A Partial-Thickness Epithelial Defect Increases the Adherence of Pseudomonas aeruginosa to the Cornea

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Some patients with infectious keratitis have no clinically demonstrable corneal abrasion predisposing them to infection. Subtle, undetectable corneal injuries may facilitate bacterial adherence to the cornea, eventually leading to keratitis. To study this concept, we have developed a rabbit model in which a partial-thickness corneal epithelial defect was induced by filter paper impression on the cornea that removed one to two layers of corneal epithelium. Following this injury, the corneas were incubated with Pseudomonas aeruginosa, washed, and the number of bacteria adhering to the injured corneas as well as to control corneas was quantitated. Corneas treated with filter paper, either ex vivo or in vivo, allowed 20 times more bacteria to adhere than did the untreated control corneas (P < 0.01). This superficial epithelial defect increased Pseudomonas adherence to the cornea for up to 72 hr after injury. When corneal injury was extended to the stroma, the adherence of Pseudomonas was further augmented as compared to adherence to the superficially injured cornea. Thus, we conclude that a clinically subtle, partial-thickness corneal epithelial injury can markedly facilitate the adherence of Pseudomonas aeruginosa, which may be an important predisposing factor for infectious keratitis.


It is well appreciated clinically that corneal abrasion is a predisposing factor to bacterial keratitis. Heretofore macroscopic injuries have been used in animal models of the disease. Nevertheless, infectious keratitis often caused by Pseudomonas aeruginosa occurs frequently in the absence of a history of corneal trauma, particularly among extended wear soft contact lens wearers and dry eye patients. Presumably disruption of the tear film (in dry eye patients), an unspecified compromise of corneal defenses, or a subtle corneal injury (in contact lens wearers) predisposes those individuals to infectious keratitis.

In this report we investigated the effect of a partial-thickness corneal epithelial injury as well as a full-thickness epithelial injury on the subsequent adherence of Pseudomonas aeruginosa. Using this approach we found that after the removal of one to two layers of corneal epithelium, the adherence of Pseudomonas to the cornea was enhanced 20-fold or greater.

Materials and Methods

Animals

Male and female adult New Zealand white rabbits were used in this study in accordance with the ARVO Resolution on the Use of Animals in Research. Enucleated eyes were also obtained from cattle and sheep at a local abbatoir and transported in phosphate buffered saline (PBS) at 4°C until use.

Bacteria

A strain of Pseudomonas aeruginosa isolated from a patient with soft contact lens-associated infectious keratitis and characterized in previous work was maintained by weekly transfer on trypticase soy agar. For adherence assays a loopful of bacteria was placed in 10 ml of Mueller–Hinton broth (MHB) and grown at 26°C with shaking for 16 hr. The bacteria were washed in phosphate-buffered saline (PBS) by centrifugation and diluted to a McFarland standard (approximately 1.5 × 10^8 bacteria/ml) in PBS.

Corneal Trauma

Ex vivo experiments: Eyes were removed aseptically from rabbits, cattle and sheep. The right eye of each animal served as a normal control. A subtle epithelial injury was induced by pressing a strip of Number 42 filter paper (Whatman, Hillsboro, OR) to the corneal surface until adherent and then gently removing it. This removed one to two layers of epithelium.

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Cellular cells from the corneal epithelium (Fig. 1A, B). Removal of epithelium was confirmed in each instance by staining the filter paper strips with 1% toluidine blue and verifying the presence of epithelium by light microscopy (Fig. 2). A severe corneal injury resulting in exposure of the stroma was induced by soaking globes in 4% cocaine hydrochloride for 30 min followed by removal of the epithelium with a spatula. This method removed all microscopic trace of epithelium (Fig. 1C).

In vivo experiments: Rabbits were anesthetized to the level of absence of pinch reflex with xylazine and ketamine intramuscularly and corneas were then treated with filter paper or cocaine as described.
above. Further anesthetic was given as needed during the experiment to maintain the appropriate level of anesthesia. No ophthalmic drops were used.

**Adherence Assay**

Conduct of adherence assay using ex vivo eyes. Ex vivo globes with or without the superficial epithelial injury or denudation to the stroma were submerged in 3 ml of PBS containing $1.5 \times 10^8$ *Pseudomonas*/ml for 90 min at 37°C without shaking. Following this, each globe was washed by immersion four times in sterile PBS and then a 7.2 mm diameter button of the central cornea was removed using a trephine. Each button was sectioned into halves which were homogenized in 1 ml MHB with a motor-driven Teflon grinder (Tri-R Instruments, Inc., Rockville Center, NY). Serial 10-fold dilutions of the homogenate or 1:1 dilutions were prepared and duplicate 0.1 ml samples of each dilution were plated onto M-H agar after the dilutions had been prepared and colony-forming units (cfu) determined the following day. Uninfected control eyes possessed no detectable cfu of bacteria.

Conduct of the adherence assay using in vivo eyes. In vivo experiments were conducted in the same manner except that *Pseudomonas* was placed in each eye by dropper every minute for 90 min. Animals were sacrificed, the eyes removed, washed, trephined, homogenized and aliquoted as above.

**Statistics**

Mean values were compared by two-tailed student t-test. In some experiments, no assumption was made about the distribution of data and Chi-square analysis was performed. There were at least three animals per experiment and each experiment repeated a minimum of three times.

**Results**

**Characterization of Injury Produced by Filter Paper Impression**

We first determined the depth of the superficial corneal injury induced by the application of filter paper to ex vivo rabbit and bovine eyes. Cross sections of ex vivo rabbit globes preserved after filter paper impression showed that epithelium was removed from the corneal surface and there was no extension of the injury to the basal layers of the epithelium (Fig. 1B). The injury was such that there were fragments of epithelial cells and intact epithelial cells present in areas of injury. Stained preparations of the filter paper showed intact corneal epithelium that appeared to be only one to two cells thick (Fig. 2). Ex vivo rabbit eyes subjected to filter paper impression and viewed with the slit lamp showed no evident corneal injury even after the application of fluorescein. Application of rose bengal demonstrated a clearly delineated central staining. Therefore, this method provided a subtle partial thickness epithelial defect.

**Adherence of Pseudomonas to the Uninjured Cornea**

In preliminary ex vivo experiments it was established that the number of *Pseudomonas* required in the inoculum in order for detectable cfu to occur in the control cornea was large: incubation of the eye for 90 min with: $1.5 \times 10^8$ bacteria/ml yielded 305 ± 91 cfu/mm² of corneal surface; $1.5 \times 10^7$ bacteria/ml yielded 198 ± 99 cfu/mm²; and $1.5 \times 10^6$ bacteria/ml yielded 46 ± 24 cfu/mm². Incubation with $10^6$ or lower bacteria/ml did not yield detectable cfu. Therefore, the remainder of the experiments were conducted with inocula of $1.5 \times 10^8$ bacteria/ml, an inoculum size which resulted in detectable cfu and was easily standardized. Adherence of *Pseudomonas* to the control ex vivo eye was a rapid process: after 15 min of incubation there were 189 ± 52 cfu/mm² of corneal surface; 30 min, 258 ± 30 cfu/mm²; and at 90 min, 344 ± 69 cfu/mm². This represented about a 100% increase in adherence from 15 to 90 min but it is noteworthy that there was considerable adherence even after 15 min of incubation.

**Adherence of Pseudomonas to Filter Paper-Injured Ex Vivo Corneas**

Control eyes and filter paper-treated eyes were then incubated with $1.5 \times 10^7$/ml *Pseudomonas aeruginosa* for 90 min, washed and coronal sections of the cornea removed, stained with toluidine blue and viewed with the light microscope. It was clearly evident that *Pseudomonas aeruginosa* adhered in abundance to defects created in the corneal epithelium by the filter paper treatment (Fig. 3), whereas in control eyes there were only a few adherent bacteria in random locations not recognizably associated with injured or abnormal epithelium. This was true of eyes from cows, sheep and rabbits.

Quantitation of the adherence of *Pseudomonas* to ex vivo filter paper-injured corneas compared to control or uninjured eyes was then performed. The results of a typical experiment are shown in Table 1. It was clear that the subtle defect greatly enhanced adherence. The control or uninjured eye had 50 ± 35 cfu/mm² whereas the filter paper-injured eye had 1398 ± 538 cfu/mm², an increase of greater than 20 times that of the uninjured eyes ($P < 0.01$). The epithelial injury was apparently repaired by 72 to 96 hr since at that time there was no significant difference
in *Pseudomonas* adherence to injured or uninjured eyes (Fig. 4).

**Adherence of *Pseudomonas* to Ex Vivo Stroma**

The level of trauma was then extended to include the removal of corneal epithelium altogether. Eyes soaked in cocaine for 30 min and then denuded to stroma with a spatula also demonstrated enhanced adherence of *Pseudomonas* (Table 1). The increase of adherence was on the order of 14 times greater than that to uninjured eyes (*P < 0.01*).

**Adherence of *Pseudomonas* to Injured Corneas In Vivo**

Having established that both partial thickness epithelial injury and full thickness epithelial injury or exposure of stroma clearly increases the adherence of *P. aeruginosa* to ex vivo eyes, we repeated these experiments in vivo to determine whether similar increases of adherence would occur in a more physiological setting. Injured corneas (whether injuries were partial or full-thickness epithelial injuries) showed greater adherence of bacteria than control or uninjured eyes similar to that obtained with ex vivo eyes. Enhanced adherence of *Pseudomonas* was seen when measured by colony forming units and by dilution determination. Multiple comparisons of the partial-thickness epithelial injury versus full-thickness epithelial injury were conducted in effort to determine which defect allowed a greater adherence of *Pseudomonas*. In every animal the cornea with stromal exposure from a full-thickness epithelial injury had greater adherence of bacteria than did the one with the partial-thickness epithelial injury. The results of a representative experiment are shown in Table 2. The

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n*</th>
<th>cfu/mm²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (control)</td>
<td>3</td>
<td>50 ± 35</td>
<td></td>
</tr>
<tr>
<td>Partial-thickness epithelial injury</td>
<td>3</td>
<td>1398 ± 538</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Normal (control)</td>
<td>3</td>
<td>285 ± 106</td>
<td></td>
</tr>
<tr>
<td>Full-thickness epithelial injury</td>
<td>3</td>
<td>4081 ± 528</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The injuries were created by removal of 1 to 2 layers of corneal epithelium with filter paper (partial-thickness epithelial injury) or by exposure of the stroma by scraping (full-thickness epithelial injury). The corneas were then incubated with 1.5 × 10⁶ *Pseudomonas*/ml for 90 min. *P* value determined from paired data using the student t-test.

* n = number of rabbits.

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**Fig. 4. Increase in *Pseudomonas* adherence to the cornea following filter paper injury. Increase in adherence was determined by the formula: mean cfu/mm² of corneal surface of filter paper-injured cornea MINUS mean cfu of control cornea DIVIDED BY mean cfu of control cornea. There were two rabbits for each time point.**
mean dilution at which the filter paper-treated cornea injury was positive (ie, visibly turbid) was 1:64, whereas eyes with the stromal exposure had a mean dilution of 1:1024. Furthermore, when colony-forming units/ml in positive dilution tubes were compared, the cornea with stromal exposure from the full-thickness epithelial injury ranged from 10–100 times greater than the eye with a partial-thickness epithelial injury.

**Discussion**

In these experiments we have established that a partial-thickness epithelial injury undetectable by a slit-lamp examination markedly enhances the adherence of *Pseudomonas aeruginosa* to the corneal surface. This was demonstrated with corneas from three mammalian species.

Previous animal models of *Pseudomonas* keratitis have used macroscopic injury in measuring adherence as well as infection: for example, by trephining to the stroma; injecting microorganisms into the cornea or by scratching the cornea (for a review of these models see Hyndiuk). In some animals no manipulation is required to induce infection, which is not considered to be the case in humans. While these manipulations of the animal corneas are successful in inducing *Pseudomonas* infections, it is questionable whether such gross trauma simulates the pathogenesis of infectious keratitis, especially as it is seen in patients wearing contact lenses or with dry eyes. Infectious keratitis in patients wearing soft contact lenses, with dry eyes or in comatose patients occurs often in the absence of a history of predisposing corneal injury. Therefore, we sought a model in which the induced corneal injury would be subtle and reversible. Having induced such an injury, we could then measure its effects upon the adherence of *Pseudomonas aeruginosa* to the corneal surface since adherence of a microorganism is a necessary first step in the pathogenesis of infectious keratitis. The model described here allows us to perform these measurements. The injury induced by impression is entirely superficial, removing only one to two layers of corneal epithelial cells (Figs. 1, 2). The defect is also reversible, with return to normal cornea after about 72 to 96 hr as determined by the ability of the bacteria to adhere to the corneal surface.

We have determined that bacteria adhere almost exclusively to areas where an epithelial defect exists (Fig. 3). Whether the bacteria are adhering to exposed extracellular matrix or surfaces of injured cells was not determined. However, work with *Candida* yeasts has demonstrated the clear preference of yeasts to adhere to extracellular matrix rather than intact endothelial cells. Extracellular matrix or epithelial cell surface glycoconjugates may be playing a similar role in the process of *Pseudomonas* adherence to the cornea as well. In the uninjured infant mouse, corneal sliacid acid may serve as a receptor whereas in vitro mannose moieties may be subserving this purpose in the adult rabbit.

Surprisingly, we found that stroma was more adherent than corneal epithelium, a finding opposite to that of Stern et al. However, in their experiment adherence was determined by scanning electron microscopy and the epithelial injury was made by scraping the cornea with a needle, hence inducing a defect through epithelium to the stroma. Spurr-Michaud et al found an enhancement of *Pseudomonas* adherence on denuded corneal basal lamina over adjacent corneal epithelium in an organ culture system. They found, however, that *Pseudomonas* adherence increased with healing whereas we found the opposite (Fig. 4). If adherence of the microorganism to target tissue is a necessary antecedent to infection, as it is believed to be in many infectious processes, then this work has demonstrated that a clinically subtle corneal lesion leads to a many-fold increase in bacterial adherence to the injured cornea. Furthermore, extending the injury to the stroma further increases bacterial adherence. Thus, if a similar partial-thickness epithelial injury occurs in soft contact lens wearers, comatose patients, or dry eye patients then this observation of enhanced bacterial adherence may help explain the pathogenesis of this enigmatic infectious disease.

**Key words:** bacterial adherence, corneal wound healing, cornea, infectious keratitis, *Pseudomonas aeruginosa*

**Table 2. Partial-thickness versus full-thickness epithelial injury in vivo in rabbits**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Partial-thickness epithelial injury</th>
<th>Full-thickness epithelial injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>1024</td>
<td>–(ND)*</td>
<td>+ (40)</td>
</tr>
<tr>
<td>512</td>
<td>–(ND)</td>
<td>+ (30)</td>
</tr>
<tr>
<td>256</td>
<td>–(ND)</td>
<td>+ (70)</td>
</tr>
<tr>
<td>128</td>
<td>–(ND)</td>
<td>+ (120)</td>
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<tr>
<td>64</td>
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<td>+ (225)</td>
</tr>
<tr>
<td>32</td>
<td>+(10)</td>
<td>+ (920)</td>
</tr>
<tr>
<td>16</td>
<td>+(15)</td>
<td>+ (2170)</td>
</tr>
<tr>
<td>8</td>
<td>+(70)</td>
<td>+(4585)</td>
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<tr>
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<td>+(140)</td>
<td>+(TNTC)</td>
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<td>+(TNTC)</td>
</tr>
<tr>
<td>1</td>
<td>+(660)</td>
<td>+(TNTC)</td>
</tr>
<tr>
<td>0</td>
<td>+(1135)</td>
<td>+(TNTC)</td>
</tr>
</tbody>
</table>

A comparison of dilutions positive (ie, turbid broth) for *Pseudomonas* and the corresponding colony-forming units in the tubes. Eyes had been incubated with 1.5 x 10⁸ *Pseudomonas* for 90 min.

* ND = none detected; TNTC = too numerous to count.
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References