Age-Related Differences in Rabbits during Experimental Staphylococcus aureus Keratitis

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PURPOSE. To analyze age-related changes in susceptibility to experimental Staphylococcus aureus keratitis and purified α-toxin in rabbits.

METHODS. Intrastromal injection of S. aureus (100 colony-forming units [CFUs]) induced keratitis in young (6–8 weeks) and aged (approximately 30 months) New Zealand White rabbits. Bacteria and polymorphonuclear leukocytes (PMNs) per cornea were quantified. Purified α-toxin at 1, 10, 25, or 50 hemolytic units (HU) or heat-inactivated α-toxin was intrastromally injected into corneas, and pathologic changes were determined by slit lamp examination (SLE) and histopathologic analysis. α-Toxin hemolysis assays were performed using erythrocytes from young and aged rabbits.

RESULTS. S. aureus keratitis produced significantly higher SLE scores in young rabbits than in aged rabbits at 15, 20, and 25 hours postinfection (PI; P ≤ 0.001); aged rabbits essentially recovered from S. aureus keratitis by 7 days PI. At 25 hours PI, numbers of CFUs and PMNs in corneas of young and aged rabbits were equivalent (P ≥ 0.6); the bacterial burden in aged rabbits declined by 5 logs per cornea from day 1 to day 7 PI. Intrastromal injection of ≥10 HU α-toxin also produced significantly more disease in young than in aged rabbit corneas (P ≤ 0.05), whereas 1 HU or heat-inactivated toxin yielded negligible pathologic changes in either group. Hemolysis assays of erythrocytes from young rabbits demonstrated greater susceptibility to α-toxin compared with those from aged rabbits.

CONCLUSIONS. Corneas and erythrocytes of young rabbits, relative to aged rabbits, are significantly more susceptible to S. aureus keratitis and to α-toxin. (Invest Ophthalmol Vis Sci. 2007;48:5125–5131) DOI:10.1167/iovs.07-0320

Staphylococcus aureus is a well-documented cause of community-acquired and nosocomial infections. Common manifestations include skin and soft tissue infections, endocarditis, pneumonia, and keratitis.1–5 Infections by Staphylococcus, an opportunistic pathogen, are often preceded by penetrating trauma, surgery, or a decline in immune function because of disease or aging.4,11 Systemic and ocular infections with S. aureus, to a much greater degree than other staphylococcal species, are associated with poor prognoses and outcomes.12–15 α-Toxin, a 34-kDa oligomeric hemolysin, is present in readily detectable amounts in approximately 75% of S. aureus strains and has been described as a primary virulence factor responsible for the heightened pathogenicity of this species.16–21 Corneal pathogenesis in rabbit and murine models of keratitis is mediated by α-toxin.22–24 Elderly persons, especially those residing in long-term care facilities—who also demonstrate a higher rate of methicillin-resistant S. aureus—are colonized by S. aureus at higher rates than younger persons.9,25–35 Increased susceptibility to staphylococcal infections has also been reported in systemic infections among the elderly.13,31,33,36 Numerous studies have attributed most ocular infections in this population to bacterial strains with which the patient was colonized on admission.37,38 The elderly are also more likely to require hospitalization for the eradication of staphylococcal infections, and they tend to present with disproportionately severe infections.34,39–41 Younger patients often have little, if any, loss in visual acuity on recovery, whereas elderly patients frequently have visual acuity at or below light perception after S. aureus ocular infections.36,37

Girgis et al.17,24 demonstrated, in the murine model of S. aureus keratitis and after corneal administration of purified α-toxin, that a direct relationship exists between increased age and extent of infection. To date, no studies have been published identifying the mechanisms responsible for increased α-toxin susceptibility with aging.

The findings in mice of an age-related change in susceptibility to staphylococcal infection and to α-toxin toxicity prompted investigations to determine whether this relationship also exists in New Zealand White rabbits. Studies examining the effects of staphylococcal keratitis and α-toxin-mediated corneal toxicity in rabbits have not been previously described. The findings illustrate a correlation between aging and decreased ocular susceptibility to staphylococcal keratitis and to decreased α-toxin-mediated ocular disease. The emerging concept is that a change in susceptibility with age is not species specific. Furthermore, altered susceptibility could fail to correlate with the innate immune status of the host and could relate to the direct action of the toxin.

MATERIALS AND METHODS

Animals

Groups of young (6–8 weeks) and aged (≥30 months) New Zealand White rabbits were obtained from Myrtle’s Rabbitry (specific pathogen-free [SPF] rabbits; Thompson’s Station, TN) or Black Creek Rabbitry (Moss Point, MS). All animals were maintained according to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Before any procedure was undertaken, each rabbit was anesthetized by subcutaneous injection of a 1:5 mixture of xylazine (100 mg/ml; Rompun; Miles Laboratories, Shawnee, KS) and ketamine hydrochloride (100 mg/ml; Ketaset; Fort Dodge Animal Health, Fort Dodge, IA). Proparacaine hydrochloride (0.5% Alcaine; Alcon Labora-
Bacterial Strains

*Staphylococcus aureus* strain 8525–4 has been previously described in rabbit and murine models of keratitis; it is known to produce α-toxin as well as β-, γ-, and δ-toxins. 17,18,22,40–45 *S. aureus* strain 60171 is a clinical isolate (generously donated by David Stroman, Alcon Laboratories) obtained from a corneal ulcer that has also been previously described in the rabbit intrastromal keratitis model.44 Strain 60171 has been identified as methicillin sensitive and fluoroquinolone resistant.

Bacteria were grown overnight in tryptic soy broth (Bacto; Becton-Dickinson, Sparks, MD) at 37°C and then were subcultured to 10^6 CFU/mL, with an optical density of 0.34 at A_650.

Experimental Keratitis

Corneas of New Zealand White rabbits (n = 4 eyes/group) were infected, as previously described, with approximately 100 CFUs of strain 8525–4 or strain 60171 administered as 10-μL intrastromal injection through a 50-gauge needle on a 100-μL gas-tight syringe (Hamilton Company, Reno, NV).19,23,44 Accuracy of the inoculum was verified by plating aliquots (100 μL) of serial dilutions in triplicate on tryptic soy agar (TSA; Difco, Becton-Dickinson).

Slt Lamp Examination

Slt lamp examination (SLE) of pathologic changes in rabbit eyes was performed by two masked observers using a biomicroscope (Topcon SL-7E; Koaku Kikai K.K., Tokyo, Japan). Each of seven parameters— injection, chemosis, iritis, hypopyon, corneal infiltrate, fibrin in the anterior chamber, corneal edema—was graded on a scale ranging from 0 (none) to 4 (severe). The sum of these grades for an eye, after averaging, determined the SLE score, which could range from 0 (normal eye) to a theoretical maximum of 28. Epithelial erosions were observed during SLE after staining with fluorescein (Fluorets; Chauvin Pharmaceuticals, Aubenas, France).

Bacterial Quantification

Infected rabbit corneas were harvested 25 hours or 7 days postinfection (PI; 60171) and were homogenized in 3 mL sterile phosphate-buffered saline (PBS; 0.1 M, pH 7.2). The homogenates and subsequent serial dilutions (1:10) were plated in triplicate on TSA. After incubation at 37°C for 24 hours, CFUs per cornea were determined and expressed as log_{10} ± SEM.

Myeloperoxidase Activity Assay

Infected corneas (*S. aureus*, 60171) of young or aged rabbits (n = 4 eyes/group) were analyzed for the number of infiltrating polymorphonuclear leukocytes (PMNs) by the myeloperoxidase (MPO) assay at 5, 20, and 25 hours PI. Aged rabbit corneas (n = 4) infected with *S. aureus* 60171 were also analyzed at 7 days PI. MPO assays were performed using a colorimetric o-dianisidine reaction, as previously described.45–47 One MPO unit of activity is equivalent to approximately 2 × 10^4 PMNs.47 Each assay was performed in triplicate, and experiments were performed twice.

Purification of α-Toxin

Commercially acquired α-toxin (Sigma-Aldrich) was further purified and concentrated with centrifugal filter devices with a 30-50KDa cutoff (Amicon Bioseparations Centricron Plus-20; Millipore, Bedford, MA). Samples were analyzed using silver-stained SDS-PAGE to confirm protein purity.

Preparation of Erythrocytes

Heparinized whole blood obtained from young or aged rabbits was centrifuged at 2000g for 10 minutes, and plasma was removed; erythrocytes were then washed twice in diluent (0.01 M PBS with 0.2% gelatin, pH 7.4). Erythrocyte pellets were subsequently resuspended in 6 vol diluent. Viable red blood cells were quantified using a hemocytometer (Bright Line; Hauser Scientific, Horsham, PA) under 400× magnification with a bright-field microscope (Nikon, Tokyo, Japan).

Hemolytic Assays

Purified α-toxin was assayed for hemolytic activity in microtiter plates by adding 10^7 erythrocytes of young rabbits to twofold serial dilutions of toxin (first well, 125 μg/mL in 0.01 M PBS with 0.2% gelatin (pH 7.4; total volume, 200 μL per well). Plates were incu- bated at 37°C for 60 minutes, followed by centrifugation at 2000g for 10 minutes at 4°C. Hemolytic titers were determined as the greatest toxin dilution at which 50% lysis of the erythrocyte suspension was observed to occur, equivalent to one hemolytic unit (HU). Erythrocyte reactions with toxin were compared with positive controls exhibiting 100% lysis, achieved by treating erythrocytes with either 1% Triton X-100 (Sigma-Aldrich) or 20 HU purified α-toxin, and they were compared with negative controls containing erythrocytes in buffer (0.01 M PBS with 0.2% gelatin, pH 7.4). In determinations of age-related differences, assays were performed in an identical manner using erythrocytes of young or aged rabbits as appropriate. Each assay was performed at least twice in triplicate.

Determination of α-Toxin Susceptibility In Vivo

Purified α-toxin containing 1, 10, 25, or 50 HU (0.015, 0.15, 0.37, or 0.74 μg in 20 μL PBS) was intrastromally injected into corneas of young and aged New Zealand White rabbits (n = 4 eyes/group) using a 50-gauge needle on a 100 μL gas-tight syringe (Hamilton Company). Also tested was toxin (0.37 μg) that had been heat inactivated at 100°C until hemolytic activity was eliminated. The activity of the toxin in the inoculum was verified by hemolysis assay.

Histopathology

Corneas injected with 25 HU α-toxin or heat-inactivated α-toxin were harvested 5 hours and 24 hours after injection (n = 4 corneas/group). Each cornea was immediately fixed in 10% neutral-buffered formalin (EK Industries, Joliet, IL) after harvest. After fixation, samples were processed as previously described.19,45,44 Briefly, fixed tissue was dehydrated in a series of ethanol baths of increasing concentration and then held in xylene. The dehydrated tissue was embedded in paraffin, cut into 5-μm sections, rehydrated, and stained with hematoxylin and eosin.

Statistical Analysis

Means of SLE scores and CFU/cornea were determined, and the SEM was calculated using statistical analysis software (SAS, Cary, NC). For SLE results, statistical analyses of intergroup differences were performed using nonparametric one-way analysis of variance. For CFU determinations and MPO comparisons, analysis of variance and Student’s t-tests between least squares means from each group were performed. P ≤ 0.05 was considered significant.

RESULTS

Bacterial Growth in the Cornea

Growth of *S. aureus* strains 8325–4 and 60171 reached approximately 7 logs CFU per cornea in young and aged groups (n = 6 eyes/group) at 25 hours PI (P ≥ 0.6). Aged animals whose corneas were harvested at 7 days PI had greatly reduced numbers of viable *S. aureus* 60171 (1.76 ± 0.425 logs).

Effects of Experimental Keratitis

Young rabbits at 15 and 25 hours PI with *S. aureus* 8325–4 or 60171 experienced substantially more pathologic changes to...
the eye than aged rabbits (Figs. 1, 2). Marked increases in corneal pathologic effects were observed in young rabbits at 15 and 25 hours PI and included chemosis, iritis, corneal epithelial erosion, and opacity caused by fibrin in the anterior chamber and PMN infiltration into the cornea (Figs. 1, 2). Conversely, aged rabbits showed fewer signs of disease than did young rabbits for the duration of the experiment. Moderate corneal ulceration, conjunctival edema, and minor accumulation of fibrin in the anterior chamber were the primary changes noted in the aged group (Figs. 1, 2). Resultant SLE scores derived from these observations indicated that the corneas of young rabbits exhibited significantly greater pathologic effects during S. aureus keratitis than the corneas of aged rabbits infected with the same strain of bacteria ($P \leq 0.01$; Figs. 1, 2).

To determine whether the pathologic effects of S. aureus keratitis in aged rabbits were merely delayed or were truly reduced overall compared with young rabbits, the infection in aged rabbits was allowed to continue to 7 days PI. SLE scores of aged rabbits increased from 25 to 35 hours PI (data not shown) but were not as severe as those in young rabbits at 25 hours PI. SLE scores of the aged rabbits steadily declined through 7 days PI. Aged eyes at 7 days PI showed moderate corneal edema and moderate amounts of infiltrate, but no epithelial defect, chemosis, injection, iritis, or fibrin in the anterior chamber was detectable (Fig. 1E).

**Evaluation of PMN Activity**

MPO assays were performed to quantify the PMN infiltration of the infected young and aged corneas at 5, 20, and 25 hours PI

**FIGURE 1.** Photomicrographs of young and aged rabbits infected with S. aureus. Young rabbits (A, C) demonstrated essentially complete corneal opacity with significant epithelial ulceration, whereas aged rabbits (B, D) showed limited pathologic effects at 25 hours PI ($n = 6$ eyes/group). Aged rabbits ($n = 4$ eyes) at 7 days PI demonstrated extensive recovery from infection (E). Strain 8525-4 (A, B). Strain 60171 (C–E).

**FIGURE 2.** SLE of experimental S. aureus keratitis in young and aged rabbits. Each cornea was intrastromally injected with S. aureus strain 8525-4 (A) or 60171 (B) 100 CFU ($n = 6$ eyes/group). Pathologic changes were graded by SLE with scores expressed as the mean ± SEM. A significant difference ($P \leq 0.006$) existed between young and aged rabbits at all time points. Young rabbits ■, aged rabbits □.

**FIGURE 3.** Effects of intrastromal injection of α-toxin. Young rabbits were more susceptible than aged rabbits to disease resulting from intrastromal injection of purified α-toxin ($n \geq 4$ eyes/group; Fig. 4). Within the first hour of intrastromal injection of 25 HU α-toxin, young rabbits exhibited overt loss of corneal epithelium, accompanying blanching of the iris, and severe conjunctival erythema. Aged rabbits at 1 hour after toxin injection showed only minimal corneal epithelial erosion and mild to moderate corneal edema. By 5 hours after toxin injection, young rabbits demonstrated almost complete loss of the corneal epithelium, severe conjunctival edema, and severe iritis with substantial influx of blood into the anterior chamber; examinations of aged rabbits at 5 hours revealed only moderate corneal ulceration and blanching of the iris (Fig. 4). SLE scores of aged rabbits 5 hours after toxin injection (25 HU) were significantly lower than those of young rabbits (Fig. 5; $P \leq 0.0026$). Injection of toxin in lower (10 HU) or higher (50 HU)
amounts showed pathologic effects similar to those seen with 25 HU toxin, and significant differences were observed between young and aged rabbits 5 hours after injection of these two toxin doses (Fig. 5; \( P \leq 0.0012 \)).

Intrastromal injection of heat-inactivated \( \alpha \)-toxin (0.37 \( \mu \)g) or 1 HU native \( \alpha \)-toxin into the corneas of young and aged rabbits \((n = 4 \text{ eyes/group})\) produced no apparent corneal changes; SLE scores of these eyes were <0.5 and were not significantly different between young and aged rabbits at any time point \((P = 0.79)\). Eyes injected with heat-inactivated toxin did demonstrate minimal iritis and injection in the first hour PI, possibly attributable to the injection itself, with no detectable abnormality 5 hours PI in either group (Fig. 4). Eyes injected with 1 HU toxin were similar to eyes injected with heat-inactivated toxin (data not shown).

Histopathologic analysis of corneas injected with \( \alpha \)-toxin (25 HU) was performed in young and aged rabbits \((n = 4 \text{ corneas/group per time point})\). Corneas injected with 0.37 \( \mu \)g heat-inactivated toxin showed no recognizable changes in either age group. However, injection of an equivalent amount (25 HU) of native \( \alpha \)-toxin resulted in severe pathologic effects in young rabbits that were not observed in aged rabbits (Fig. 6). Five hours after injection, the corneas of young rabbits exhibited mild corneal edema and trace amounts of PMN infiltration with a significant loss of intercellular attachment and extensive erosion of corneal epithelium. In contrast, the corneas of aged rabbits 5 hours postinjection with 25 HU native toxin showed mild corneal edema, with no additional abnormality of significance (Fig. 6). Twenty-four hours after injection, the corneas of young rabbits were essentially denuded of corneal epithelium and had moderate to severe PMN infiltration, whereas the aged rabbits at 24 hours after injection still displayed only trace to moderate amounts of corneal edema and mild to moderate infiltration of PMNs into the corneal stroma, with only minor erosion of corneal epithelium (Fig. 6).

**Assays of \( \alpha \)-Toxin–Mediated Hemolysis**

Erythrocytes obtained from young and aged rabbits displayed differences in susceptibility to \( \alpha \)-toxin that correlated with the differences in susceptibility observed in corneal infection and toxin after intrastromal injection. Twofold serial dilutions of

\( \alpha \)-toxin produced lysis of erythrocytes from young rabbits at a fourfold lower concentration than that required to lyse erythrocytes of aged animals \((P \leq 0.01; \text{Fig. 7})\).

**DISCUSSION**

This study in rabbit eyes demonstrated an age-related reduction in susceptibility to \textit{S. aureus} keratitis and to the administration of purified \( \alpha \)-toxin. \( \alpha \)-Toxin has been found, in several studies, to be responsible for most of the bacterial virulence in the rabbit keratitis model, causing corneal epithelial cell sloughing, corneal edema, corneal infiltration, and severe iritis.\textsuperscript{10,20,22,25,43} It is estimated that \( \alpha \)-toxin accounts for between 50\% and 70\% of the ocular damage in this model of keratitis.\textsuperscript{22,24} A reduction in the susceptibility to \( \alpha \)-toxin could explain the less severe outcome of the corneal infection in
The interaction between α-toxin and the cell surface has been analyzed in some detail, but it is not yet fully understood. α-Toxin is known to be a monomer of 34 kDa that can bind to high-affinity receptors on rabbit cells or to a wide spectrum of low-affinity receptors on the same cell surface. To form toxin heptamers, the monomers must react with a membrane protein, caveolin 1, that is found in lipid rafts. The interaction between the toxin and caveolin 1 allows the heptamer to undergo conformational changes that facilitate toxin penetration of the membrane, forming a lethal pore in the cell. The chemistry of the lipid raft (e.g., cholesterol content) is known to change with age, and such changes could alter the susceptibility of the host cell to toxin action.

Injection of 10, 25, or 50 HU α-toxin into rabbit corneas demonstrated significantly greater pathologic effects in young than in aged rabbits. Each of these three quantities of toxin produced similar pathologic effects. The lack of a dose-response effect for the toxin between 10 and 50 HU implies that these doses were saturating in terms of their ability to induce maximal pathologic effects. In contrast to active toxin injected in doses at or above 10 HU, injection into the cornea of heat-inactivated toxin in substantial quantities or active toxin at only 1 HU caused essentially no pathologic effects.

The effect of age on the susceptibility to S. aureus keratitis and to toxin administration is not specific to one bacterial strain or to one animal species. The effect of age on the symptoms of keratitis was found herein to be associated with a well-characterized laboratory strain and a clinical corneal isolate. Furthermore, a second laboratory strain (strain Newman) produced a similar effect (data not shown). With regard to animal species, studies of S. aureus keratitis in mice have shown an age-related effect on the symptoms of keratitis. Girgis et al. demonstrated that the advanced age of mice caused an increase in susceptibility, an effect opposite that of aging in rabbits. The increased susceptibility of the mouse with age suggested a possible linkage of toxin susceptibility to a declining immune response, a decline noted to be important in Pseudomonas keratitis. However, the rabbit is expected to undergo a decline in the immune system similar to that found in the mouse. In addition, animals used in this series of experiments included aged rabbits lacking protective antibody (data not shown). Thus, the reduced α-toxin susceptibility in the aged rabbits suggested a possible decline in the immune response.
aged rabbit is opposite that of the aged mouse, indicating that toxin interactions with cell surfaces could be a more important mechanism of pathogenesis than a decline in the innate immune system. The findings with \textit{S. aureus} in the rabbit are also unlike those for \textit{Pseudomonas} in the mouse. Hazlett et al.\textsuperscript{34,55} have shown that the corneas of mature mice (1 year old) fail to recover from \textit{Pseudomonas} keratitis, whereas the corneas of young mice (6–8 weeks old) almost completely recover.

The ability to essentially recover from \textit{S. aureus} keratitis was observed in aged, but not in young, rabbits. This difference occurred with equivalent numbers of PMNs present in the corneas of both groups of animals. The effects of purified native toxin on the eyes of young and aged rabbits suggest that a difference in the direct action of the toxin in young versus aged rabbits determines the outcome of the infection. This concept is supported by the difference in toxin susceptibility in the erythrocytes of young and aged rabbits.

The present study describes differences between young and aged rabbits in their susceptibility to \textit{S. aureus} keratitis, to corneal administration of \(\alpha\)-toxin, and to erythrocyte lysis by \(\alpha\)-toxin in vitro. These results are consistent with the concept that the toxin causes direct pathologic cellular effects, the extent of which could determine both the amount of direct tissue damage and the amount of immunopathology induced by \textit{S. aureus} keratitis.

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References


