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Pseudomonas aeruginosa Invades Corneal Epithelial Cells during Experimental Infection

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***Pseudomonas aeruginosa* is considered an extracellular pathogen. Using assays to determine intracellular survival in the presence of gentamicin, we have demonstrated that some strains of *P. aeruginosa* are able to invade corneal cells during experimental bacterial keratitis in mice. Although intracellular bacteria were detectable 15 min after inoculation, the number of intracellular bacteria increased in a time-dependent manner over a 24-h period. Levels of invasion were similar when bacteria were grown as a biofilm on solid medium and when they were grown in suspension. Intracellular bacteria survived in vitro for at least 24 h, although only minimal bacterial multiplication within cells was observed. *P. aeruginosa* PAK and *Escherichia coli* HB101 did not cause disease in this model and were not isolated from corneas after 24 h even when an inoculum of 10^8 CFU was applied. Transmission electron microscopy of corneal epithelium from eyes infected for 8 h revealed that intracellular bacteria were present within membrane-bound vacuoles, which suggests that bacterial entry was an endocytic process. At 24 h, the observation of many bacteria free in the cytoplasm indicated that *P. aeruginosa* was able to escape the endocytic vacuole. The ability of some *P. aeruginosa* strains to invade corneal epithelial cells may contribute to the pathogenesis or to the progression of disease, since intracellular bacteria can evade host immune effectors and antibiotics commonly used to treat infection.**

Pseudomonas aeruginosa is the pathogen most commonly involved in bacterial keratitis associated with the use of contact lenses (1). This form of corneal infection is rapidly progressive and difficult to treat and can cause vision impairment.

P. aeruginosa has traditionally been thought to be an extracellular bacterium. After the organism adheres to host tissues, it is believed that the infectious process involves destruction of the underlying tissue by release of extracellular toxic substances and that stimulation of the host immune response can exacerbate tissue damage, leading to erosive disease (17). For these reasons, research has focused on adherence of this organism to the cornea and other tissues and the host immune response to infection (17).

The ability to invade host cells is a virulence factor of some bacteria, including *Salmonella*, *Shigella*, *Yersinia*, and *Neisseria* species (4, 20). Some of these bacteria are also able to replicate inside host cells, either within vacuoles or in the cytoplasm (4). Invasion follows adherence and generally involves metabolic activity of both host and bacterial cells. Intracellular survival is thought to contribute to the infectious process, since bacteria are then able to persist despite host defenses against infection.

In recent years, there has been anecdotal evidence by transmission electron microscopy of *P. aeruginosa* inside cells. *P. aeruginosa* has been observed within epithelial cells from injured canine tracheas in an in vitro assay (25) and inside keratocytes of athymic nude mice during infection (11). There have been two separate but contradictory reports of *P. aeruginosa* inside rabbit corneal epithelial cells during infection (16, 21). The first of these reports described gradual engulfment of bacteria with no evidence of cell destruction, suggesting that the bacteria were in the process of transcellular migration. The authors of the second article also noted pocket formation and embedding of the bacterium in the cell membrane but thought that this led to destruction of epithelial cells by bacterial exotoxins.

It was recently reported that *P. aeruginosa* invades cultured A549 pneumocytes (transformed respiratory epithelial cells) and that a proportion of these intracellular bacteria remain viable for at least 4 h (2). However, the viability of *P. aeruginosa* and of epithelial cells during invasion in the course of an actual infection has not previously been explored.

In this report, we demonstrate in a murine model for *P. aeruginosa* keratitis that a significant number of bacteria survive inside viable corneal cells during infection and that this phenomenon begins early in the infectious process. For these reasons, we propose that the term association be used to define the sum of adherent and intracellular bacteria when describing *P. aeruginosa* interaction with the cornea. Invasion of corneal cells may explain why *P. aeruginosa* infection is often refractory to the host defense system and to certain types of antibiotic therapy.

MATERIALS AND METHODS

Preparation of bacteria. Bacteria were grown overnight at 37°C on a tryptic soy agar (TSA) plate covered with a dialysis membrane of 12,000- to 14,000-molecular-weight pore size (5). The inoculum was prepared by resuspension of bacteria from the membrane into saline (0.9% NaCl in distilled water) or 1% proteose peptone until the appropriate optical density was achieved. The *P. aeruginosa* strains used included a corneal isolate (6294, serogroup O6), a strain from a bacteremic patient (9376-80, serogroup O11), and strain PAK (serogroup O6). A noninvasive strain of *Escherichia coli* (HB101) was used as a negative control. In order to confirm that bacterial invasion was not a function of the manner in which bacteria

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were prepared, another series of experiments were performed with *P. aeruginosa* 6294 grown in suspension. Bacteria were grown overnight in 5% peptone with 0.25% tryptic soy broth (TSB) and then washed twice after centrifugation at 3,000 × *g* in saline for 10 min. This method has been used by other investigators studying *P. aeruginosa* infection in mice (13).

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 (Parke Davis, Morris Plains, N.J.) and 1.3 mg of xylazine (Haver, Shawnee, Kans.) per ml. 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.

Tissue culture medium. Modified SHEM medium was prepared as described previously (15) but with bovine pituitary extract (10 μg/ml) instead of cholera toxin. All reagents apart from nutrient mixture F-12 Ham's (HyClone Laboratories, Logan, Utah) and Eagle's minimal essential medium (Whittaker Bioproducts, Walkersville, Md.) were obtained from Sigma Chemical Co., St. Louis, Mo.

Bacterial interaction with the mouse cornea during infection. After the mice had been anesthetized, three full-thickness epithelial abrasions were produced on the left cornea with a 26-gauge needle. The cornea was immediately inoculated with 5 μl of bacterial suspension. At various time points after inoculation, the animals were killed, and the left corneas were removed and rinsed in three washes of 5 ml of PBS each to remove nonadherent bacteria. The infected corneas were divided into two groups. The first group was used to determine the total number of viable bacteria within infected corneas. Each of these corneas was homogenized in 1 ml of TSB with 0.5% Triton X-100 (Sigma Chemical Co.), which was added to ensure lysis of corneal cells and release of intracellular bacteria into solution. After a 15-min exposure to the detergent, a count of viable bacterial cells in the homogenate was performed in duplicate to determine the number of bacteria that had been associated with the infected cornea.

The second group of corneas was used in gentamicin survival assays to quantify bacterial invasion of corneal cells. These assays have been used extensively by other investigators studying intracellular bacterial invasion and survival (4). The procedure involves exposing eukaryotic cells to bacteria for several hours to allow bacteria to enter and then killing extracellular bacteria with gentamicin, which does not affect the viability of intracellular organisms (24). Thus, intracellular bacteria survive treatment and may be detected by viable-cell counting after removal of gentamicin and lysis of host cells with Triton X-100 (4). Corneas were suspended in 60 μl of SHEM medium containing 200 μg of gentamicin (Whittaker Bioproducts) per ml for 2 h at 37°C to kill extracellular bacteria and then rinsed in two washes of 5 ml of PBS each to remove the antibiotic. The number of surviving bacteria in these corneas was determined by viable-bacterial-cell counts after homogenization of the cornea in Triton X-100 as described above.

In other experiments, the outer epithelial layer of infected corneas was separated from other layers by scraping the ocular surface with a blunt spatula and suspending the epithelial tissue in SHEM medium. The epithelia from at least 10 infected eyes were pooled for each experiment and divided into three series of three samples each. One series was used to calculate the number of viable bacteria associated with the cells by viable-bacterial-cell counting after lysis of cells with 0.5% Triton X-100 for 15 min. The second series was used to confirm that gentamicin was able to neutralize all associated bacteria once they were all extracellular. Cells were lysed with Triton X-100, washed after centrifugation at 12,000 rpm for 3 min, and treated with 200 μg of gentamicin per ml for 2 h. The antibiotic was washed from these samples after centrifugation at 12,000 rpm for 3 min, the pellets were resuspended in SHEM medium, and a viable-cell count was performed to determine the number of surviving bacteria (those resistant to gentamicin). The third series of samples was used to quantify bacterial invasion into epithelial cells. Gentamicin (200 μg/ml of medium) was added for 2 h. When the cells had been washed after centrifugation and resuspension in SHEM medium, cells were lysed with 0.5% Triton X-100, and a viable-cell count was performed to quantify survivors (intracellular bacteria).

To determine whether bacteria were able to multiply within cells, samples containing epithelium removed from infected corneas that had been treated with 200 μg of gentamicin per ml were allowed to incubate for a further 3 or 24 h in the presence of 10 μg of gentamicin per ml (to neutralize bacteria released from within cells). Samples were then treated again with 200 μg of gentamicin per ml for 2 h, the cells were washed and lysed, and surviving bacteria were enumerated.

Transmission electron microscopy. Infected and control uninfected mouse eyes were removed immediately after death and prepared for transmission electron microscopy by standard methods. Briefly, 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 in graded series of ethanol and embedded. Thin sections of the cornea were examined with a Zeiss EM 100A electron microscope set at 60 kV. These sections were compared with samples prepared from six mouse corneas that were not infected.

Statistics. For each experiment, at least five mice were assigned to each group. The results are presented as the mean ± standard error for each set of data points in the text and tables. Initially, parametric statistics were used to analyze the data, including the unpaired *t* test for comparing two groups of data and analysis of variance for comparing three or more groups of data, and all *P* values presented in this report were determined in this manner. Since it was not certain whether the invasion data were normally distributed, all statistical comparisons were also performed by the use of nonparametric statistics, including the Mann-Whitney test for comparing two groups of data and the Kruskal-Wallis test for three or more groups of data. With $P < 0.05$ as the level of statistical significance, all data that were found to be significantly different by parametric tests were also shown to differ by nonparametric tests.

RESULTS

Bacterial interaction with the mouse cornea during infection. Significant numbers of bacteria invaded corneal cells during infection with *P. aeruginosa* 6294, as demonstrated by gentamicin survival assays. The results obtained with an infectious dose of 10^8 CFU of *P. aeruginosa* 6294 are presented in Table 1. Invasion began early in the infectious process — intracellular bacteria were noted as early as 15 min after inoculation. Both the number of bacteria associated with the cornea and the number of bacteria that invaded cells increased steadily with time, as demonstrated by the results obtained after 4 and 24 h ($P = 0.0001$ for association and $P = 0.0011$ for invasion) (Table 1). In control experiments, it was found that 10^7 CFU of *P. aeruginosa* 6294 per ml was 100% susceptible to killing by 200 μg of the antibiotic per ml of medium. Samples from infected corneas in which cells were lysed with Triton

TABLE 1. Bacterial association with and invasion of corneal cells in vivo[a]

Strain	Duration of infection	Mean CFU of bacteria/cornea[b] ± SE		
		Adherent plus invading	Invading	% Invading
P. aeruginosa				
6294	15 min	$8.9 \times 10^4 \pm 0.2 \times 10^4$	$3.3 \times 10^3 \pm 1.7 \times 10^3$	3.76
	4 h	$4.9 \times 10^5 \pm 0.6 \times 10^5$	$3.6 \times 10^4 \pm 1.6 \times 10^4$	7.25
	24 h	$9.2 \times 10^5 \pm 2.9 \times 10^5$	$2.0 \times 10^5 \pm 0.8 \times 10^5$	22.22
PAK	24 h	0	0	0
E. coli HB101	24 h	0	0	0

[a] The inoculum was 10^8 CFU.

[b] The total number of adherent plus invading bacteria was determined by viable-cell counts. The number of invading bacteria was determined by survival after a 2-h treatment with 200 μg of gentamicin per ml. The percent invading is the percentage of associated (adherent plus invading) bacteria that invaded.

X-100 before antibiotic treatment contained no viable bacteria; these results confirmed that the antibiotic killed all bacteria that were in the corneas once they were extracellular and excluded the possibility that the bacteria had developed resistance to gentamicin as a result of growing in the infected eye. In other control experiments, we confirmed that bacterial adherence to corneas did not increase resistance to killing by gentamicin and that 0.5% Triton X-100 — which was used to lyse cells prior to enumerating surviving intracellular organisms — did not affect the ability to pellet bacteria by centrifugation (data not shown). Experiments were also performed to test whether prior exposure to Triton X-100 affects susceptibility to gentamicin. For these experiments, a bacterial suspension of 10^8 organisms per ml was used, a concentration that was determined to be 10-fold higher than the maximum number of organisms that could be effectively killed by 200 μg of gentamicin per ml in 2 h at 37°C. The number of bacteria surviving 200 μg gentamicin per ml was not affected when bacteria were pretreated with 0.5% Triton X-100 for 30 min (9.3×10^5/ml without pretreatment and 9.6×10^5/ml after exposure to the detergent).

Macroscopic examination of eyes infected with strains 6294 and 9376-80 revealed the classical signs of infection and inflammation that have been described previously for *P. aeruginosa* keratitis (13). As a negative control, two other organisms that do not cause disease in this murine model, *P. aeruginosa* PAK and *E. coli* HB101, were tested for their ability to invade corneal cells in vivo. Neither of these strains was present in the cornea 24 h after inoculation (Table 1).

Gentamicin assays of whole corneas do not distinguish between invasion of epithelial cells and invasion of other corneal cells, such as keratocytes and endothelial cells. In another series of experiments, the epithelium was removed from the corneas of infected animals with a blunt spatula and investigated separately from the other corneal layers to determine whether epithelial cell invasion had occurred. The number of bacteria associated with each epithelial sample (each sample contained epithelium removed from two infected corneas) was $1.1 \times 10^6 \pm 0.2 \times 10^6$; $7.0 \times 10^3 \pm 2.0 \times 10^3$ (0.65%) of these bacteria survived gentamicin treatment, which demonstrates that *P. aeruginosa* was able to enter corneal epithelial cells. Similar results were noted with *P. aeruginosa* 9376-80. There were no surviving bacteria in samples in which epithelial cells were first lysed and then treated with gentamicin, a result confirming that the antibiotic killed all bacteria associated with the samples once they were extracellular.

We did not observe multiplication of *P. aeruginosa* inside infected mouse corneal epithelial cells in vitro. In these experiments, $2.0 \times 10^3 \pm 1.0 \times 10^3$ CFU of intracellular bacteria were found immediately after removal of the epithelium from the eye. After 3 h of incubation in vitro, $2.6 \times 10^3 \pm 1.0 \times 10^3$ CFU of intracellular bacteria were recovered, and at 24 h there were $5.9 \times 10^3 \pm 1.9 \times 10^3$ CFU inside cells. This small increase in the number of intracellular bacteria with longer incubation in vitro was not statistically significant ($P = 0.13$).

To determine the effect of inoculum size on bacterial association and invasion, eyes were infected with smaller doses of strain 6294 (Table 2). Similar numbers of bacteria associated with the cornea when an inoculum of 10^8 or 10^5 CFU was used (Tables 1 and 2, $P = 0.18$), suggesting that there was a saturation point for association of strain 6294 with abraded corneas. When an inoculum of 10^4 or 10^5 CFU was used, the number of bacteria associated with corneas after 24 h was greater than the number that were added to the eye to initiate infection, indicating that strain 6294 multiplied in the eye during infection. Indeed, when 10^4 CFU were added, about twice this number were isolated from within cells and about 20 times as many were associated with the cornea. For this reason, the efficiency of invasion is presented in the tables as the percentage of associated bacteria that invaded, rather than as a percentage of the inoculum added.

There was no significant difference between the two methods of preparing bacteria in the number of bacteria that were

TABLE 2. Effect of inoculum size and method of growth on *P. aeruginosa* 6294 association with and invasion into corneal cells 24 h after infection in vivo[a]

Inoculum (CFU)	Growth method	Mean CFU of bacteria/cornea ± SE		
		Adherent plus invading	Invading	% Invading
10^4	Biofilm	$2.4 \times 10^5 \pm 1.1 \times 10^5$	$1.8 \times 10^4 \pm 0.6 \times 10^4$	8.0
10^5	Biofilm	$1.7 \times 10^6 \pm 0.5 \times 10^6$	$4.5 \times 10^4 \pm 1.2 \times 10^4$	2.6
10^5	Planktonic	$1.9 \times 10^6 \pm 1.0 \times 10^6$	$7.8 \times 10^4 \pm 3.1 \times 10^4$	4.0

[a] Bacteria were grown either as a biofilm on a dialysis membrane covering TSA or in a planktonic manner in 5% peptone–0.25% TSB and were washed twice by centrifugation at 3,000 × *g* in saline for 10 min in the same medium. Bacteria grown by both methods were resuspended in 1% proteose peptone. For other details, see Table 1, footnote *b*.

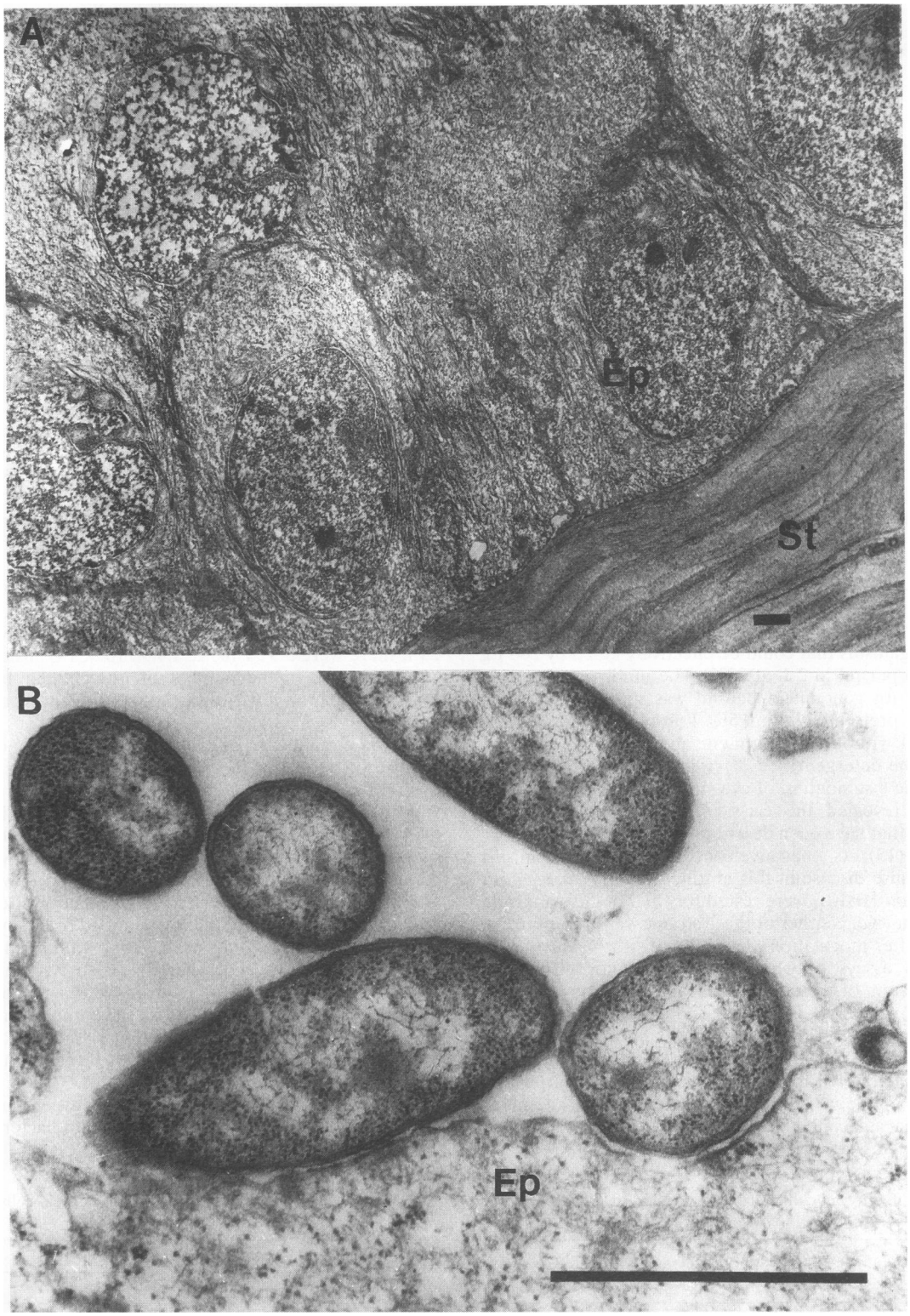

FIG. 1. Transmission electron micrographs of corneas from C57BL/6 mice. (A) Normal cornea demonstrates appearance of healthy epithelium (Ep) and the underlying stroma (St). To produce infection, corneas were scratch-injured and inoculated with 10^8 CFU of *P. aeruginosa* 6294. After 8 h of infection, bacteria were found in various stages of entry into epithelial cells. (B) Some bacteria were adherent to the membrane and were only beginning to penetrate the cell. Bars, 1 μm.

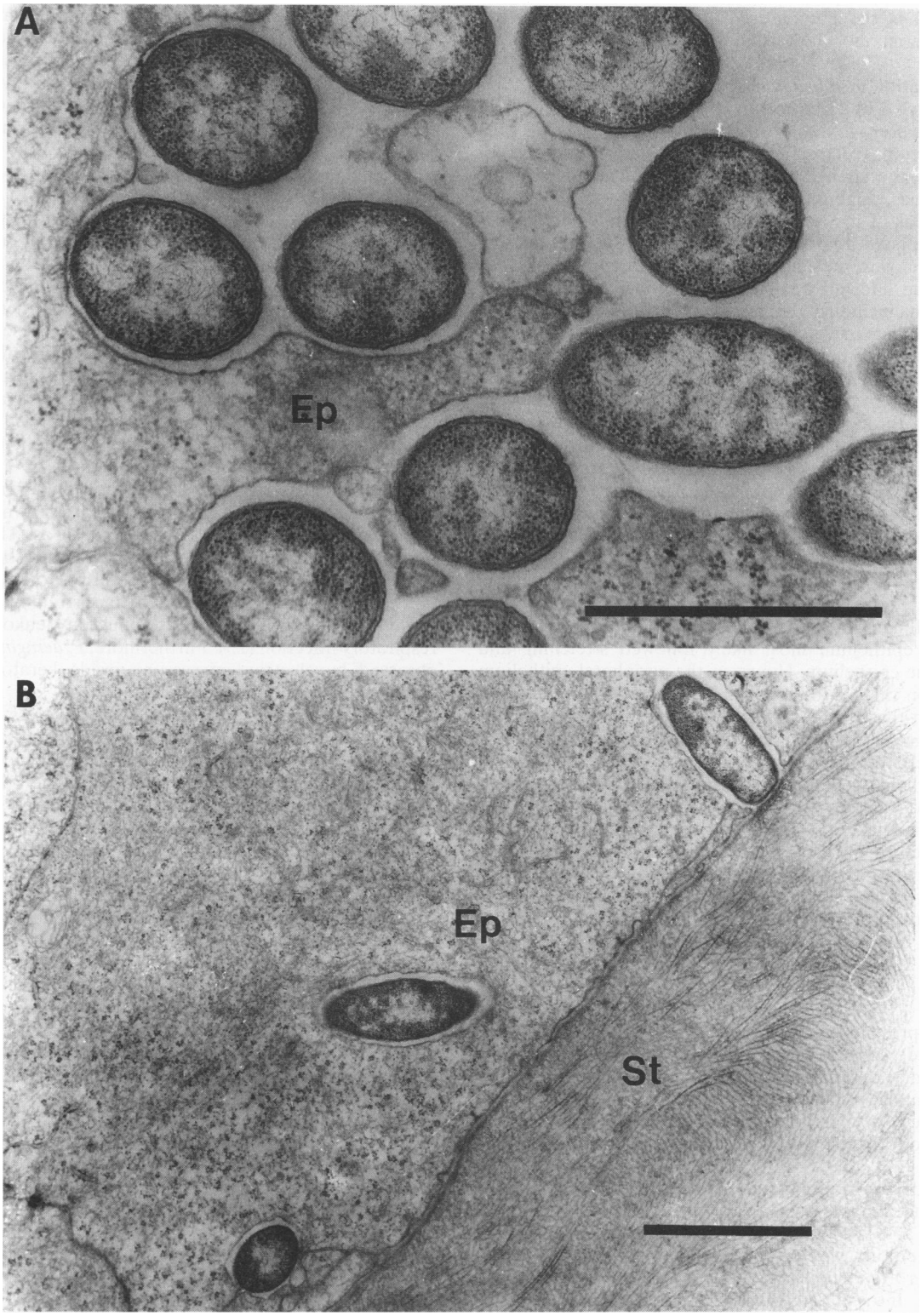

FIG. 2. Transmission electron micrographs of C57BL/6 mouse corneas after infection for 8 h with *P. aeruginosa* 6294. Many bacteria were partially surrounded by cell membranes; sometimes several bacteria were invading in one area of the membrane (A). Bacteria that were enclosed by the membranes of two adjacent cells were observed (B), as were others that were entirely within cells inside membrane-bound vacuoles (B). Ep, epithelium; St, stroma. Bars, 1 μm.

associated with corneas (Mann-Whitney U test, $P = 0.86$) or in the number of bacteria that invaded cells ($P = 0.35$) at 24 h (Table 2). Thus, the method used for growth and preparation of the bacterial inoculum is not necessarily important for invasion.

Demonstration of *P. aeruginosa* invasion of corneal epithelial cells in vivo by electron microscopy. To confirm visually that *P. aeruginosa* was able to invade corneal epithelial cells during infection, electron microscopy was performed on 12 mouse corneas that had been infected with strain 6294 for various periods ranging from 15 min to 24 h.

The corneas of mouse eyes that were not infected with *P. aeruginosa* appeared normal by transmission electron microscopy; bacteria were not detected in these samples (Fig. 1A). Corneas from infected eyes showed all the classical signs of inflammation, including the presence of polymorphonuclear leukocytes and massive corneal edema. A small number of bacteria were noted in the stroma of these corneas. Bacteria in various stages of penetration into epithelial cells were observed at 8 h (Fig. 1B, 2A, and 2B). In many instances, bacteria were seen passing through intercellular junctional complexes and were enclosed by two adjacent epithelial cells (Fig. 2B). Cells that were penetrated by bacteria were mostly basal cells, near the basal lamina. *P. aeruginosa* cells that were entirely intracellular were present within a membrane-bound vacuole (Fig. 2B). These observations showed that, like other intracellular bacteria, *P. aeruginosa* was probably taken up into cells by a form of endocytosis. By 24 h, the epithelial layer from the central infected area was lost from all corneas. Since there was massive swelling of the underlying stroma during infection, the loss of the epithelium most likely followed stretching and tearing of the epithelium. However, the peripheral epithelium was intact on these 24-h-infected corneas, and bacteria were noted within these epithelial cells (Fig. 3A and B). Many of these organisms were no longer within membrane-bound vacuoles but were free in the cytoplasm of apparently normal corneal epithelial cells (Fig. 3A and B). It appeared that *P. aeruginosa* was able to escape from the endocytic vacuole.

Intracellular bacteria were noted in all layers of epithelial cells by electron microscopy, including superficial cells and deeper-layer (basal) cells (Fig. 2B, 3A, and 3B). Intracellular organisms were not noted by electron microscopy before 8 h, probably because of the smaller number of bacteria that were intracellular early in the infection (Table 1).

DISCUSSION

The data presented in this report show that *P. aeruginosa* is able to invade corneal cells during infection, as was demonstrated for mice by gentamicin survival assays and by transmission electron microscopy. Both the number of bacteria associated with the cornea and the percentage of associated bacteria that invaded cells increased with time after the onset of infection.

We have demonstrated that as many as 10^3 bacteria survived gentamicin treatment as early as 15 min after the onset of infection (Table 1). Using the same murine model of *P. aeruginosa* keratitis, we have found that as few as 10^2 CFU of *P. aeruginosa* are sufficient to initiate infection with virulent strains (18). Intracellular bacteria can evade host immune factors and many forms of antibiotic therapy. Thus, in an injured cornea, where deeper-layer cells that do not exfoliate are exposed, bacterial invasion of cells may contribute to the persistence of infection.

Two organisms that were not virulent in this model for murine bacterial keratitis, *P. aeruginosa* PAK and *E. coli* HB101, were not isolated from within corneal cells 24 h after inoculation. It has been noted in a different murine model for corneal infection that strain PAK is not as virulent as other *P. aeruginosa* strains (12). *P. aeruginosa* PAK has been found to efficiently invade transformed respiratory epithelial cells (A549 pneumocytes) (2). Here we demonstrate that PAK does not invade corneal cells in vivo, and we have confirmed this in vitro by using epithelial cell cultures (data not shown); this result suggests that transformed respiratory cells and cells of the cornea differ with respect to their susceptibility to *P. aeruginosa* invasion.

Bacteria in various stages of penetration into epithelial cells were observed at 8 h by electron microscopy, and in many instances bacteria were seen between intercellular junctional complexes enclosed by two adjacent epithelial cells. Indeed, some bacteria were observed abutting the basement membrane and/or separating desmosomes (Fig. 2B). These phenomena may reflect intercellular migration, which could also be important in the pathogenesis of *P. aeruginosa* keratitis. This effect has been observed as part of the process of invasion of ileal epithelium by *Salmonella typhimurium* (22). *S. typhimurium* eventually becomes entirely enclosed within one of the lateral plasmalemmas and becomes intracellular.

At least a proportion of the bacteria that invaded corneal cells were present inside epithelial cells, as shown by transmission electron microscopy of infected corneas. The possibility remains that a percentage of bacteria may have survived gentamicin in infected corneas by entering other types of cells. A recent study found that one particular strain of *P. aeruginosa* could survive inside polymorphonuclear leukocytes during respiratory infection (23). Furthermore, *P. aeruginosa* has been observed by electron microscopy inside keratocytes during corneal infection in mice (11).

Bacterial uptake by host cells usually involves envelopment of the bacteria in the cell membrane, so that a vacuole forms around each bacterium (4). Some bacteria, such as *Yersinia* spp., remain within these vacuoles (3), while others, such as *Shigella flexneri*, can escape from the vacuole and survive in the cytoplasm (19). Other studies have found what appeared, by electron microscopy, to be bacterial uptake by epithelial cells on injured rabbit corneas during infection with *P. aeruginosa* (21). The authors described fusion of the epithelial and bacterial cell membranes 15 min after inoculation with bacteria, the formation of depressions or pockets in the epithelial membrane at 30 min, and the filling-in of these depressions with cellular material after 45 to 60 min. Since there was no evidence of cellular destruction, the authors suggested that what they had observed represented transcellular migration of bacteria. Another report of *P. aeruginosa* interaction with the cornea, this time after contact lens overwear in rabbits, described similar pocket formation and embedding but observed that the epithelial cell was gradually destroyed during this process (16). Our results at 8 h showed that intracellular *P. aeruginosa* cells were present within a membrane-bound vacuole, which suggests that *P. aeruginosa* was probably taken up into cells by a form of endocytosis. Invasion of *P. aeruginosa* into cultured A549 pneumocytes was also found to involve membrane-bound vacuoles 3 h after inoculation (2). After 24 h, most bacteria were no longer inside membrane-bound vacuoles, which suggests that *P. aeruginosa*, like *Shigella* spp., was able to escape from the endocytic vacuole.

The fact that *P. aeruginosa* invasion of corneal epithelial cells has not been noted previously in mice after abrasion injury may indicate that differences in the method used to infect eyes affects *P. aeruginosa* invasion. The method of anesthesia used in our experiments may have promoted bacterial invasion of

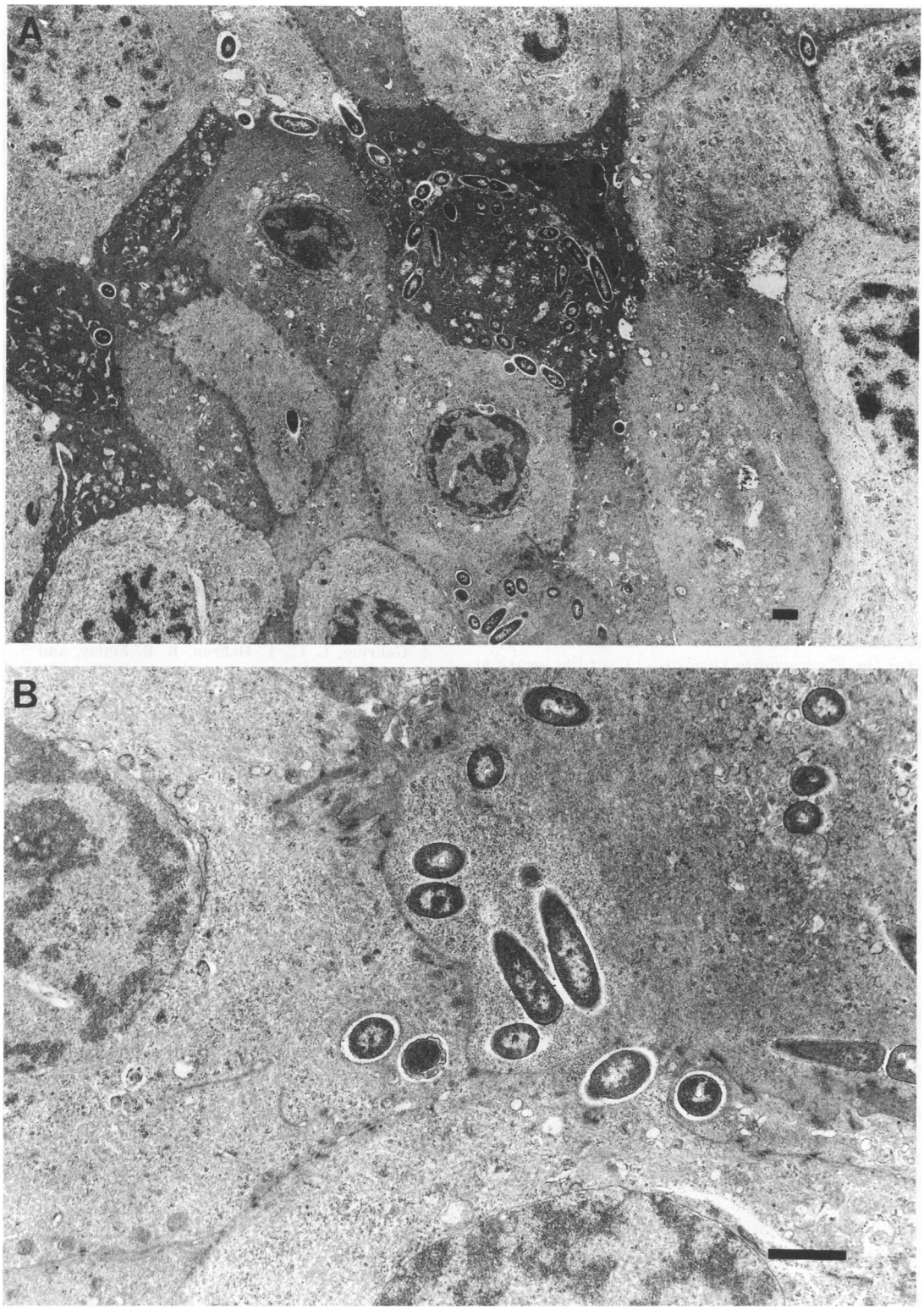

FIG. 3. Transmission electron micrographs of C57BL/6 mouse corneas after infection for 24 h with *P. aeruginosa* 6294. By 24 h, the epithelial layer from the central infected area was shed from all corneas. This was most likely a result of stretching and tearing of the epithelium, since there was massive swelling of the underlying stroma. The peripheral epithelium (adjacent to the central infected area) was intact, and bacteria were present within these epithelial cells (A and B). Many intracellular organisms were no longer present within membrane-bound vacuoles but were free in the cytoplasm (A and B). Bars, 1 μm.

cells. Other investigators have used ether to anesthetize mice, which lasts for approximately 1 min (13). Anesthesia by our method lasts for at least 20 min; thus, there was prolonged contact time between bacteria and the cornea before the animal could blink. Ocular mucus inhibits *P. aeruginosa* adherence to the cornea (7), and this may be a mechanism for removal of bacteria from the ocular surface in the blinking eye. Since the mouse eye remains open without blinking for the period of anesthesia, this and possibly other clearance mechanisms of the eye may not operate effectively, thus altering the manner in which bacteria infect the cornea.

We observed multiplication of *P. aeruginosa* within the cornea but not within cells. *S. flexneri* and *S. typhimurium* are able to multiply inside host cells and can spread from cell to cell to expand the focus of infection (8, 19). Other bacteria, such as *Yersinia enterocolitica*, do not replicate inside cells (3). Studies of *P. aeruginosa* PAK invading A549 pneumocytes revealed that the number of viable intracellular bacteria decreased by 30% over a 4-h period (2). In contrast, we observed that the number of viable bacteria inside corneal cells remained stable in vitro for at least 24 h with *P. aeruginosa* 6294. This finding suggests that the bacteria did not multiply inside corneal cells. However, if a portion of the epithelial cells died during the 24-h period in vitro, then bacterial multiplication within other epithelial cells could have occurred while the total number of intracellular bacteria remained constant.

It is clear that overt corneal injury predisposes to infection (9), a phenomenon which we and others have used to produce experimental infections. Contact lens wear is the major predisposing factor for *P. aeruginosa* keratitis (1), yet the wearing of these devices does not normally cause overt corneal injury. The impact of bacterial invasion of cells found on a healthy cornea, such as under a contact lens, is likely to differ from that of bacterial invasion of cells in an injured cornea. On an intact healthy cornea, bacteria will first encounter surface epithelial cells, which are continuously sloughed from the cornea. We have demonstrated that *P. aeruginosa* can invade these superficial exfoliating corneal cells (6). Sloughing of cells may be a means by which bacteria that have invaded cells are removed from the corneal surface and in this manner may serve as a defense against infection. Contact lens wear has been found to increase epithelial cell residence time on the corneal surface (10), and once exfoliated, an epithelial cell may become trapped under a contact lens. Stagnation of intracellular bacteria could contribute to the pathogenesis of contact lens-related *P. aeruginosa* keratitis. Indeed, soft contact lenses are associated with the highest risk of infection, and stagnation is more marked under these lenses than under other lens types. We have previously determined that contact lens wear enhances *P. aeruginosa* association with human corneal epithelial cells removed by irrigation (5). Whether this increased bacterial binding to cells leads to increased levels of bacterial invasion during contact lens wear is yet to be explored.

Although methods of inhibiting bacterial adherence to corneal cells may eventually lead to methods of preventing infections, the inhibition of adherence is unlikely to be useful for the treatment of infection. Furthermore, bacterial adherence to host cells is difficult to inhibit, since adherence normally involves multiple interactions that are both specific and nonspecific in nature (14). Alternative methods of infection control might be developed from studies of host and bacterial factors involved in *P. aeruginosa* invasion of corneal cells.

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