Effects of Toxin Production in a Murine Model of Staphylococcus aureus Keratitis

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PURPOSE. To investigate the corneal virulence of toxin-deficient mutants of Staphylococcus aureus in young and aged mice in a topical inoculation model of keratitis.

METHODS. Corneas of young and aged A/J mice were scarified and topically inoculated with a log phase S. aureus parent strain (8325-4), an α-toxin-deficient mutant (DU1090), or an Agr-defective mutant (ISP546) deficient in production of multiple toxins or with purified α-toxin. Slit lamp examination (SLE) and histopathology were performed, and bacterial colony-forming units (CFU) and myeloperoxidase (MPO) activity were determined.

RESULTS. The infection of young mice with the mutant strains demonstrated significantly lower SLE scores (P ≤ 0.0001) and reduced histopathologic changes compared with infections with the parent bacterial strain. Either mutant strain of S. aureus produced SLE scores in aged mice through 9 days after infection (PI) that were significantly lower than those of aged mice similarly infected with the toxin-producing parent strain (P ≤ 0.0001). Despite use of identical inocula, the CFU per eye were greater for the parent than the mutant strains from 1 to 5 days PI in the young mice (P = 0.0372) and from 1 to 3 days PI in the aged mice (P = 0.0018). MPO activities were at the maximum at day 1 PI and were similar overall for all infections. Administration of purified α-toxin caused greater gross and histopathologic changes in eyes of aged mice than in those of young mice.

CONCLUSIONS. Bacterial toxins, and especially α-toxin, can mediate corneal disease in mice. Differences in severity of S. aureus keratitis in aged versus young mice correlates with their susceptibility to α-toxin. (Invest Ophthalmol Vis Sci. 2005;46:2064–2070) DOI:10.1167/iovs.04-0897

The bacterium Staphylococcus aureus is an opportunistic pathogen that is frequently associated with bacterial conjunctivitis1 and is a leading cause of bacterial keratitis in the United States.2,3 Tissue damage associated with S. aureus keratitis results from exotoxin production4–6 as well as from the host inflammatory response to infection.7 Bacterial keratitis can cause irreversible corneal scarring, resulting in loss of visual acuity or even blindness.2

Staphylococcus aureus can produce numerous toxins, including α-, β-, γ-, and δ-toxins. α-Toxin is a pore-forming hemolytic toxin that causes membrane damage to many types of mammalian cells8–10 and is capable of producing extensive corneal disease. It is not produced, or is produced in low amounts, by approximately 25% of isolates.11 α-Toxin-producing strains have been shown in the experimental rabbit model of keratitis to cause significantly greater ocular inflammation and corneal damage than α-toxin-deficient mutant strains.5

The expression of multiple proteins potentially involved in virulence is controlled by the accessory gene regulator (Agr) system.12–14 S. aureus secretes cell-wall–associated products and adhesins (i.e., clumping factor, fibrinectin binding protein, and protein A) during the logarithmic phase of growth, whereas most extracellular virulence factors (i.e., toxins, proteases, and lipases) are secreted in the stationary phase.12,13 Mutants defective in Agr demonstrate reduced expression of multiple proteins normally produced in the stationary phase, including α-, β-, and γ-toxins.14 Several studies have demonstrated that mutation of the agr locus results in a decrease in pathogenesis in nonocular15–18 as well as ocular5,6 models of infection (keratitis or endophthalmitis).

We19 have shown that aging in mice is associated with increased pathologic changes during S. aureus keratitis, a difference that could be attributed to a greater susceptibility of aged animals to the effects of staphylococcal virulence factors. Age-related changes in the susceptibility of mice to toxins could explain why aged mice demonstrate more severe corneal damage than young mice during Staphylococcus keratitis.19 To date, however, no studies have been conducted to analyze the effects of S. aureus toxin on the mouse eye. Thus, the purpose of this study was to determine the contribution of toxins to corneal damage in the mouse eye—a study based on the comparison of corneal virulence in both young and aged mice of a toxin-producing parent strain with that of an α-toxin-deficient mutant and an Agr mutant deficient in multiple toxins.

MATERIALS AND METHODS

Mice

Young A/J male mice (6–7 weeks of age) were purchased from the National Cancer Institute (Frederick, MD). Male A/J retired breeders (36–48 weeks of age) were purchased from the National Cancer Institute and Harlan Sprague-Dawley (Indianapolis, IN). A/J mice were chosen because they are the most susceptible to S. aureus keratitis, a trait possibly related to their lack of group II phospholipase A.19 All animals were maintained according to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Bacteria

S. aureus strain 8325-4, a well-characterized laboratory strain with a positive phenotype for toxin production (α, β, γ, and δ-toxins), was used in the study and has been described for use in rabbit intrastromal injection and topical inoculation models of keratitis, as well as the topical murine model of keratitis.4,19–22 These strains have been shown to grow at equivalent rates.5 To determine the role of toxins in corneal...
virulence, isogenic mutants of parent strain 8325-4 were used: an α-toxin-deficient mutant strain (DU1090) and an Agr-defective mutant strain (ISP546). The α-toxin-deficient mutant (DU1090) was constructed by inactivation of the structural gene for α-toxin (bbp) by allele-replacement mutagenesis with bby::Emr. The Agr-defective mutant of 8325-4 (ISP546) was generated by the insertion of transposon Tn551. Bacteria were grown overnight in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) at 37°C and then subcultured to log phase under the same conditions.

**Infection of Mice**

Mice were challenged with *S. aureus* strain 8325-4, as previously described. To anesthetize the mice, a 1.5-mL volume of xylazine (100 mg/mL; Rompun; Miles Laboratories, Shawnee Mission, KS) was combined with 10 mL ketamine HCl (100 mg/mL; Ketaset; Bristol Laboratories, Syracuse, NY), diluted 1:4 with saline, and injected subcutaneously at a dose of 0.1 mL/20 g body weight. Each cornea was scarified with a 30.5-gauge needle by making four parallel incisions to the corneal surface that did not penetrate beyond the superficial stroma. A 5-μL suspension of bacteria containing 1.0 × 10^9 CFU/mL was applied to each scarified cornea. The eyes of control mice were neither scarified nor inoculated with bacteria. Mice (n = 7 mice/group per time point) were monitored by slit lamp examination (SLE) and killed at 1, 3, 5, 7, and 9 days after infection (PI). After death, the eyes of noninfected and infected mice were enucleated and homogenized, and CFU/eye and myeloperoxidase (MPO) activity were determined. The eyes of two mice per group underwent histopathologic analysis. All experiments were performed in triplicate.

**Quantification of Viable Bacteria**

Eyes were enucleated and homogenized in 1.0 mL sterile phosphate-buffered saline (PBS) with sterile, disposable tissue grinders (Kontes Scientific, Vineland, NJ). To quantify viable bacteria, we used 0.1 mL aliquot of the homogenate serially diluted 1:10 in PBS. Serial dilutions (0.1 mL/plate) were plated onto tryptic soy agar (TSA; Difco Laboratories) and mannitol salt agar (MSA; EM Science, Gibbstown, NJ) in triplicate, and incubated at 37°C for 24 to 48 hours. Colonies were counted and CFU/eye were expressed as logarithmic values.

**Slit Lamp Examination**

Ocular disease was evaluated both macroscopically and microscopically with a slit lamp biomicroscope (Topcon Biomicroscope SL-5D; Kogaku Kikai KK, Tokyo, Japan) up to 9 days PI. Mouse eyes infected with *S. aureus* were graded by using a modification of the scale previously described by Hazlett et al.: 0, clear and normal; +1, readily detectable opacity; +2, dense opacity or opacity partially covering the corneal surface over pupil; +3, dense opacity covering entire corneal surface over pupil; and +4, moderate to dense opacity covering entire corneal surface with corneal erosion. Mean SLE scores were calculated by summation of the scores for each group of mice divided by the total number of mice graded at each time point. Corneal erosions were detected with fluorescein (Fluor-I-Strip AT; Wyeth-Ayerst Laboratories, Inc., Philadelphia, PA).

**Histopathology**

Eyes from control and infected mice (n = 2 mice per group) were enucleated at 1, 3, 5, 7, and 9 days PI. Whole eyes of noninfected and infected mice were fixed immediately after enucleation in 10% neutral buffered formalin (EK Industries, Joliet, IL). After fixation, eyes were bisected and processed as previously described. Briefly, fixed tissue was dehydrated in a series of ethanol baths of increasing concentration. Once dehydrated, sections were held in xylene. The dehydrated tissue was then embedded in paraffin and cut into sections of 5 μm. Sections were then rehydrated and stained with hematoxylin and eosin.

**Toxin Production in a Model of *S. aureus* Keratitis**

**MPO Activity Assay**

To assess the activity of polymorphonuclear leukocytes (PMNs) in the mouse eye, the amount of MPO was quantified in eye homogenates of noninfected and infected mice, as previously described. Briefly, hexadecyltrimethylammonium bromide (CTAB; Sigma-Aldrich, St. Louis, MO) was added to each sample at a final concentration of 0.5% and an MPO microtiter assay, based on an α-diamisidine-induced colorimetric reaction, was used. Reactions were incubated at room temperature, and the change in optical density at 450 nm was determined every 2 minutes for 12 minutes. The units of MPO were calculated as previously described for the microtiter plate assay. One unit of MPO activity has been reported to be equivalent to approximately 2 × 10^5 PMNs. All assays were performed in triplicate.

**Hemolysin Assay**

Hemolytic titration was performed to determine the potency of the α-toxin administered to rabbit eyes. The hemolytic activities of α-toxin preparations were assayed by their ability to lyse rabbit erythrocytes, as previously described. Briefly, each suspension of rabbit erythrocytes was adjusted to 10^8 erythrocytes/mL by adding toxin diluent (10 mM PBS with 0.2% gelatin, pH 7.4). α-Toxin preparations were serially diluted twofold in PBS with gelatin. Erythrocytes (equal volume) were added to each dilution, incubated for 30 minutes at 37°C, and centrifuged (1000g for 5 minutes) to pellet the erythrocytes. An aliquot was then placed in a microtiter plate, and the optical density was measured at 570 nm with a spectrophotometer. The hemolytic titer was established as the lowest dilution with 50% lysis of erythrocytes. Complete (100%) lysis was obtained for each erythrocyte suspension by adding 5 μL Triton X-100 (Sigma-Aldrich) to a mixture of equal volumes of erythrocytes and water (200 μL).

**Purification of α-Toxin**

α-Toxin was obtained from Sigma-Aldrich and purified to homogeneity by isoelectric focusing (Rotofor; Bio-Rad, Hercules, CA) as previously described. Samples of α-toxin (2 mg/mL) were isoelectrically focused (using 3/10 ampholytes), and fractions were tested for pH and protein activity. Fractions were tested for hemolysis of rabbit erythrocytes, and active fractions were refocused. Fractions with peak hemolytic activity were recovered and dialyzed against PBS. Fractions were analyzed by SDS-PAGE and silver stained to ensure homogeneity of the protein.

Previous studies have shown by slit lamp examination that intrastromal injection of purified α-toxin into the rabbit cornea is toxic in concentrations ranging from 0.002 to 10 μg. For the present studies, purified α-toxin (0.005 μg) was used. Toxic (5 μL) was topically applied to the scarified eyes of young and aged mice (n = 4 mice/group). Each cornea was scarified with a 30.5-gauge needle by making five parallel incisions into the corneal surface that did not penetrate beyond the superficial stroma. Control studies included eyes of mice that were untreated as well as scarified eyes to which heat-inactivated α-toxin in PBS was applied. α-Toxin was heated at 60°C for 2 hours, causing inactivation of the toxin, as determined by its inability to lyse rabbit erythrocytes. Eyes were monitored up to 24 hours by SLE, and then the mice were killed. All eyes underwent histopathologic analysis as described earlier.

**Statistical Analysis**

The mean ± SEM of the SLE scores, CFU/eye, and units of MPO were determined on computer (SAS, Cary, NC). Statistical analyses were performed using a one-way nested analysis of variance on each group. Protected *t*-tests were then determined between least-square means derived from each variance analysis on each group. *P* ≤ 0.05 was considered significant.
RESULTS

Ocular Disease in Infected Eyes

Young Mice. Infection of young mice with either mutant S. aureus strain (DU1090 or ISP546) demonstrated significantly lower SLE scores compared with mice infected with the parent strain. The difference between either of the mutant strains and the parent strain appeared as early as 5 days PI and was apparent up to 9 days PI (Fig. 1A, $P \leq 0.0001$). The α-toxin-deficient strain (DU1090) produced significantly higher SLE scores than in mice infected with the Agr-deficient mutant (ISP546). This difference was observed from 7 to 9 days PI in young mice (Fig. 1A). The specific pathologic changes observed in young mice infected with the parent strain on day 1 PI included moderate corneal opacities (Figs. 2A, 2B). Young mice infected with either mutant strain (DU1090 or ISP546) demonstrated only mild to moderate corneal opacities without corneal erosions in eyes of mice infected with either mutant S. aureus strain by 9 days PI (Fig. 1B, $P \leq 0.0001$).

Aged Mice. Infection of aged mice also demonstrated a significant reduction in corneal disease in the mutant strains compared with the toxin-producing parent strain. Aged mice challenged with the parent strain (8325-4) demonstrated such severe keratitis that by day 3 they had to be killed. However, infection with either mutant strain (DU1090 or ISP546) demonstrated significantly lower SLE scores at 1 and 3 days PI than in mice infected with the parent strain (Fig. 1B, $P \leq 0.0001$). The infection of aged mice by the mutant strains, unlike that of the parent bacterial strain, could be allowed to continue through day 9 PI. The infections in aged mice caused by the α-toxin-deficient strain (DU1090) were more severe than that of the Agr-deficient strain (ISP546), as evidenced by the significantly higher SLE scores from 3 to 9 days PI (Fig. 1B, $P \leq 0.04$).

The specific pathologic changes observed in aged mice infected with either mutant strain (DU1090 or ISP546) demonstrated only mild to moderate corneal opacity at 3 days PI (Figs. 3A, 3B). Aged mice infected for 3 days with the parent strain included dense infiltrate covering the entire cornea and complete corneal erosions in most eyes (Fig. 3D). However, aged mice infected with either mutant strain (DU1090 or ISP546) demonstrated only mild to moderate corneal opacity at 3 days PI (Figs. 3E, 3F).

Histopathologic Analysis of Infected Eyes

Young Mice. Histopathologic analysis of infections of young mice with either mutant strain (DU1090 or ISP546) confirmed the SLE findings by demonstrating that the mutant strains caused less severe infections compared with that of the parent strain. Whereas young mice infected with the parent strain (8325-4) demonstrated moderate edema and PMN accumulation in the central cornea at 9 days PI (Fig. 4A), mice infected with either mutant strain demonstrated only minimal pathologic changes (Figs. 4B, 4C).

Aged Mice. Histopathologic differences between the infection of mice with either the parent or mutant strains were...
greater in aged mice than in young mice. Aged mice infected with the parent strain (8325-4) at 3 days PI demonstrated detachment of the corneal epithelium from the basement membrane, PMN infiltration, and severe edema (Fig. 4D). In contrast, infection with the α-toxin-deficient strain (DU1090) at 3 days PI demonstrated corneal edema, but no erosion of the corneal epithelium or PMN accumulation (Fig. 4E). Infection with the Agr-deficient strain (ISP546) at 3 days PI failed to produce many of the changes caused by the parent strain, including inflammatory infiltrate, edema, and vascular congestion (Fig. 4F).

**CFU in the Infected Eyes**

To characterize further the course of infection with parent or toxin-deficient mutant strains, the number of viable *S. aureus* (CFU) recovered from infected mouse eyes was quantified. Despite the use of an equivalent number of CFU in the inocula, infection with either mutant strain (DU1090 or ISP546) produced significantly lower CFU/eye than in mice infected with the parent strain (8325-4). This difference was observed from 1 to 5 days PI in the young mice (Fig. 5A, *P* ≤ 0.0372) and from 1 to 3 days PI in the aged mice (Fig. 5B, *P* ≤ 0.0018). Bacterial loads in eyes infected with the α-toxin-deficient mutant (DU1090) remained equivalent to that produced by the Agr-deficient strain (ISP546) from 3 to 9 days PI in the young mice and from 5 to 9 days PI in the aged mice. **MPO Activity in Infected Eyes**

PMN infiltration into normal and infected mouse eyes was determined by MPO assays at various times PI. Noninfected control eyes had minimal MPO activity (Fig. 6), and scarification did not significantly affect the MPO activity of noninfected eyes. Maximum MPO activity was observed at 1 day PI in both young (Fig. 6A) and aged (Fig. 6B) mice infected with any of the three strains of *S. aureus*. However, the parent strain infection produced greater MPO activity than infection with either mutant strain on day 3 PI in aged mice (Fig. 6B, *P* ≤ 0.0138). This difference between parent and mutant strains was not observed in young mice or at subsequent times in either young or aged mice.

**Pathologic Changes in α-Toxin-Treated Eyes**

**Young Mice.** As early as 2 hours after topical application of α-toxin to scarified eyes, mild iritis and the first indications of epithelial erosion became apparent (Fig. 7A). By 10 hours after toxin application, moderate epithelial sloughing as well as slight corneal infiltrate was observed in the eyes of young mice (data not shown). By 24 hours after toxin application, the epithelial erosions had healed, and moderate iritis and edema were observed as well as an accumulation of infiltrate in the eyes of young mice (Fig. 7C). Histopathologic analysis of the eyes of young mice at 24 hours demonstrated PMN infiltrate in the corneal stroma, concentrated near the corneal epithelium (Fig. 8A). Young mice showed no pathologic effects after topical application of heat-inactivated α-toxin.

**Aged Mice.** The effects of α-toxin were more severe in the eyes of aged mice than in those of young mice. At 2 hours after toxin application to the eyes of aged mice, mild iritis and moderate corneal erosions were observed (Fig. 7B). By 10 hours, moderate iritis and corneal infiltrate, as well as severe corneal erosions were observed (data not shown). By 24 hours, corneal disease in aged mice was so severe that perforation was imminent. All eyes demonstrated iritis, severe corneal edema, and complete corneal erosion (Fig. 7D). Histopathologic analysis of the eyes of young mice at 24 hours demonstrated PMN infiltrate in the corneal stroma, concentrated near the corneal epithelium (Fig. 8A). Young mice showed no pathologic effects after topical application of heat-inactivated α-toxin.
logic analysis confirmed the increased severity of disease due to α-toxin in eyes of aged mice. In addition to corneal erosion, PMNs in aged mice were found to span the full thickness of the cornea, with accumulations noted deep to Bowman’s membrane and between the endothelium and Descemet’s membrane (Fig. 8B). Topical application of heat-inactivated α-toxin produced no pathologic effects in the eyes of aged mice.

**DISCUSSION**

The findings of this study indicate for the first time that aging in mice is associated with an increased susceptibility to the action of α-toxin, either when the toxin is produced by *S. aureus* in the eye or applied in a pure form topically to the eye. The extent of pathogenesis of keratitis in aged mice infected with either toxin-deficient mutant strain of *S. aureus* was greatly reduced compared with infection with the parent strain that produces the full spectrum of toxins. α-Toxin, in concentrations of only 5 ng, applied topically to the scarified eyes of aged mice caused more severe pathologic changes than those observed in toxin-treated eyes of young mice. Aged mice treated with α-toxin demonstrated PMN infiltration throughout the entire thickness of the cornea and extensive PMN accumulation below Descemet’s membrane, changes that did not occur in eyes of young mice treated similarly with α-toxin.

In this study, eyes of young mice infected with either mutant strain of *S. aureus* demonstrated much less severe disease than eyes infected with the parent strain. These results were even more dramatic in eyes of aged mice, suggesting that toxin susceptibility may increase with age. In addition, the eyes of aged mice were far more susceptible to the damaging effects of topically administered α-toxin than were those of young mice, as evidenced by SLE and histopathologic analysis. These findings are consistent with the studies of *Staphylococcus* toxins in mice in nonocular sites that suggest that aged mice may be more susceptible to staphylococcal toxins.32

The mutant *S. aureus* strains analyzed in this study were engineered to differ from the parent strain in only a single genetic locus.23–25 Based on this fact, the finding of a reduced population of bacteria in corneas infected with toxin-deficient mutants, compared with the parent strain, suggests that toxins facilitate *Staphylococcus* infection. The fact that *S. aureus* strain DU1090 is only deficient in α-toxin suggests that α-toxin...
promotes the infection of the cornea as well as causes extensive pathologic changes. α-Toxin can provide enhanced bacterial translocation through the epithelium, an increase in nutrients at the site of infection due to degradation of cornea components, or protection of bacteria in the inflamed cornea. The higher SLE scores for α-toxin-deficient bacterial infections compared with infections with the Agr-deficient strain suggest that a toxin other than α-toxin also contributes to corneal damage in the mouse. Dajcs et al. found that γ-toxin is a corneal virulence factor in the rabbit model of keratitis, suggesting that production of γ-toxin by the α-toxin-deficient mutant could explain its greater virulence relative to the Agr-deficient mutant. This role of γ-toxin should be further studied in this model.

Both histopathologic analyses and MPO assays demonstrated that the PMN infiltrate into eyes infected with the parent strain was greater than that of infections with either mutant strain. The reduced infiltrate observed in infection with the α-toxin-deficient strain demonstrate that this toxin is responsible for a chemotactic response in the cornea. This point was substantiated by the histopathologic analysis of eyes treated with purified α-toxin.

Overall, there are very few studies that have analyzed the prevalence and course of microbial keratitis in the elderly. However, microbial keratitis appears to be significantly more prevalent in the elderly population than in the general population, perhaps due to decreased host resistance associated with aging. Aged patients are at high risk for keratitis caused by Staphylococcus aureus or other ocular pathogens, including P. aeruginosa. In the study conducted by Ormerod, the symptoms of keratitis were more severe in aged than in young patients. The rates of corneal ulcer formation appeared to be higher in the elderly population than in younger patients. Also, higher rates of descemetocele and corneal perforation were noted in patients >65 years of age. Several studies have indicated that anatomic or physiological changes may mediate this apparent increased predisposition to infection in the elderly including a decline in tear production, impairment of healing processes, such as epithelial cell regeneration, and a decline in a wide range of immunologic responses. However, the role of bacterial virulence factors in keratitis in elderly patients has not been analyzed. The results of the present study indicate that aged mouse eyes are more susceptible to Staphylococcus α-toxin, suggesting that a similar increased susceptibility to toxin could explain the observed increased severity of keratitis in the aged human eye. Not only is the toxin responsible for more severe pathologic changes, but it appears to aid in the ability of the bacteria to invade or survive in the cornea.

The findings of this study illustrate for the first time that the eyes of aged mice are more susceptible to staphylococcal α-toxin than young mice. These results suggest that the elderly population may be at a greater risk for more severe keratitis caused by Staphylococcus than are younger individuals and that inhibition of the action of α-toxin in the cornea is essential to limiting the damage associated with S. aureus keratitis, particularly in the aged. The availability of the murine model for the identification of inhibitors of α-toxin could be very beneficial and could provide further insight into the use of immunotherapy targeted to α-toxin for Staphylococcus keratitis.

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