Pathogenesis of experimental Pseudomonas keratitis in the guinea pig: bacteriologic, clinical, and microscopic observations

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Uniformly severe corneal infections were produced in guinea pigs by intracorneal injection of about 10 viable Pseudomonas aeruginosa. After a brief lag period, multiplication of bacteria was rapid, reaching geometric means of 250,000 after 24 hr and of 5 million after 48 hr. Within 8 hr after inoculation, polymorphonuclear leukocytes (PMNs) began to infiltrate the anterior two thirds of the stroma. Stromal cells adjacent to the injection site became necrotic and appeared to be engulfed by PMNs. By 14 to 16 hr, an abscess containing a dense aggregate of PMNs and multiplying bacteria developed in the central stroma. By 16 to 24 hr, collagen breakdown was apparent within and around the abscess. Ultrastructural evidence of collagen breakdown included loss of intact collagen fibrils, tactoid formation, and accumulation of amorphous electron-dense material. The area of liquefactive necrosis gradually enlarged, and many corneas perforated after 3 to 4 days. Because the course of infection is highly reproducible, this model should prove useful for many studies of experimental Pseudomonas keratitis.

Key words: bacterial keratitis, cornea, histopathology, Pseudomonas aeruginosa, Pseudomonas keratitis, ultrastructure

Pseudomonas aeruginosa produces a severe keratitis which is difficult to treat and rapidly progressive and often leads to partial loss of vision in humans. One of the factors that has hindered progress in research on experimental Pseudomonas keratitis has been the absence of a highly reproducible model of infection. We have developed such a model in guinea pigs and have used it to evaluate efficacy of chemotherapy.

Our purpose in this study was to determine the sequence of events in untreated experimental Pseudomonas keratitis in guinea pigs by quantitative bacteriology, clinical inspection, biomicroscopy, and light and electron microscopy.

Materials and methods

Male Hartley strain guinea pigs (Camm Laboratories, Wayne, N. J., and Hilltop Laboratories, Scottdale, Pa.), weighing 300 to 350 gm, were fed guinea pig food and tap water ad lib. They were anesthetized and infected intracorneally with 20 μl of a dilute broth of P. aeruginosa strain. To determine the number of colony-forming units in the cornea, animals were sacrificed with anes-
Table I. Multiplication of *Pseudomonas* strain 107 in guinea pig cornea*

<table>
<thead>
<tr>
<th>Time</th>
<th>Corneal colony counts</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>0 hr</td>
<td>0.92 ± 0.39</td>
</tr>
<tr>
<td>4 hr</td>
<td>0.74 ± 0.42</td>
</tr>
<tr>
<td>8 hr</td>
<td>1.92 ± 0.93</td>
</tr>
<tr>
<td>16 hr</td>
<td>4.88 ± 0.86</td>
</tr>
<tr>
<td>1 days</td>
<td>5.45 ± 0.70</td>
</tr>
<tr>
<td>2 days</td>
<td>6.70 ± 0.41</td>
</tr>
<tr>
<td>3 days</td>
<td>6.16 ± 0.65</td>
</tr>
<tr>
<td>4 days</td>
<td>6.26 ± 0.76</td>
</tr>
<tr>
<td>6 days</td>
<td>6.22 ± 0.83</td>
</tr>
<tr>
<td>8 days</td>
<td>4.81 ± 1.49</td>
</tr>
<tr>
<td>10 days</td>
<td>4.36 ± 1.64</td>
</tr>
<tr>
<td>12 days</td>
<td>0.96 ± 1.82</td>
</tr>
<tr>
<td>14 days</td>
<td>0.83 ± 1.40</td>
</tr>
<tr>
<td>16 days</td>
<td>No growth</td>
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</tbody>
</table>

* Data were pooled from three trials. Numbers of viable bacteria are expressed as common logarithms (base 10).

† n = number of corneas at each time.

Pathogenesis of experimental *Pseudomonas keratitis*

For the clinical observations, seven animals were examined grossly and biomicroscopically at frequent intervals for 48 hr after infection by two experimenters (T. A. and R. A. H., ophthalmologists), who had no knowledge of whether the individual corneas had been injected with live or heat-killed organisms. More than 80 animals were used in the complete study, and the clinical course was similar in all eyes.

For light and electron microscopy, more than 20 animals were injected with *Pseudomonas* and sacrificed at 8, 10, 12, 14, 16, 24, 32, and 48 hr after injection. At least two animals were sacrificed at each time period. The corneas were removed, and then one of each pair was fixed in buffered formalin for light microscopy and the other, in buffered glutaraldehyde for electron microscopy. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin, periodic acid–Schiff, the AFIP modification of Mowry's colloidal iron stain for acid mucopolysaccharides (AMP), Masson's trichrome, and Van Gieson's stains.

For electron microscopy, the corneas were fixed overnight in 2.67% phosphate-buffered glutaraldehyde (pH 7.2, 330 mOsm) and postfixed in the AFIP modification of Mowry's colloidal iron stain for acid mucopolysaccharides (AMP), Masson's trichrome, and Van Gieson's stains.

Figs. 1 to 3. Histological sections of guinea pig corneas inoculated with virulent strain of *P. aeruginosa*. PMNs can be seen migrating across anterior stroma at 8 hr (Fig. 1), accumulating in midstroma at 12 hr (Fig. 2), and forming a dense abscess "battle zone" at 16 hr (Fig. 3). (x100.)
Table II. Light microscopic observations of guinea pig corneas infected with *P. aeruginosa*

<table>
<thead>
<tr>
<th>Time after infection (hr)</th>
<th>Corneal thickness (mm)</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-10</td>
<td>0.30</td>
<td>Intact</td>
<td>Many PMNs in anterior 2/3 of stroma and in and around blood vessels; also small mononuclear cells</td>
<td>Intact; no inflammatory cells</td>
</tr>
<tr>
<td>14-16</td>
<td>0.40-0.60</td>
<td>Intact</td>
<td>Dense accumulation of inflammatory cells in mid stroma (PMNs and mononuclear cells); necrosis of mid stroma</td>
<td>Clumps of inflammatory cells on endothelium</td>
</tr>
<tr>
<td>24</td>
<td>0.60</td>
<td>Intact</td>
<td>Same dense accumulation of PMNs as at 16 hr. Almost continuity between inflammatory cells in anterior and periphery</td>
<td>No endothelial cells; clumps of inflammatory cells</td>
</tr>
<tr>
<td>48</td>
<td>1.10</td>
<td>Ulcer with loss of epithelium; also small micro abscesses extending into anterior stroma</td>
<td>Necrosis and fibillation of anterior stromal collagen in areas of intense infiltration</td>
<td>No endothelial cells; clumps of inflammatory cells</td>
</tr>
</tbody>
</table>

for 2 hr in 2.7% phosphate-buffered osmium. The tissue was dehydrated in a graded series of ethanol followed by propylene oxide and flat-embedded in a low-viscosity epoxy resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate and were viewed with an RCA-EMU-4C transmission electron microscope. Corneal thickness was measured under a light microscope with a micrometer, using 1 μm sections stained with azure II.

**Results**

**Quantitative bacteriology.** Groups of animals were infected intracoronally with a dilute broth suspension containing about 10 viable bacteria. Animals were sacrificed at intervals, and the number of viable bacteria in the cornea was determined (Table I). The data presented were derived in three experiments on a total of 50 animals. The bacteria had a lag phase in multiplication of more than 4 hr, but subsequent multiplication was rapid. The number of bacteria reached a plateau of about 5 million organisms (geometric mean) at 2 days and remained fairly constant for 4 days. After 10 days, the number of bacteria in the cornea rapidly declined. Four corneas contained no *Pseudomonas* upon subculture after 16 days.

**Clinical observations**

*Heat-killed bacteria injected—two eyes (controls).* Fine, punctate epithelial keratitis was observed in both eyes at 16 hr, and mild to moderate epithelial edema and a linear area of stromal edema corresponding to the inoculation tract were noted in both eyes at 4, 8, 16, 24, and 48 hr after the injection. An anterior chamber flare ranging from mild to moderate persisted for 24 hr in one eye, whereas the anterior chamber reaction in the other eye remained normal.

*Live Pseudomonas injected—five eyes.* Fine to medium punctate epithelial keratitis and mild to severe epithelial edema and haze were noted in all eyes at 4, 8, 16, and 24 hr.
Fig. 4. Epithelium (Epi), Basal lamina (BL), keratocyte (KC), and anterior stroma of guinea pig cornea 14 hr after intracorneal inoculation with virulent strain of P. aeruginosa. A PMN is migrating between the stromal lamellae. (×7,500.)

At the injection site, diffuse central stromal edema was noted at 16 hr in all eyes. Three of these corneas also showed small yellowish stromal infiltrates centrally.

By 24 hr, yellow infiltrates were present in the central midstroma of all eyes, with edema of the overlying anterior stroma. The red reflex was barely perceptible in three eyes as a result of the density of the stromal infiltrate. All the anterior chambers had a mild inflammatory reaction at 4 hr. By 16 hr, the anterior chamber inflammatory reaction was moderate to severe.

At 48 hr, all five eyes had moderate to se-
Fig. 5. Bacteria (anterior to injection site) lie with their long axes parallel to the collagen fibrils and are surrounded by clear halos. (x 17,000.)

vere chemosis and purulent discharge. The epithelium in all cases was extremely hazy and frequently edematous. A central ulceration was present in two, and mild central thinning without overlying ulceration was present in the other three. Four corneas had a dense, annular yellow infiltrate surrounding a gray area that earlier had demonstrated denser infiltration. One cornea still had a diffuse-yellow central infiltrate, and all five corneas had scattered peripheral foci of inflammation. The limbal cornea was uniformly edematous, with variable amounts of infiltrate. No red reflex was discernible, nor could the anterior chamber be evaluated.

Systematic observations by “blinded” observers were not made beyond 48 hr. After that time, the corneas were so severely damaged as to be virtually destroyed.

Light and electron microscopy

Heat-killed bacteria injected. Minimal lac-
ing was seen between the collagen lamellae and there was minimal infiltration of anterior stroma with polymorphonuclear leukocytes (PMNs) at 16, 24, and 48 hr. No degradation of collagen was seen.
Fig. 6. Battle zone between degenerating PMNs and bacteria 14 hr after inoculation. Seven bacteria are seen with electron-lucent halos. The collagen fibrils appear to be intact, although they are loosely packed in some areas. (×10,000.)

*Live Pseudomonas injected.* Inflammatory cells migrated through the anterior stroma and gradually accumulated in central stroma at the injection site (Table II and Figs. 1 to 3). The infiltrate was composed primarily of PMNs, which seemed to migrate between the collagen lamellae and around the stromal cells without producing any damage in the periphery (Fig. 4). The stromal cells near the injection site, underwent necrosis and appeared to be engulfed by the PMNs.

The bacteria multiplied in central stroma
and seemed to move between collagen fibrils with their long axes parallel to the long axes of the collagen fibers (Fig. 5). The individual bacteria was surrounded by clear halos (Figs. 5 and 6).

A "battle zone" composed of a dense aggregation of bacteria and PMNs was noted in central stroma by 14 hr (Table II and Figs. 3 and 6). The collagen fibrils remained intact in some areas of the abscess at 16 hr, although the PMNs were undergoing degeneration and dissolution and many bacteria were present (Fig. 6). By 24 hr, necrosis and liquefaction of collagen was visible by light micros-
copy, and electron microscopy revealed the breakdown of collagen fibers, tactoid formation, and extensive accumulations of amorphous electron-dense material (Figs. 7 and 8) when compared to an area of normal stroma (Fig. 9). Undigested bacteria were observed in PMNs that were completely degranulated (Fig. 7). Between 24 and 32 hr, ulceration of the epithelium developed over the dense central infiltrate (Table II).

Corneal thickness began to increase by 14 to 16 hr and had quadrupled (from control values of 0.30 mm to greater than 1.0 mm) by 48 hr (Table II). At 16 hr, inflammatory cells
had settled on the endothelium, and much of the endothelium was damaged or absent by 24 hr.

Discussion

Intracorneal injection of about 10 viable _P. aeruginosa_ strain 107 was uniformly followed by a progressive infection, inflammation, and destruction of the cornea in guinea pig. After a lag phase, the number of bacteria increased rapidly. About 48,000 bacteria were present 16 hr after infection and about 280,000 were present 24 hr after infection. Electron microscopic observations could be correlated with
clinical, biomicroscopic, and light microscopic observations of the inflammation and progressive destruction of the corneas at various times after infection. For example, the dense yellow infiltrates seen by biomicroscopy and light microscopy after 16 to 24 hr were shown by electron microscopy to be abscesses composed of degranulating PMNs interacting with multiplying bacteria. Liquefactive necrosis and loss of collagen staining by light microscopy were shown by electron microscopy to be due to degradation of collagen fibers. Collagen breakdown began in association with the degranulating PMNs in the abscess. In animals that were not sacrificed, the area of liquefactive necrosis gradually enlarged until the corneas perforated after 3 to 4 days.

The ultrastructural changes seen in guinea pig corneas infected with *P. aeruginosa* are remarkably similar to those described by Rowsey et al. In rabbit cornea 9 days after the intracorneal injection of concentrated polymorphonuclear leukocyte lysosomal (PMNL) preparations. After the injection of lysed PMNL, collagen fibrils were replaced by amorphous deposits of electron-dense material, and tactoidal formation occurred. Tactoids have also been described in in vitro studies of collagen degradation. In contrast, Gray and Kregar emphasized the etiologic importance of breakdown of proteoglycan ground substance rather than collagen in experimental *Pseudomonas* keratitis in rabbit. They reported that loss of the characteristic weblike pattern of the proteoglycan ground substance resulted in dispersal of ultrastructurally normal collagen fibrils, weakening of the cornea, and subsequent perforation. They suggested that a bacterial or host-derived collagenase is not required for extensive corneal damage. Kessler et al. purified an extracellular protease of a virulent strain of *P. aeruginosa* which had little or no collagenolytic activity but solubilized 60% of the total proteoglycans from rabbit corneas following incubation with the enzyme. It is possible that the clear halo around each bacterium is due to activity of such a bacterial enzyme. Kessler et al. have also studied the host response to the intrastromal injection of heat-inactivated *P. aeruginosa* cells in rabbit cornea. They described an extensive PMN infiltration which progressed to ulceration in 1 week and found collagenase and proteolytic enzymes capable of degrading proteoglycans in the ulcerated corneas. Iglewski et al. reported that *Pseudomonas* exotoxin A injected into the rabbit corneas produces death of all cell types, infiltration with PMNs, and necrosis of the cornea.

We cannot conclude therefore that the corneal ulceration and perforation in experimental *Pseudomonas* keratitis in the guinea pig is due only to collagenolysis. The accumulation of electron-dense material and tactoids is most likely due to the proteoglycan breakdown products of collagen, but the presence of this material makes it impossible to tell whether or not the proteoglycan-ground substance is present or absent in electron microscopic sections.

By electron microscopy, organisms that appeared to be intact were observed in PMNs that appeared to have completely degranulated. We speculate that this sequestration might be an explanation for the persistence of *Pseudomonas* in the cornea despite chemotheraphy. Relapse of *Pseudomonas* keratitis has been reported in humans after apparently adequate chemotheraphy.

Van Horn et al. described ultrastructural alterations in a pair of human donor corneas that had been infected with *P. aeruginosa*. Cellular infiltration was seen in both corneas, and extensive degradation of collagen was described in one cornea that had perforated. The observations made in our experimental model in guinea pigs are therefore compatible with those previously reported in human corneas. Because the course of infection produced in our experimental model is highly reproducible, we believe that it will prove to be a useful model for many types of studies of experimental *Pseudomonas* keratitis.

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REFERENCES