An Organ Culture System For Study of Adherence of Pseudomonas aeruginosa to Normal and Wounded Corneas

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An organ culture system has been developed to study the adherence of Pseudomonas aeruginosa to unwounded corneas and to corneas healing after a 3 mm central epithelial debridement. The Pseudomonas strain was isolated from a human corneal ulcer; suspensions containing 1 × 10^6 colony-forming units/ml (CFU/ml) of bacteria were incubated with the corneas for the last 30 min of the 18 hr culture period. The distribution pattern and number of adherent bacteria on the ocular surface were determined by morphometric analysis of scanning electron micrographs. Few bacteria (25 ± 15/mm²) adhered to the apical cells of unwounded corneas. There was a definite region-specific distribution of adherent bacteria on healing corneas. Most bacteria were found on the denuded basal lamina in front of the leading edge of the migrating epithelium (360,700 ± 49,900/mm²). Appreciable but lower numbers adhered to the apical membrane of leading-edge cells (37,700 ± 6,100/mm²) and to the central portion of the denuded basal lamina (28,800 ± 10,700/mm²). No bacteria were found adherent to the apical cells of the stratified epithelium behind the leading edge of the epithelium migrating to cover the wound. A similar region-specific distribution of adherent bacteria was found when corneas were inverted in the bacterial suspension and when corneas were incubated in the bacterial suspension for 15 rather than 30 min. Corneas preincubated with the lectin, succinyl-concanavalin A, showed significantly decreased bacterial adherence, indicating a possible role for mannose moieties of wound surface glycoconjugates in bacterial adherence. Invest Ophthalmol Vis Sci 29:379–386, 1988

Corneal infections caused by gram-negative bacilli, particularly Pseudomonas aeruginosa, appear to be increasing in frequency and are a cause of growing concern. These infections are found especially in patients with corneal trauma or disease and are highly destructive and difficult to treat.1,2 Adherence of bacteria to an epithelial surface is generally accepted to be the first event in the establishment of an infection.3,4 This adherence is affected by several factors,3,5,6 including strain of bacteria, species of host, type of tissue, physiological state and developmental stage of host cells.7 The bonds between bacteria and host cells may arise from direct molecular interactions, ie, adhesion-receptor or bridging ligands,3,5,6,8 hydrophobic bonds,9 or interactions due to surface charge.10 Hazlett et al7 showed that corneas of newborn Swiss-Webster mice were more susceptible to Pseudomonas infection than were adult corneas. Other workers reported that P. aeruginosa adheres to wounded corneal surfaces more avidly than to non-wounded surfaces.11–13 Ramphal et al11 and Stern et al13,14 showed by scanning electron microscopy of wounded corneas that P. aeruginosa adheres to the injured epithelium and bare stroma along the edges of a linear corneal abrasion.

Cell surface glycoconjugates may play a role in adherence of P. aeruginosa to the ocular surface. The data of Hazlett et al12 suggest that sialic acid might be an ocular receptor for P. aeruginosa in immature mice. The ocular mucin of adult mice contains abundant sialic acid, which could serve to protect the ocular surface from infection by trapping the bacteria before they can reach the apical cell membrane.16 Iida et al17 have evidence indicating that mannose and galactose molecules on cultured rabbit corneal cells may serve as the receptor for P. aeruginosa.

We have developed an organ culture system to study the adherence of P. aeruginosa to wounded and unwounded corneas. It is based on an in vitro model designed to determine factors involved in epithelial migration during wound healing.18 Data from our previous studies with the model demonstrated that
cell surface glycoconjugates are altered during healing such that the surfaces of migrating cells bind higher amounts of the lectin concanavalin A (Con A) than do apical cells of unwounded corneas. In addition, denuded basal laminae in the wound area also bind Con A avidly. Considering the propensity of wounded corneas to Pseudomonas infection and the potential role of glycoconjugates in bacterial adherence, our wound-healing model seems an appropriate system to examine the events involved in Pseudomonas adherence. Such a system allows for standardization of assay conditions and correlation of regional variations in adherence with the known distribution of cell surface glycoconjugates.

Materials and Methods

Preparation of Bacterial Suspensions

To determine the optimal conditions for the bacterial adherence assay, we conducted preliminary experiments in which we compared the effect of the following elements on adherence: four different strains of P. aeruginosa at four densities of bacterial suspension (10^6, 10^7, 10^8 and 10^9 colony-forming units (CFU/ml), varying the temperature and number of washes of the bacteria after growing them in nutrient broth, and different incubation times (15, 30 and 60 min) of the bacterial suspension with the corneas. The strain that adhered most avidly was chosen for development of the assay; it was obtained from a human corneal ulcer. Bacteria were grown overnight in trypticase soy broth (Difco, Detroit, MI), centrifuged and washed three times in phosphate-buffered saline (PBS), pH 7.2, at room temperature, and resuspended to a density of 1 x 10^8 CFU/ml in a completely defined culture medium containing no antibiotic or antimiycotic agents but with L-glutamine, nonessential amino acids, and trace elements. Bacterial density in the suspension was confirmed for each experimental condition. Bacteria adhering to four regions of the cornea were examined: (1) surface of the cells peripheral to the original wound margin; (2) surface of the cells at the leading edge of the migrating epithelium; (3) denuded basal lamina immediately in front of the migrating epithelium; and (4) denuded basal lamina at the wound center. Adherent bacteria were counted and expressed as number per mm^2 using a Zeiss Videoplan Digitizer (Rainin Instruments, Woburn, MA). This method is similar to that used by Ramphal and Pyle for quantitating bacteria adherent to trachea.

Culture Conditions

These studies conformed to the ARVO Resolution on the Use of Animals in Research. Adult Sprague-Dawley rats were killed with an overdose of sodium pentobarbital. Unwounded corneas or corneas with a 3 mm central epithelial debridement were used. The debridement area was gently outlined with a 3 mm trephine. Then a small scalpel, made with a blade breaker and razor blade fragment, was used to remove the epithelium from within the trephined area. Electron microscopy of this scrape wound demonstrates an intact basement membrane (as can be seen in Fig. 2D, inset). The corneas were excised, pinned on paraffin posts and cultured for 18 hr at 35°C in c-MEM. The bacterial suspension was added for the last 30 min of culture. Two to 4 hr prior to incubation with the bacterial suspension, the corneas were washed three times in c-MEM without antibiotic-antimiycotic to eliminate the antibacterial and antimiycotic agents present in c-MEM, and culture was continued in this medium.

To determine if specific sugar moieties of cell surface glycoconjugates are involved in the adherence of P. aeruginosa, corneas healing in MEM containing fructose instead of glucose (which binds s-Con A) were preincubated at room temperature for 30 min with 50 μg/ml of succinyl-concanavalin A (s-Con A; Polysciences, Warrington, PA) or 50 μg/ml s-Con A plus 0.2 M α-methyl mannoside (αMM; Gibco, Grand Island, NY) before addition of the bacterial suspension. Divalent s-Con A was used rather than tetravalent Con A in order to reduce the possibility of open valencies on the lectin acting as a bridging ligand for the bacteria. s-Con A binds specifically to mannose moieties in glycoconjugates, and αMM provides the specificity control.

Scanning Electron Microscopy and Quantitation of Adherent Bacteria

After incubation with the bacterial suspension, the corneas were washed three times in MEM and fixed in half-strength Karnovsky’s fixative. They were dehydrated through graded ethanol, critical point-dried using liquid CO_2 as the transitional fluid, coated with gold, and viewed on an Amray (Bedford, MA) 1000A scanning electron microscope. All corneas were photographed with a working distance of 12 mm, at an angle of 45°, and with a magnification of ×2000. Every fourth field was photographed across one axis from central to peripheral cornea. Six to eight corneas from two separate trials were photographed for each experimental condition. Bacteria adhering to four regions of the cornea were examined: (1) surface of the cells peripheral to the original wound margin; (2) surface of the cells at the leading edge of the migrating epithelium; (3) denuded basal lamina immediately in front of the migrating epithelium; and (4) denuded basal lamina at the wound center. Adherent bacteria were counted and expressed as number per mm^2 using a Zeiss Videoplan Digitizer (Rainin Instruments, Woburn, MA). This method is similar to that used by Ramphal and Pyle for quantitating bacteria adherent to trachea.
Results

Very few bacteria (25 ± 15/mm²) were seen adherent to apical cell membranes of normal, unwounded epithelial surfaces of corneas (Fig. 1, Table 1). Most adherent bacteria appeared to be on desquamating cells.

After a 3 mm diameter central epithelial debridement in which the basal lamina is left intact, the epithelium surrounding the wound moves centripetally as a unified sheet. In cross-section, the migrating epithelium tapers from its normal stratification peripheral to the wound to a single cell layer at the tip of the leading edge. After 18 hr of healing in vitro, a small central defect remains (Fig. 2, inset). More bacteria adhered to surfaces of wounded than unwounded corneas (Table 1). Also, there was a distinct regional variation in the distribution of adherent bacteria on healing corneas (Figs. 2, 3, Table 1). Bacteria adhered in largest numbers to the denuded basal lamina immediately in front of the migrating epithelium (Figs. 2C, 3). The number of bacteria found adherent to cells at the leading edge of the migrating epithelium (Fig. 2B) was significantly greater than that adhering to cells peripheral to the wound (Fig. 2A) but similar to that on the central basal lamina (Fig. 2D).

The avid adherence of *Pseudomonas* to the denuded basal lamina in front of the leading edge was unexpected. Therefore, three types of experiments were done to determine if the regional distribution of adherent bacteria was due to artifact. (1) To examine the possibility that the bacteria had simply collected

Table 1. Bacteria on surfaces of unwounded and wounded corneas

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<tr>
<th>Cornea</th>
<th>Adherent bacteria/mm²*</th>
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<tr>
<td>Unwounded</td>
<td>25 ± 15</td>
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<tr>
<td>Wounded</td>
<td></td>
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<tr>
<td>Surface of cells peripheral to wound</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Surface of cells at leading edge of migrating epithelium</td>
<td>37,700 ± 6,100</td>
</tr>
<tr>
<td>Denuded basal lamina in front of migrating epithelium</td>
<td>360,700 ± 49,900</td>
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<tr>
<td>Denuded basal lamina at wound center</td>
<td>28,800 ± 10,700</td>
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* n = 8 corneas; mean ± SEM.
Fig. 2. Scanning electron micrographs of wounded corneas after healing for 18 hr in vitro, the last 30 min in the Pseudomonas suspension.

(A) Epithelial cells peripheral to original wound. Note absence of bacteria and presence of microplicae. (Inset) Whole cornea illustrating the four areas examined in this figure. (B) Bacteria adherent to leading-edge cells of the migrating epithelium. Note adherent bacteria (arrows). (C) Basal lamina in front of the leading-edge cells (arrows). Bacteria adhered avidly to this region. (D) Adherent bacteria on denuded basal lamina at center of wound. (Inset) Transmission electron micrograph demonstrating intact basal lamina (arrow) after epithelial debridement. Bars: (A-D) 5 μm; (A, Inset) 750 μm; (D, Inset) 0.48 μm.
Fig. 3. Regional variation of adherent bacteria on the wound surface. Area at far right (center) is central cornea. Cell surfaces of the leading edge (LE) have fewer bacteria than the area in front of the LE. Bar: 33.3 μm.

by gravity in a depression in front of the leading edge cells, six healing corneas were incubated inverted in the bacterial suspension for 30 min. (2) To determine the effect of a shorter incubation time, eight healing corneas were incubated upright in the bacterial suspension for 15 min. Although the overall numbers of adherent bacteria were smaller than those shown in Table 1, the pattern of regional distribution was duplicated in both experiments (Table 2). (3) To examine the effect of the migrating epithelium on the regional pattern, six corneas with total limbal-limbal epithelium debridement were incubated upright for 30 min in the bacterial suspension. A uniform distribution of adherent bacteria was seen on the denuded basal lamina across the surface of the cornea in the absence of migrating epithelium. The numbers of adherent bacteria per mm² on four equidistant regions of the cornea from limbus to wound center were 33,000 ± 9,800, 26,500 ± 11,100, 34,300 ± 12,700 and 28,800 ± 8,200, respectively.

Having determined the optimal conditions for the adherence assay, we were able to evaluate the contribution of sugars as bacterial ligands by comparing bacterial adherence to corneas that had been preincubated with either 50 μg/ml s-Con A or 50 ng/ml s-Con A plus 0.2 M αMM (the specificity control). The two areas of greatest adherence, the denuded basal lamina in front of the migrating epithelium and the cells at the leading edge of the migrating epithelium, were examined. There were significantly fewer

<table>
<thead>
<tr>
<th>Region</th>
<th>Corneas incubated inverted for 30 min</th>
<th>Corneas incubated upright for 15 min</th>
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<tbody>
<tr>
<td>Surface of cells peripheral to wound</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Surface of cells at leading edge of migrating epithelium</td>
<td>14,400 ± 3,600</td>
<td>26,800 ± 7,200</td>
</tr>
<tr>
<td>Denuded basal lamina in front of migrating epithelium</td>
<td>140,800 ± 31,300</td>
<td>144,400 ± 38,500</td>
</tr>
<tr>
<td>Denuded basal lamina at wound center</td>
<td>9,400 ± 6,000</td>
<td>39,700 ± 16,900</td>
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* n = 6 corneas; mean ± SEM.  † n = 8 corneas; mean ± SEM.
bacteria adherent to corneas pretreated with s-Con A (Fig. 4A,B) as compared to controls (Fig. 4C,D, Table 3).

Discussion

The organ culture system for the study of adherence of *P. aeruginosa* to wounded and unwounded corneas reported here yields quantifiable data and permits experimental manipulation. Using whole corneas preserves the normal apical to basal stratification within the epithelium and the relationship between the epithelium and basal lamina of healing corneas. Such preservation of spatial relationships is impossible with cells in culture. In addition, the scanning electron microscopic assay provides data on regional variations in adherence. Our results confirm previous reports that *P. aeruginosa* adheres poorly to unwounded corneal epithelium.\textsuperscript{11-13} In the case of healing corneas, we found a distinct regional pattern of adherence. By far the greatest numbers of bacteria adhered to the denuded basal lamina in front of the migrating epithelium. The next most avid areas of adherence were the leading-edge cells of the migrating...
epithelium and the central denuded basal lamina. Although our results appear to contradict those of Stern et al.\textsuperscript{13} who showed higher adherence to injured mouse epithelial cells than to denuded basal lamina of corneas with limbal-limbal debridement, the cells in our model are uninjured migrating cells whose cell surface glycoproteins have been modified during transition to the migrating state.\textsuperscript{19,23} This model therefore mimics the clinical condition of corneas healing after an epithelial scrape (either accidental or surgical) rather than that of bacterial adherence to damaged cells immediately after an accidental scratch or refractive keratoplasty wound.

Preservation of the regional distribution of adherent bacteria on corneas inverted in the bacterial suspension demonstrates that the result is not a culture artifact caused by gravitational settling of the bacteria in a depression in front of the leading-edge cells. Similarly, preservation of the regional distribution pattern after only 15 min of culture with the bacterial suspension confirms that the pattern was not due to the bacterial invasion into selected areas of the stroma, which, as shown by Stern et al.,\textsuperscript{13} can begin to occur in injured rabbit corneas by 30 min of co-culture. The uniform pattern of bacterial adherence that we found following a total epithelial debridement indicates that the corneal epithelium is necessary for expression of the regional pattern. It is possible that the migrating corneal epithelium secretes some factor, possibly along the basal lamina in advance of its movement, and that this factor is responsible for increased bacterial adherence. Alternatively, a factor secreted by the keratocytes of the wounded cornea could be extruded preferentially at the surface of the stroma in front of the migrating epithelium because of hydrostatic pressure resulting from edema of the cultured, wounded corneas. Future studies investigating the molecular basis underlying the regional distribution of adherent bacteria on healing corneas may provide additional insights into the mechanisms of adherence of \textit{P. aeruginosa}.

We previously showed that cell surfaces of corneal epithelium migrating to cover a scrape wound, as well as the denuded basal lamina at the wound surface, bind much more Con A than do apical cell surfaces of stationary corneal epithelium or epithelium behind the leading edge of the migrating sheet.\textsuperscript{19,22} Since the zones of greatest bacterial adherence correspond to zones of increased Con A binding, the use of Con A seems to be an appropriate experimental manipulation in our bacterial adherence assay. We found a significant decrease in bacterial adherence when mannose moieties on ocular surface glycoconjugates were blocked by s-Con A, suggesting that mannose moieties may play a role in initial \textit{Pseudomonas} adherence. Other studies support this hypothesis. Gilboa-Garber et al.\textsuperscript{24} demonstrated mannose-binding hemagglutinins in \textit{Pseudomonas} extracts. Iida et al.\textsuperscript{17} found that pretreatment of \textit{Pseudomonas} with mannose or galactose inhibits their adherence to cultured rabbit corneal cells and that exposure of cultured rabbit corneal cells to mannosidase or galactosidase inhibited \textit{Pseudomonas} adherence.

Hazlett et al.\textsuperscript{17,15} showed that \textit{Pseudomonas} adherence to the unwounded cornea is much greater in newborn than in adult mice and that adherence may be mediated by sialic acid residues. We previously demonstrated a relative paucity of mannose moieties in glycoconjugates on apical cell membranes of normal, adult corneal epithelium.\textsuperscript{19} These two studies suggest that the normal cornea of the newborn animal and the wounded cornea of the adult possess sugar moieties that may serve as receptors for adherence of gram-negative bacteria, but that such moieties are not found in appreciable concentration on the normal cornea of the adult animal. Further study of the chemical nature and the conditions leading to the display of these receptors could provide insights into the basis for the increasing clinical problem of \textit{Pseudomonas} infection of the cornea.

\textbf{Key words:} bacterial adherence, corneal wound healing, cornea, \textit{Pseudomonas aeruginosa}, concanavalin A

\section*{References}


