Acute Inflammation of the Eyelid and Cornea in Staphylococcus Keratitis in the Rabbit

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PURPOSE. The inflammatory response during Staphylococcus keratitis was analyzed biochemically and histologically to determine the source of the neutrophils infiltrating the tear film and cornea.

METHODS. Rabbit eyes were swabbed and then examined by slit-lamp microscopy at 0, 5, 10, 15, 20, and 25 hours after intracorneal inoculation with Staphylococcus aureus. Bacterial colony-forming units were quantified in the cornea, eyelid, and acute inflammatory exudate. Myeloperoxidase activity of ocular swabs of acute inflammatory exudate, corneal homogenates, and eyelid homogenates was determined. Gross and microscopic examinations of corneas and eyelids were performed.

RESULTS. The colony-forming units per cornea exceeded $10^7$ after 10 hours, whereas no bacteria were cultured from the eyelid until 15 hours postinfection. Slit-lamp examination revealed progressive pathology, and the myeloperoxidase activities of ocular swabs, corneas, and eyelids increased markedly by 15 hours postinfection. Corneas showed a wave of neutrophils moving from the tear film toward bacteria in the central corneal stroma and early neutrophil migration from the limbus into the stroma. In the eyelid, neutrophils migrated from the stromal vessels to the tear film.

CONCLUSIONS. Staphylococcus keratitis in the rabbit causes acute inflammation in the overlying eyelid. Neutrophils of the acute inflammatory exudate interact with the infected cornea, whereas neutrophils migrating through the cornea from the limbus remained distant from the site of infection. (Invest Ophthalmol Vis Sci. 1999;40:385–391)

The infection of the rabbit cornea with Staphylococcus aureus or the application of pure staphylococcal α-toxin to the cornea results in a marked inflammatory response, including severe corneal edema and an influx of neutrophils into the corneal stroma.1–3 The present study investigates the inflammatory processes inherent in this infection; such processes are possibly predictive of the events occurring in other ocular inflammations.

In vascularized tissue, inflammatory mediators and leukocytes arrive at the site of inflammation directly from blood vessels. However, an alternative mechanism is necessary for the avascular cornea. Perhaps the most widely held opinion is that the neutrophils in acute keratitis originate in the limbus and bulbar conjunctiva4 and that they reach the cornea by chemotaxis through the stroma5 or the tear film.6,7 In contrast, Weissman et al.8 and Majno and Joris9 have emphasized the role of the eyelid in corneal inflammation. The eyelid could be a source of substantial humoral and formed elements in the immune response because it is relatively large and has a richer vascular supply than the bulbar conjunctiva. The central cornea is also closer to the eyelid than to the bulbar conjunctiva, especially when the eye is closed.10 Thus, the eyelid is more likely to respond earlier and more profoundly to a stimulus from the central cornea.

Soluble chemotaxins of bacterial origin, such as N-formylmethionyl peptides or toxins, mediate inflammation.3–11 Chemotaxins of host origin such as complement and cytokines are also known mediators of ocular inflammation.11,12 These soluble mediators could diffuse through the tear film and elicit a host response in the overlying eyelid and in the limbus. Despite the attractive features of this hypothesis, histopathologic documentation of the inflammatory response in the eyelid during Staphylococcus keratitis is lacking. The aim of the present study was to examine the inflammatory response occurring in the cornea and eyelid during Staphylococcus keratitis. Emphasis has been given to the role of the overlying eyelid in the inflammatory process.

METHODS

Bacterial growth and corneal inoculation. S. aureus 8325-4 was grown to log phase in tryptic soy broth (TSB; Difco, Detroit, MI), and the culture was diluted to approximately $10^8$ colony-forming units (cfu) per milliliter, as described previously.13 An aliquot of this bacterial suspension (10 μl, 100 cfu) was injected into the corneal stroma of both eyes of New Zealand white rabbits. Previous studies have shown that injection of media does not result in any ocular changes other than the very

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transient changes associated with the needle’s insertion and placement of liquid into the corneal stroma. All rabbits were maintained according to the guidelines put forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Quantification of S. aureus per Cornea
The replication of S. aureus 8325-4 in the rabbit cornea was measured by determining the number of colony-forming units per cornea at 0, 5, 10, 15, 20, and 25 hours postinfection. Rabbits intrastromally injected with 8325-4 were killed, and their corneas were harvested (four per time point) and assayed for colony-forming units at the times described above. Quantification of viable S. aureus per cornea has been previously described. Briefly, whole corneas were aseptically removed, dissected, and homogenized. Aliquots of corneal homogenates were serially diluted in sterile phosphate-buffered saline (PBS, 0.1 M, pH 7), plated onto tryptic soy agar (0.1 ml, Difco), and incubated at 37°C for 24 hours. Colonies were counted and colony-forming units per cornea expressed as log base10 values.

Quantification of S. aureus in the Tear Film
The number of S. aureus colony-forming units in the acute inflammatory exudate was determined by rubbing sterile cotton swabs across the conjunctiva and cornea at 0, 5, 10, 15, 20, and 25 hours postinfection (swabs of 4 to 12 eyes were cultured per time point). The swabs were immersed in 2 ml sterile PBS for 10 minutes. After agitation, the swabs were removed; three 100-μl aliquots were plated onto tryptic soy agar (Difco) and incubated at 37°C for 24 hours. Colonies were counted and colony-forming units per swab expressed as log base10 values.

Quantification of S. aureus per Eyelid
The number of S. aureus colony-forming units per eyelid was determined by harvesting the eyelids at 0, 5, 10, 15, 20, and 25 hours postinfection (8 eyelids per time point). Each eyelid was bisected parallel to the conjunctiva using aseptic technique. The inner half of each eyelid was then homogenized in sterile PBS, and dilutions were cultured on mannitol salt agar (Difco). Each mannitol-positive colony was identified as S. aureus by determining its coagulase activity using rabbit plasma (Sigma Chemical Company, St. Louis, MO).

Myeloperoxidase Activity of Ocular Swabs, Corneal Homogenates, and Eyelid Homogenates
Sterile cotton swabs were rubbed across the conjunctiva and cornea and then immersed in a sterile solution of CTAB (0.5% in water, hexadecyltrimethyl ammonium bromide; Sigma Chemical). Swabs submerged in CTAB were frozen at −20°C. At the time of assay, the immersed swabs were thawed, frozen in liquid nitrogen, and thawed again to lyse neutrophils. Swabs were discarded, and the CTAB solution was assayed for myeloperoxidase activity as described previously. Corneal homogenates were prepared in sterile PBS (3 ml per cornea) and assayed as described previously. Eyelids were homogenized in sterile PBS, and CTAB (0.5%; Sigma) was added. Eyelids were homogenized again, frozen, thawed, and assayed for myeloperoxidase activity as described previously for corneal homogenates. There were 72 samples assayed in triplicate: 24 swab samples, 24 cornea samples, and 24 eyelid samples. The myeloperoxidase assay has a sensitivity of 0.01 units, which has been estimated to be equivalent to 103 neutrophils.

Slit-Lamp Examination
Ocular pathology was graded by slit-lamp examination (SLE) with a Topcon SL-5D slit-lamp biomicroscope (Koaku Kikai K.K., Tokyo, Japan) using a scoring system that has been previously described. Seven parameters were scored on a scale of 0 (absent) to 4 (severe), including conjunctival injection, conjunctival chemosis, corneal infiltrate, corneal edema, fibrin in the anterior chamber, hypopyon, and iritis. Two masked observers performed all SLE scoring.

Histopathologic Examination
All rabbits were killed by intravenous injection of sodium pentobarbital solution (100 mg/ml; The Butler Co, Columbus, MO). Upper and lower eyelids from each eye (eight per time point) were harvested at 0, 5, 10, 15, 20, and 25 hours after injection and immediately placed in formalin (10%; EK Industries, Joliet, IL). Gross pathologic examination was performed on each eyelid. The left upper eyelid from each rabbit was then bisected and processed for histopathologic examination using hematoxylin and eosin staining as previously described. Corneas were harvested at 15 and 20 hours postinfection for histologic examination using hematoxylin and eosin staining as described previously.

Statistical Analysis
The SEM of Staphylococcus per cornea, SLE scores, and myeloperoxidase activity was determined using a Statistical Analysis Systems program.

RESULTS
Slit-Lamp Examination
Inflammatory changes in the conjunctiva, cornea, aqueous humor, and iris were reflected by the SLE scores, which increased throughout the course of infection reaching a maximum of 19.75 ± 0.18 by 25 hours postinfection. SLE showed that corneal epithelial sloughing began in the central cornea by 10 hours postinfection and extended peripherally. Gross examination of the infected rabbit eyes revealed a viscous acute inflammatory exudate after 10 hours postinfection.

Myeloperoxidase Activity
The myeloperoxidase activity of ocular swabs, corneal homogenates, and eyelid homogenates of infected eyes increased from 10 to 25 hours postinfection (Fig. 1). The myeloperoxidase activity of ocular swabs was approximately six- to eightfold greater than that of the corneal homogenates and approximately twofold greater than eyelid homogenates. The myeloperoxidase activity of eyelid homogenates was approximately threefold to fourfold greater than that of the corneal homogenates.

Bacteriology
The replication of Staphylococcus in the cornea was exponential from 0 to 10 hours postinfection, reaching a maximum of
Ocular Inflammation in *Staphylococcus* Keratitis

**Cornea**

- **DSwab**
- **Eyelid**

**Hours Postinfection**

- **FIGURE 1.** Myeloperoxidase activity of corneal homogenates, ocular swabs, and eyelid homogenates of eyes with *Staphylococcus aureus* keratitis. Myeloperoxidase activity, expressed in total recovered units of enzyme activity, was determined for corneal homogenates, ocular swabs, and eyelid homogenates of eyes with *Staphylococcus* keratitis. All values are the means; error bars are the SEM.

- Approximately 7.0 log base 10 cfu/cornea after 10 hours postinfection and maintaining 7.0 log base 10 cfu/cornea to 25 hours postinfection. Despite the large number of colony-forming units in the cornea, the tear film and eyelid contained less than 100 cfu at all time points until 25 hours postinfection (Fig. 2).

**Corneal Histopathology**

Histopathologic examination of corneas at 15 and 20 hours postinfection revealed corneal ulceration and marked edema, a mass of cocci in the central corneal stroma, and migration of

- **FIGURE 2.** Colony-forming units of *Staphylococcus aureus* in rabbit eyes with keratitis. The cornea, acute inflammatory exudate on the corneal surface, and eyelid of eyes with *Staphylococcus* keratitis were cultured to determine the colony-forming units at each site. Bacterial colony-forming units are expressed as log base 10 values. All values are the means; error bars are the SEM. In some samples the error bar is imperceptible when compared with the same value.

- **Neutrophils** from the anterior toward the central cornea (Figs. 3, 4). At 20 hours postinfection (Fig. 4), the neutrophils in the anterior cornea were approximately 0.3 mm from the cocci. Migration of neutrophils from the limbus to the central cornea was also seen. The advancing edge of this wave of neutrophils was 1.3 mm from the cocci. Sections of the limbus revealed marked edema and moderate acute inflammatory exudate (Fig. 3).

**FIGURE 3.** Inflammation in the rabbit cornea at 15 hours after injection of *Staphylococcus aureus*. Top: a cornea infected with S. aureus for 15 hours shows a wave of neutrophils (open arrowhead) migrating into the stroma from the limbus toward the mass of bacteria (arrow) in the central cornea and adhesion of neutrophils to the denuded anterior cornea (closed triangle; magnification, X44). Middle: higher magnification of the central cornea shows the association of neutrophils in the tear film with the denuded central corneal stroma; also seen is the mass of bacteria in the deeper portion of the central cornea (magnification X220). Bottom: higher magnification of the pithery shows neutrophils migrating from the limbus into the peripheral corneal stroma (magnification X220). Sections were stained with hematoxylin and eosin.
unlike those harvested immediately after bacterial injection (0 hours postinfection), revealed extensive edema and erythema of the mucous membrane. Only mild redness and edema were noted by gross examination of eyelids harvested at 5 hours. These changes became progressively more pronounced with an increase in time after corneal injection.

**Eyelid Histopathology**

Microscopic examination of eyelids harvested immediately after injection of bacteria (0 hours postinfection) into the underlying cornea revealed very rare isolated neutrophils within the stroma. Neither vascular congestion nor adhesion of neutrophils to vascular endothelium was seen in these eyelids (Fig. 6). By 5 hours after injection of bacteria into the cornea, inflammatory changes in the eyelids were noted (Fig. 6). These changes progressed with time through 15 and 25 hours postinfection (Fig. 6). The inflammatory changes included vascular congestion and acute inflammatory exudation. A gradient in the number of neutrophils was noted, such that neutrophils in the eyelid stroma became more numerous toward the epithelium of the eyelid. Pavementing of neutrophils on the endothelial surface of blood vessels nearest the eyelid epithelium was obvious (Fig. 7). In all cases, neutrophil migration through a nonulcerated eyelid epithelium into the tear film was observed (Fig. 8). No bacteria or ulceration of the eyelid was seen.

5). Migration of neutrophils into the scleral epithelium was noted.

**Eyelid Gross Examination**

Gross examination of eyelids from eyes with infected corneas revealed general concordance in degree of change between the right and left eyes and between the upper and lower lids. For those eyelid pairs in which mild discordance was evident, pathologic changes were more pronounced in the upper lid. Eyelids harvested at 25 hours after injection of *Staphylococcus*,
at any time in histologic sections of the eyelid. In the sclera and the eyelid, neutrophils could be seen accumulating on the stromal aspect of the epithelial basement membrane (Fig. 9).

**DISCUSSION**

This study demonstrates that experimental *Staphylococcus* keratitis in the rabbit is associated with acute inflammation of the overlying eyelid, which contributes to the neutrophil population of the tear film. The histopathologic data suggest that neutrophils of the tear film reach the central cornea faster than those migrating through the cornea from the limbus. Also, the myeloperoxidase data demonstrate that neutrophils from the tear film are more numerous than those migrating into the cornea from the limbus. An intense neutrophilic response was observed previously in normal eyes injected with purified staphylococcal α-toxin.

The histopathologic evidence indicates that the inflammation seen in the eyelid is a response to the associated corneal infection and not a result of an infection of the eyelid itself. Inflammation was noted in the eyelid 10 hours before bacterial cultures of the eyelid became positive. In the eyelid, the lack of ulceration, the adherence of neutrophils to the capillary endothelium nearest the cornea, the concentration gradient of neutrophils toward the cornea, the migration of neutrophils through a nonulcerated epithelium, and their exudation into the tear film all suggest that this inflammation was elicited by a stimulus from the cornea, not the eyelid. Furthermore, the basement membrane is a physical barrier to neutrophil movement, so accumulation of neutrophils along the stromal aspect...
Figure 8. Exudation of neutrophils from the eyelid to the tear film. The movement of neutrophils (arrows) through the eyelid epithelium can be seen at 5 (top) and 25 (bottom) hours postinfection. Sections were stained with hematoxylin and eosin; magnification, X1063.

of the epithelial basement membrane in both the eyelid and the sclera suggests that neutrophils originating in the stroma of both tissues are migrating into the tear film. The contribution of the limbal vasculature to the exudate in the tear film has previously been reported.6

The microbiologic data also suggest that the inflammatory reaction in the eyelid is a response to the keratitis and not to an infection of the eyelid itself. The low number of bacteria cultured from the eyelid is more consistent with contamination from the heavily infected cornea than infection of the eyelid itself. Bacteria shed from the ulcerated cornea could have populated both the tear film and the inner surface of the eyelid, causing the observed result. In contrast to the cornea, in which abundant bacteria were easily visible, no bacteria were noted on microscopic examination of the eyelid. Overall, these data indicate that the inflammatory process observed in the eyelid is a response to the associated corneal infection.

Histopathologic examination of the cornea in this model of Staphylococcus keratitis suggests that neutrophils from the tear film may be more prominent in the central cornea than neutrophils migrating from the limbus. Neutrophils from the tear film reach the central cornea by 15 hours postinfection and by 20 hours are seen entering the superficially eroded stroma of the central cornea. Basu and Minta18 also observed the penetration of neutrophils into a denuded corneal stroma. Like Chusid and Davis,19 we also noted neutrophil migration from the limbus toward the central cornea. However, neutrophils traveling across the corneal stroma from the limbus did not reach the central cornea by 25 hours, suggesting that migration from the tear film is a faster route of neutrophil passage to the site of infection early in the disease process. This observation is supported by the relative myeloperoxidase activity of the tear film, cornea, and eyelid. The myeloperoxidase activity of the eyelid and the tear film, as measured by swabbing the anterior eye surface, was multi-fold greater than that of corneal homogenates. Thus, these results confirm that neutrophils from the tear film are involved in inflammation of the cornea, as has previously been reported.6,7

Taken together, the observations that bacterial infection of the cornea is associated with inflammation of the overlying eyelid and that neutrophils from the tear film are present in the cornea during keratitis suggest that the eyelid has an integral role in corneal inflammation. This is perhaps not surprising.

Figure 9. Neutrophils adhering to the stromal aspect of the basement membrane in the sclera and eyelid. Top: neutrophil morphology is clearly seen in neutrophils accumulating on the stromal aspect of the epithelial basement membrane in the scleral limbus (arrowhead). Bottom: neutrophil morphology is clearly seen in neutrophils accumulating on the stromal aspect of the basement membrane of the eyelid epithelium (arrowhead). Intraepithelial neutrophils are also seen (arrow). Sections were stained with hematoxylin and eosin; magnification, X2126.
because there is no obvious reason why soluble inflammatory mediators, which have been implicated in a variety of other ocular inflammatory processes\textsuperscript{4,12,20} including \textit{Pseudomonas} keratitis,\textsuperscript{21} should not elicit acute inflammation in the eyelid. The elucidation of this source of neutrophils has important implications for management of ocular inflammation. A mechanical blockade, which limits the passage of neutrophils from the eyelid to the cornea, could be a beneficial adjunct to bactericidal antimicrobials in the management of bacterial keratitis.

In summary, this study has shown that experimental \textit{Staphylococcus} keratitis is associated with an inflammatory response in the overlying eyelid and that neutrophils from the tear film are more prominent than those migrating from the limbus in early inflammation of the central cornea. These observations suggest that the eyelid has an important role in corneal inflammation.

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