledge of these factors is critical for understanding the pathological process and designing therapies to lessen the occurrence and severity of *P. aeruginosa* corneal infections.

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