Corneal Pathogenesis of *Staphylococcus aureus* Strain Newman

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Purpose. To determine the pathogenic role of γ- and α-toxin in a rabbit model of *Staphylococcus aureus* keratitis.

Methods. *S. aureus* strains Newman (expressing γ-toxin), Newman Δ*hlg* (deficient in γ-toxin), Newman Δ*hlg*/pCU1 *hlg*$^+$ (chromosomal γ-toxin–deficient mutant rescued by a plasmid encoding γ-toxin), and Newman Δ*hla* (α-toxin–deficient) were intrastromally injected into rabbit corneas. Eyes were scored by slit lamp examination (SLE), and bacterial colony-forming units (CFU) per cornea were determined at 15, 20, and 25 hours after infection. Histologic examination of corneas was performed. Rabbits were immunized against α-toxin and subsequently challenged with *S. aureus* strain Newman. Western blot analyses of culture supernatants were performed to detect α-toxin production.

Results. All strains grew equivalently, producing approximately 7 log CFU per cornea at 25 hours after infection. SLE scores at 20 and 25 hours after infection revealed that strains Newman Δ*hlg* and Newman Δ*hla*, although virulent, caused significantly less ocular damage and inflammation than their parent or the γ-toxin genetically rescued strain ($P \leq 0.0006$). Histologic and SLEs revealed that all strains except Newman Δ*hla* produced corneal erosions. Rabbits immunized actively or passively to α-toxin had reduced SLE scores ($P \leq 0.0003$ and $P \leq 0.0033$, respectively) and no epithelial erosions when infected with strain Newman. Western blot analysis demonstrated that strains Newman and Newman Δ*hlg*, but not Newman Δ*hla*, produced α-toxin.

Conclusions. These results illustrate that the virulence of strain Newman involves both α- and γ-toxin, with α-toxin mediating corneal epithelial erosions. An additional uncharacterized toxin could also be active in damaging the cornea. (*Invest Ophthalmol Vis Sci.* 2002;43:1109–1115)

The pathogen *Staphylococcus aureus* is a leading cause of bacterial keratitis in the United States.[1,2] Tissue damage during *Staphylococcus* keratitis results from the action of bacterial products on ocular tissues[3,4] and from the host inflammatory response to infection.[5] *Staphylococcus* keratitis can cause irreversible corneal scarring, resulting in loss of visual acuity or blindness.[2]

S. aureus can produce a variety of toxins, including the hemolytic exoproteins α-, β-, δ-, and γ-toxins.[6,7] In the rabbit keratitis model, strains producing α-toxin have been shown to cause extensive tissue damage and ocular inflammation.[3,4] Purified α-toxin injected into the rabbit cornea in nanogram quantities causes corneal epithelial erosions, marked edema, and ocular inflammation.[4,8] β-Toxin has been shown to induce edema in rabbit eyes during keratitis and when purified toxin is injected into the cornea.[4] δ-Toxin has not been specifically analyzed as a virulence factor for keratitis. However, strains producing δ-toxin, but not other hemolysins, produce minimal corneal virulence, suggesting that δ-toxin is not an important virulence factor in keratitis.[4]

Currently, α-toxin is the only known virulence factor capable of producing extensive corneal disease[4]; however, approximately 25% of *S. aureus* strains isolated from human infections are reported to show no α-toxin activity in vitro.[7,9–11] Thus, it is possible that α-toxin–deficient isolates contain one or more corneal virulence factors that have yet to be identified. One toxin that could account for corneal damage is γ-toxin. Piemont[12] and Siqueira et al.[13] have reported that γ-toxin can mediate inflammatory reactions and tissue disruption in the rabbit eye after injection of purified toxin into the midvitreous cavity. Supersac et al.[14] analyzed the virulence of strain Newman and its γ-toxin–deficient mutant. Strain Newman reportedly has no α- and β-toxin production.[15] Supersac et al.[14] found that the mutant deficient in γ-toxin had reduced virulence relative to its γ-toxin–producing parent. However, the γ-toxin–deficient strain retained considerable virulence, the cause of which was not determined.

The γ-toxin locus occurs in 99% of *S. aureus* isolates.[14,16] γ-Toxin consists of two separately secreted proteins, one class F protein (HlgB, 36 kDa) and one of either of two class S proteins (HlgA or HlgC, each 32 kDa) that act synergistically to lyse target cells.[6,7,14–18] γ-Toxin is a pore-forming toxin[7,16,19] and the HlgC component is a protein kinase A recognition protein.[7,16,20] The toxin has activity in human and rabbit polymorphonuclear cells, monocytes, and macrophages, and is able to lyse human, rabbit, sheep, and horse erythrocytes.[7,14,16,18]

The purpose of this study was to investigate the corneal virulence of strain Newman, a strain virulent for the rabbit cornea and reportedly deficient in α-toxin. Because α-toxin is the main staphylococcal toxin responsible for severe corneal damage,[3,4,8] this study was undertaken to determine whether γ-toxin production by strain Newman mediates corneal virulence. The corneal virulence of the parent strain was compared with that of isogenic mutants in the genes coding for γ- or α-toxin and to a genetically rescued form of the γ-toxin–deficient mutant. A major finding of this study is that the γ-toxin–deficient mutant, but not the complemented form of the mutant, had reduced corneal virulence. These results also suggest that α-toxin is produced by strain Newman and its

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Supported by National Eye Institute Grant EY-10974 (RJO) and The Wellcome Trust Grants 061617 and 052320 (TJF).

Submitted for publication June 29, 2001; revised November 6, 2001; accepted November 21, 2001.

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production, even in relatively small quantities, apparently contributes significantly to corneal virulence.

Materials and Methods

Rabbits

New Zealand White rabbits (2.0–3.0 kg) were treated and maintained in strict accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All rabbits were anesthetized by subcutaneous (SC) injection of a 1:5 mixture of xylazine (100 mg/mL; Rompum; Miles Laboratories, Shawnee, KS) and ketamine hydrochloride (100 mg/mL; Ketaset; Bristol Laboratories, Syracuse, NY). Proparacaine hydrochloride (0.5% Alcaine; Alcon Laboratories, Fort Worth, TX) was topically applied to each eye before intrastromal injection. Rabbits were killed with an overdose of pentobarbital (Sigma, St. Louis, MO).

Bacteria

A brief list of properties of all strains analyzed are summarized in Table 1. Strains Newman, Newman Δ*hlg* (γ-toxin–deficient mutant), and Newman Δ*hlg*/pCU1 *hlg*$^+$ (a genetically rescued mutant containing a plasmid encoding γ-toxin) have been described previously.[6,14] Newman Δ*hlg* contains an insert coding for tetracycline resistance[6,14] and was grown on tryptic soy agar (TSA; Difco Laboratories, Inc., Detroit, MI) containing tetracycline (5–10 μg/mL). Newman Δ*hlg*/pCU1 *hlg*$^+$ contains a plasmid that expresses genes for γ-toxin and chloramphenicol resistance. This genetically rescued strain was grown on TSA containing chloramphenicol (5–10 μg/mL) and tetracycline (5–10 μg/mL). Strain 8325-4 produces α-, β-, γ-, and δ-toxins and has been studied previously in the rabbit keratitis model.[3,4,6,16,17]

Newman Δ*hla* has the α-toxin gene disrupted with an erythromycin resistance insert and was grown on TSA with 10 μg/mL erythromycin. The Newman Δ*hla*::*erm* mutation was transduced from strain 8325-4 *hla*::*erm* (DU1090)[21] using phage 85, selecting for resistance to erythromycin. The structure of the mutated *hla* locus was verified by Southern blot hybridization, as previously described.[21]

The corneal inoculum for each strain was grown to log phase in tryptic soy broth (TSB; Difco Laboratories, Inc.) then diluted to approximately 10,000 colony forming units (CFU) per milliliter. Each cornea ($n \geq 4$ per strain) was intrastromally injected with 10 μL containing approximately 100 CFU per cornea, as previously described.[3,4]

Hemolysin Assay

Hemolytic titers were performed using sheep or rabbit erythrocytes. Erythrocytes were washed twice in phosphate-buffered saline with gelatin (pH 7.4, 0.2% gelatin, 0.145 M NaCl, 0.039 M NaH_2PO_4, 0.062 M $Na_2HPO_4 \cdot 7\ H_2O$; Sigma) and resuspended to approximately 10^8 erythrocytes/mL. Culture supernatants were serially diluted twofold in phosphate-buffered saline with gelatin. Erythrocytes (equal volume) were added to each dilution, incubated for 30 minutes at 37°C, and centrifuged (1000*g* 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. Erythrocytes were added to water and 5 μL Triton X-100 (Sigma) for complete lysis (100% hemolysis).

Table 1. Characteristics of *Staphylococcus aureus* Strains

S. aureus Strain	Hemolysins Reportedly Produced*	Reference
Newman	γ-Toxin and δ-toxin‡	14, 15
Newman Δ*hlg*	δ-Toxin	14, 15
Newman Δ*hlg*/pCU1 *hlg*†	γ-Toxin and δ-toxin	14, 15
Newman Δ*hla*	γ-Toxin† and δ-toxin	This work
8325-4	α-, β-, δ-, and γ-toxins‡	15, 21

* The hemolysins included are those reported in the literature as described in the reference listed.

† Newman Δ*hla* had a reduced hemolytic titer for rabbit erythrocytes as compared with Newman (titers 2 and 256, respectively).

‡ δ-Toxin is not believed to be a corneal virulence factor.

Western Blot Analysis

Bacterial culture supernatant (10 μL) was added to 10 μL sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS [wt/vol], 20% glycerol [vol/vol], 10% β-mercaptoethanol [vol/vol], and 0.002% bromophenol blue [wt/vol]) and boiled for 5 minutes. SDS-polyacrylamide gel electrophoresis was performed by standard methods. The gel was electrophoretically transferred to polyvinylidene difluoride membranes (Roche Molecular Biochemicals, Indianapolis, IN). Membranes were incubated overnight at 4°C in 10% milk powder as a blocking reagent. Polyclonal rabbit antibodies to α-toxin were used at a dilution of 1:2000 for a 1-hour incubation at room temperature. Protein A–conjugated horseradish peroxidase (1 mg/mL stock diluted 1:500) was used to detect bound antibody by incubation for 1 hour at room temperature. The membrane was reacted with chemiluminescent substrate (LumiGLO; New England Biolabs, Beverly, MA), according to the manufacturer's instructions, and exposed to x-ray film.

Bacterial Quantification

To determine the number of viable *S. aureus* per cornea, corneas were cut in half, and one half was homogenized. The corneal homogenate dilutions were cultured in triplicate, as previously described.[3,4] CFUs were expressed as base 10 logarithms. Bacterial colonies ($N = 50$) recovered from eyes infected with each strain were tested for hemolysin production and for the ability to grow on TSA medium containing chloramphenicol (5 μg/mL), erythromycin (5 μg/mL), or tetracycline (5 μg/mL).

Immunization

Specific pathogen-free New Zealand White rabbits were immunized SC with 50 μg heat-inactivated α-toxin (Sigma) mixed with complete Freund's adjuvant (Sigma). Rabbits were subsequently immunized (boosted) monthly with 50 μg α-toxin toxoid mixed with incomplete Freund's adjuvant (Sigma). Rabbits were bled before all immunizations. Antibody titers to α-toxin were determined by ELISA. The eyes ($n = 6$ per group) of immune and nonimmune rabbits were subsequently challenged with 100 CFU *S. aureus* strain Newman and examined by slit lamp at 15, 20, and 25 hours after infection. For the passive immunity studies, rabbits were intrastromally injected (20 μL) with equal volumes of strain Newman plus immune sera to α-toxin, strain Newman plus normal rabbit serum, or strain Newman alone.

Slit Lamp Examinations

Slit lamp examinations (SLEs) of rabbit eyes were performed by two masked observers using a biomicroscope (Topcon; Koaku Kikai K.K., Tokyo, Japan). Each of seven ocular parameters (injection, chemosis, corneal infiltrate, corneal edema, fibrin in the anterior chamber, hypopyon formation, and iritis) was graded on a scale of 0 (none) to 4 (severe). The parameter grades were totaled to produce a single SLE score ranging from 0 (normal eye) to a theoretical maximum of 28, as previously described.[3] Corneal erosions were detected using fluorescein (Fluor-I-Strip AT.; Wyeth Ayerst Laboratories Inc., Philadelphia, PA) and the diameters measured and expressed in millimeters.

Tissue Embedding and Sectioning

Corneas were harvested at 25 hours after infection. They were cut in half and one half was fixed immediately in formalin (10%; EK Industries, Joliet, IL) for histopathologic studies. A tissue processor

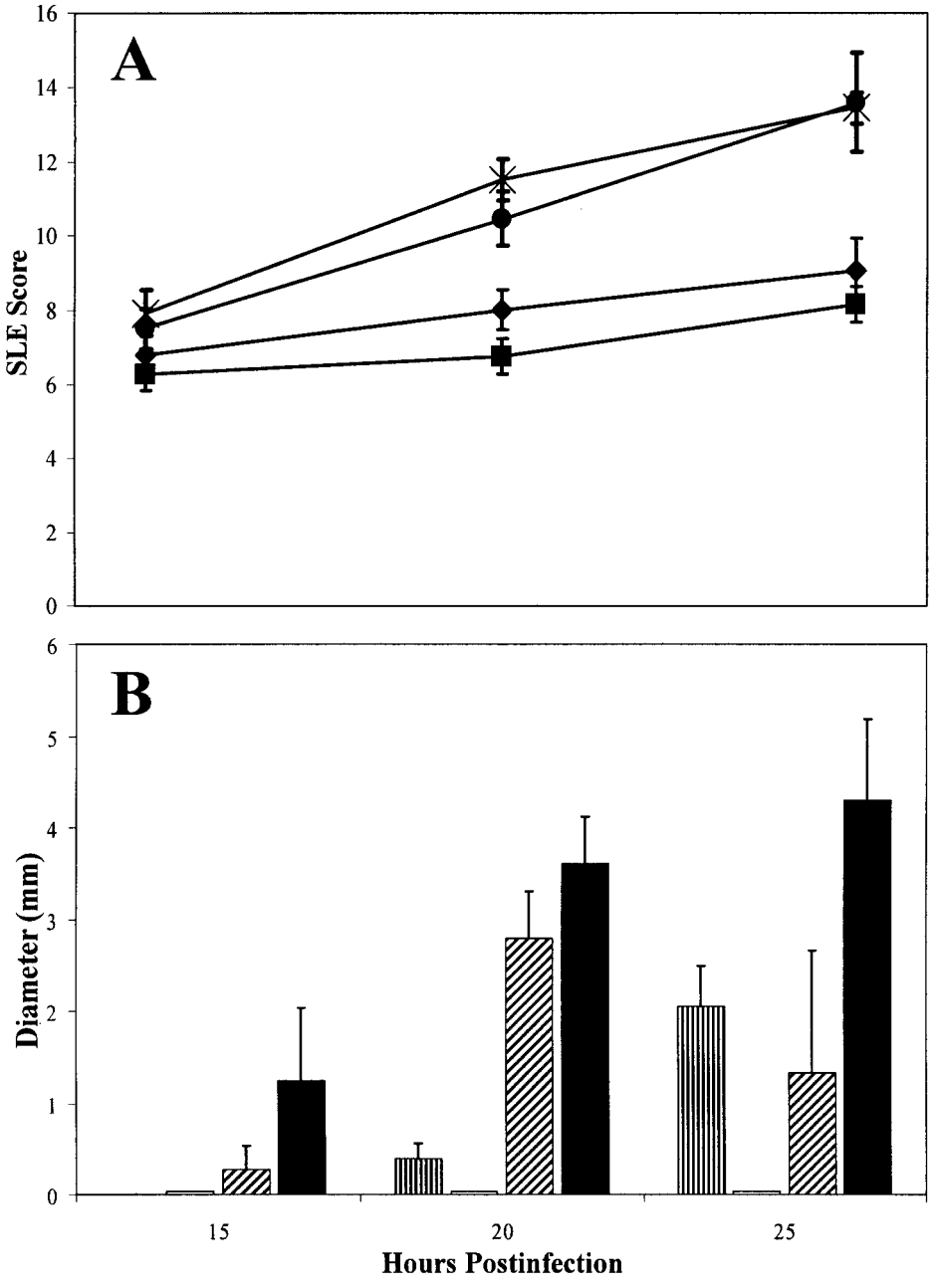

FIGURE 1. Pathologic course of rabbit corneas infected with *S. aureus* Newman Strains differing in their toxin production. Bacteria grown to log phase in TSB were injected intrastromally (approximately 100 CFU) into rabbit corneas. (**A**) Groups of eyes ($n \geq 4$ eyes per group) infected with each strain were scored at 15, 20, and 25 hours after infection. SLE scores are the sum of seven parameters each graded on a scale of 0 (normal) to 4 (severe) by two masked observers. The data points represent the average SLE score and the error bars represent the SEM. (✳), Newman; (●), Newman Δ*hlg*pCU1 hlg^+; (◆), Newman Δ*hlg*; (■), Newman Δ*hla*. (**B**) Corneal epithelial erosions were determined by measuring the diameter of the area of the erosion. Data points, average erosion diameter; error bars, SEM. (■), Newman; (▥), Newman Δ*hlg* pCU1hlg^+; (▨), Newman Δ*hlg*; (□), Newman Δ*hla.*

(Hypercenter XP Processor; Shandon, Pittsburgh, PA) was used to process the corneal tissue as follows: Tissues were immersed overnight in zinc formalin (10%), dehydrated in alcohol (70%, 80%, and 95%, and three changes of absolute alcohol), and immersed in xylene three times to clear the tissue. Corneal tissues were embedded in paraffin. The resultant paraffin blocks were then cut with a rotary microtome into 4-μm-thick sections and stained with hematoxylin and eosin.

Statistical Analysis

Data were analyzed by computer (SAS software; SAS, Cary, NC).[22] For CFU determination, analysis of variance and protected Student's *t*-tests between the least-squared mean from each group were performed. For SLE scores, nonparametric one-way analysis of variance (Kruskal-Wallis test) and Wilcoxon's test were used for comparison among groups. By conventional standards, the type I error is 0.05 and type II error is 0.20.

RESULTS

Bacterial Growth in the Cornea

Corneas injected with strain Newman, Newman Δ*hlg*, Newman Δ*hlg*/pCU1 hlg^+, or Newman Δ*hla* contained equivalent numbers of bacterial CFU per cornea at 25 hours after infection (7.36 ± 0.05; 7.0 ± 0.12; 7.03 ± 0.09; 7.25 ± 0.13; $P \geq 0.0618$). Bacterial colonies recovered from eyes infected with mutant strains of Newman grew in subculture on TSA plates containing appropriate antibiotics (5–10 μg/mL tetracycline, chloramphenicol, or erythromycin).

Role of γ-Toxin in the Corneal Virulence of Strain Newman

Corneal virulence of strains Newman, Newman deficient in γ-toxin (Newman Δ*hlg*), and the γ-toxin rescued strain (New-

man Δ*hlg*/pCU1 *hlg*$^+$) was compared (Fig. 1A). SLE scores of eyes infected with the γ-toxin–deficient Newman Δ*hlg* were significantly lower at 20 and 25 hours than scores in eyes infected with its parent strain ($P \leq 0.0001$) or the genetically rescued strain (Newman Δ*hlg*/pCU1 *hlg*$^+$; $P \leq 0.0001$), both of which produced γ-toxin. The γ-toxin–rescued strain (Newman Δ*hlg*/pCU1 *hlg*$^+$) produced SLE scores that were statistically equivalent to the Newman parent strain at 15, 20, and 25 hours after infection ($P \geq 0.1066$; Figs. 1A, 2). The virulence of Newman (parent), Newman Δ*hlg*, and the rescued strain correlated with the hemolytic titers of these organisms for sheep erythrocytes (titers of 256, 2, and 1024 for Newman, Newman Δ*hlg*, and Newman Δ*hlg*/pCU1 *hlg*$^+$, respectively).

Corneas infected with Newman, the γ-toxin mutant, or the rescued strain showed development of epithelial erosions that began at 15 hours after infection and increased in size throughout the period of infection analyzed (Figs. 1B, 2).

Corneas infected with Newman or the rescued strain demonstrated conjunctival injection and chemosis, as well as corneal edema, corneal infiltrate, fibrin accumulation, hypopyon formation, and iritis. Corneas infected with the γ-toxin–deficient mutant (Newman Δ*hlg*) demonstrated all the same changes, but the extent of each change was less.

Role of α-Toxin in the Corneal Virulence of Strain Newman

Strain Newman was compared with Newman Δ*hla*, an α-toxin–deficient mutant, for corneal virulence. SLE scores of corneas infected with strain Newman were significantly higher than those caused by infection with the α-toxin–deficient mutant, Newman Δ*hla*, at 15, 20, and 25 hours after infection ($P \leq 0.0031$; Fig. 1A). The differences in inflammation between infections caused by Newman and Newman Δ*hla* were quantitative—that is, similar inflammatory events occurred but the extent and speed of development were greater for strain Newman. However, an important difference was that the Newman parent strain produced corneal epithelial erosions but the α-toxin–deficient strain (Newman Δ*hla*) failed to produce corneal erosions (Figs. 1B, 2).

Histopathology of Infected Corneas at 25 Hours after Infection

Sections of corneas infected with strain 8325-4, an α-toxin–producing strain, showed bacteria and numerous neutrophils in the central stroma, as well as extensive corneal epithelial erosion (Figs. 3A, 3B). Corneas infected with strain Newman showed bacteria and numerous neutrophils in the central stroma as well as significant epithelial erosion (Figs. 3C, 3D). Corneas infected with Newman Δ*hlg*, which is deficient in γ-toxin, showed bacteria in the central stroma and epithelial erosion, but these corneas demonstrated markedly reduced numbers of neutrophils compared with corneas infected with strain 8325-4 or Newman (Figs. 3E, 3F). Corneas infected with Newman Δ*hla*, which has a mutation in its α-toxin gene, show bacteria in the central stroma and a limited number of neutrophils, but no corneal epithelial erosion (Figs. 3G, 3H).

Active and Passive Immunizations

Rabbits immunized with purified α-toxin toxoid had serum antibody titers of 2560 ± 809 whereas nonimmune rabbits had no detectable antibody to α-toxin. Rabbits were challenged with *S. aureus* strain Newman and underwent SLE at 15, 20, and 25 hours after infection. Immune rabbits had significantly less corneal disease than the nonimmune rabbits at 25 hours after infection ($P \leq 0.0003$; Table 2). Epithelial erosions were not evident in immune rabbits at 25 hours after infection;

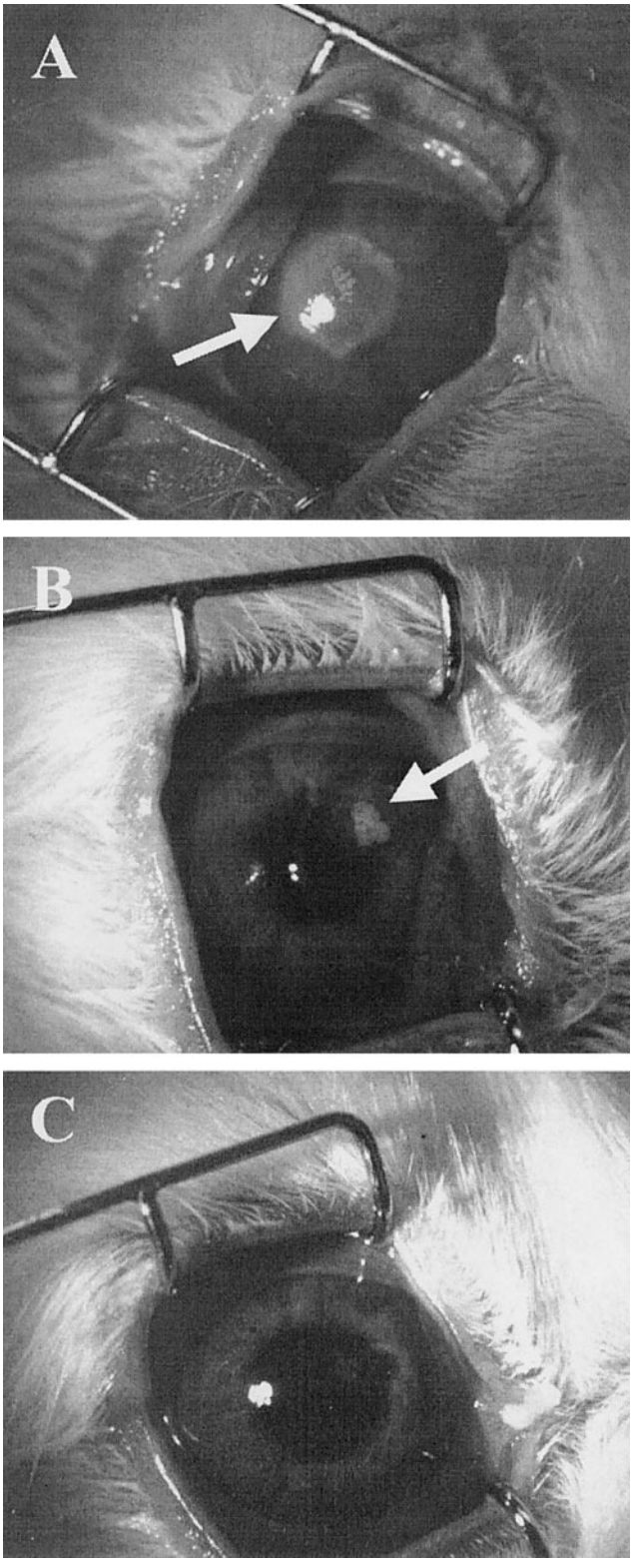

FIGURE 2. Rabbit eyes infected with *S. aureus* strain Newman, a γ-toxin–deficient mutant (Newman Δ*hlg*), or an α-toxin–deficient strain (Newman Δ*hla*). Rabbit eyes were injected with approximately 100 CFU of log phase bacteria and photographed at 25 hours after infection. (**A**) Rabbit eye infected with *S. aureus* strain Newman shows moderate to severe disease. An epithelial erosion (*arrow*) encompasses approximately 25% to 30% of the cornea. (**B**) Rabbit eye infected with *S. aureus* Newman Δ*hlg* (γ-toxin–deficient) shows moderate disease and epithelial erosion (*arrow*). (**C**) Rabbit eye infected with *S. aureus* Newman Δ*hla* (α-toxin–deficient) shows moderate disease and no epithelial erosion.

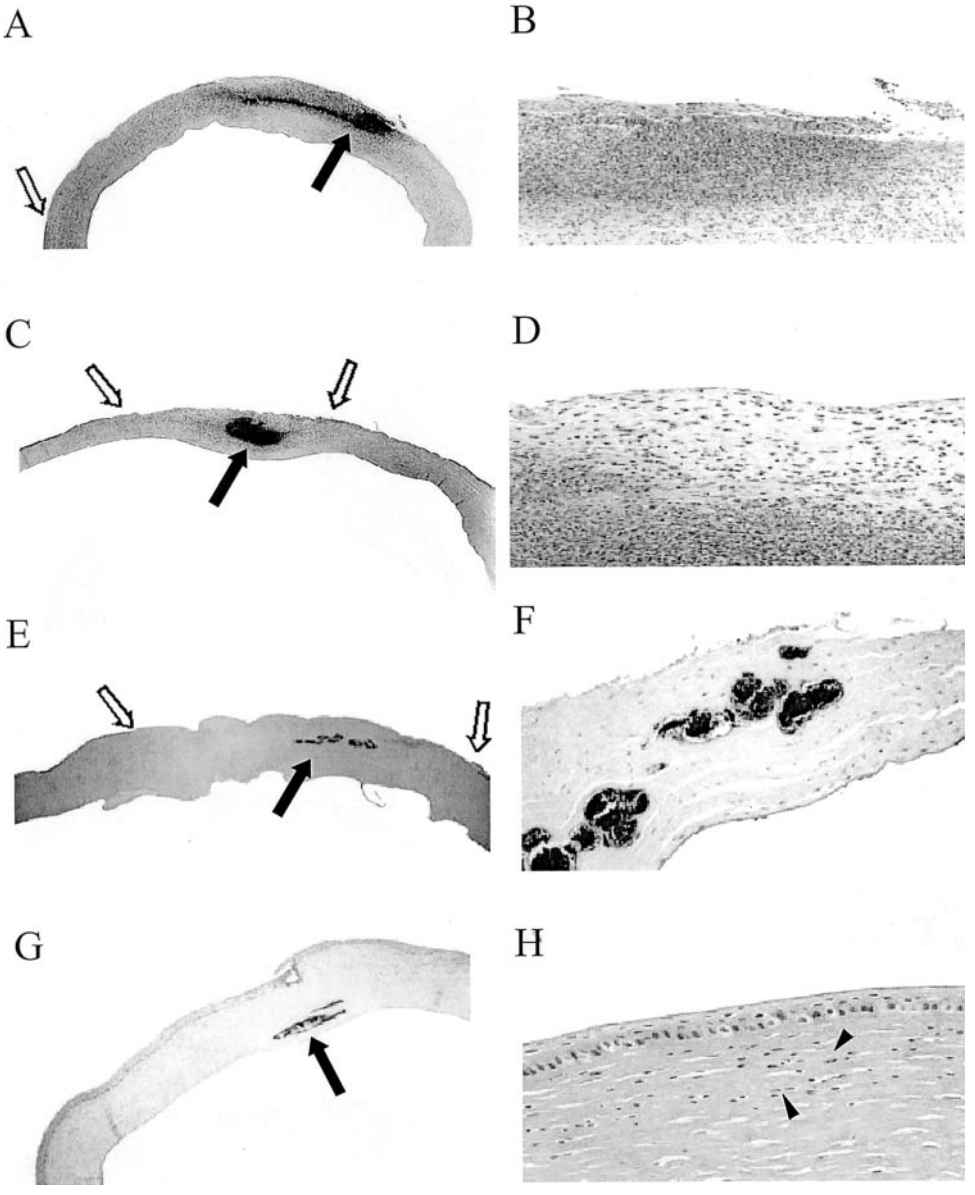

FIGURE 3. Histopathology of corneas infected with *S. aureus* at 25 hours after infection. (**A**, **B**) *S. aureus* 8325-4-infected cornea. (**A**) Low-magnification photomicrograph shows diffuse acute inflammatory exudate present in a cornea infected with *S. aureus* strain 8325-4. *Open arrow*: edge of the epithelial erosion; *filled arrow*: bacteria in the central corneal stroma. (**B**) High magnification shows denudation of the corneal epithelium and exudate in the overlying tear film. (**C**, **D**) *S. aureus* strain Newman–infected cornea. (**C**) Low-magnification photomicrograph of a cornea infected with *S. aureus* strain Newman shows a mass of bacteria (*solid arrow*) in the corneal stroma and the edges of an epithelial erosion (*open arrows*). (**D**) High-power photomicrograph of the cornea shows extensive neutrophil infiltrate and denudation of the corneal epithelium. (**E**, **F**) *S. aureus* strain Newman Δ*hlg* (γ-toxin–deficient) infected cornea. (**E**) Low-power photomicrograph of a cornea infected with Newman Δ*hlg* shows bacteria in the central stroma (*solid arrow*), a sparse inflammatory infiltrate, and the edges of a corneal epithelial erosion (*open arrows*). (**F**) High-power photomicrograph of the Newman Δ*hlg*–infected cornea shows bacteria and epithelial erosion. (**G**, **H**) *S. aureus* strain Newman Δ*hla* (α-toxin–deficient) infected cornea. (**G**) Low-power photomicrograph of a cornea infected with Newman Δ*hla* shows a mass of bacteria present in the stroma (*solid arrow*). (**H**) High-power photomicrograph illustrates sparse neutrophil infiltrate (*arrowheads*) and an intact corneal epithelium. Magnification, (**A**, **C**, **E**, **G**) ×40; (**B**, **D**, **H**) ×200; (**F**) ×100.

however, nonimmune rabbits exhibited epithelial erosions (no erosion and erosion of 1.5 ± 0.67 mm, respectively).

Rabbit corneas injected with a combination of strain Newman plus immune sera to α-toxin had significantly less disease at 25 hours after infection (SLE score) than the corneas injected with Newman plus normal rabbit serum or with Newman alone ($P \leq 0.0033$; Table 2). Rabbit corneas injected with Newman plus immune sera did not show development of epithelial erosions, whereas rabbits injected with bacteria plus normal sera or bacteria alone showed erosions (no erosion compared with erosion of 3.67 ± 1.23 and 3.83 ± 0.28 mm; Table 2).

Western Blot Analysis

Western blot analysis demonstrated that that culture supernatants of strains Newman and Newman Δ*hlg* produced α-toxin (Fig. 4). Culture supernatants of strain Newman Δ*hla*, however, did not demonstrate the production of α-toxin.

DISCUSSION

γ-Toxin is the major hemolysin of strain Newman,[14] and rendering the bacterium deficient in this toxin significantly reduces corneal virulence. In comparing γ-toxin–producing and –deficient strains of Newman, there was a direct correlation between the hemolytic titer attributable to γ-toxin and the extent of virulence produced during infection. The effects of γ-toxin activity on ocular virulence were not expressed in any specific ocular change; rather, the toxin correlated with quantitatively enhanced toxicity in multiple ocular parameters, including conjunctival chemosis, injection, and corneal inflammation. The absence of any specific ocular change attributable to γ-toxin is unlike the situation with α-toxin, for which there were one or more specific qualitative differences between eyes infected with the toxin-deficient mutant or those infected with the corresponding parent strain (e.g., epithelial erosion).[3,4]

This study also suggests that α-toxin is active in producing ocular changes in eyes infected with strain Newman. All the Newman strains, except the one deficient in α-toxin, produced corneal epithelial erosions that were readily visible on gross

examination and in histologic sections of corneas. Corneal erosions produced by the γ-toxin–deficient Newman strain, but not the α-toxin–deficient mutant, are evidence for a role for α-toxin in corneal epithelial erosion produced by strain Newman. Also, supporting a role for α-toxin in the corneal virulence of strain Newman was the protection against corneal damage, especially epithelial erosion, afforded by either active or passive immunization to α-toxin. The epithelial erosions caused by Newman strains developed more slowly and were smaller throughout the 25 hours of infection than those caused by strain 8325-4, a strain in which α-toxin is the key hemolytic toxin. This difference in the rate of erosion formation correlates with the relatively small amount of α-toxin that appears to be produced by strain Newman. In fact, strain Newman has been previously considered to be devoid of α-toxin production.[15] In the intrastromal model of *Staphylococcus* keratitis, as used in the present study, bacterial products are secreted in a nearly enclosed area of the corneal stroma, allowing accumulation of the products throughout the course of the infection. Erosion of the rabbit corneal epithelium can be mediated by as little as 2.0 ng α-toxin.[4] Thus, erosion of the rabbit corneal epithelial cells could be a very sensitive assay for biologically active α-toxin. Western blot analysis also demonstrated that culture supernatants of strains Newman and Newman Δ*hlg* produced α-toxin, but not Newman Δ*hla*.

Previous work from this laboratory has shown that mutations of the α-toxin gene (Δ*hla*) in strain 8325-4 (i.e., strain DU1090) result in a decline in the SLE score from the parental score of approximately 20 to a mutant score of 9 to 10 at 25 hours after infection.[4] A mutation in both the α- and β-toxin genes (i.e., strain DU5720) or in the *agr* locus (i.e., strain ISP546) results in similar declines in the SLE score to 5 to 6 at 25 hours after infection. The *agr* locus is required for expression of α-, β-, and γ-toxins and the similarity of the SLE scores of strains DU5720 and ISP546 suggests that γ-toxin is not an important corneal virulence factor in strain 8325-4. Production of γ-toxin by strain 8325-4 is not readily measurable in terms of hemolytic activity. These findings for strain 8325-4 are inverse to those of the present study on strain Newman, in which γ-toxin appears to be the major hemolysin and α-toxin is produced only in small quantities. A study by Nilsson et al.[16] concluded that both α- and γ-toxins may work in synergy to produce virulence. Our data also support their hypothesis by demonstrating that disrupting either the α-toxin or γ-toxin gene reduced corneal disease to approximately equivalent scores (Fig. 1). Possibly supporting this hypothesis is our finding in a strain with a mutation in both the α- and γ-toxin genes. This strain with both mutations had the same corneal virulence as the strains with a mutation in either the α- or γ-toxin gene (data not shown).

TABLE 2 Effect of Immunization with α-Toxin Toxoid on Corneal Damage in Eyes Infected with Strain Newman

Treatment	Antibody Titer	SLE Score*	Erosions† (mm)
Actively immunized‡	2560 ± 809	8.79 ± 0.77	0.0 ± 0.0
Nonimmune	0	10.77 ± 0.66	1.5 ± 0.67
Immune sera + bacteria§	2560 ± 809	8.63 ± 0.41	0.0 ± 0.0
Nonimmune sera + bacteria§	0	13.35 ± 0.82	3.67 ± 1.23
Bacteria alone	0	13.90 ± 1.04	3.83 ± 0.28

* Rabbits underwent SLE examination at 25 hours after infection by two masked observers.

† Rabbit corneas were examined for epithelial erosion using fluoroscein at 25 hours after infection.

‡ Rabbits ($n = 3$) were immunized with α-toxin toxoid over a period of 4 months and subsequently challenged with strain Newman. The SLE scores of actively immunized rabbits were significantly less than nonimmune rabbits ($P \leq 0.0003$). The SLE scores of eyes injected with bacteria plus immune sera were significantly lower than that of eyes injected with bacteria plus non-immune sera or bacteria alone ($P \leq 0.003$).

§ Sera from actively immunized rabbits or normal rabbits were passively administered with bacteria (100 CFU of strain Newman) by intrastromal injection ($n = 6$ eyes per group).

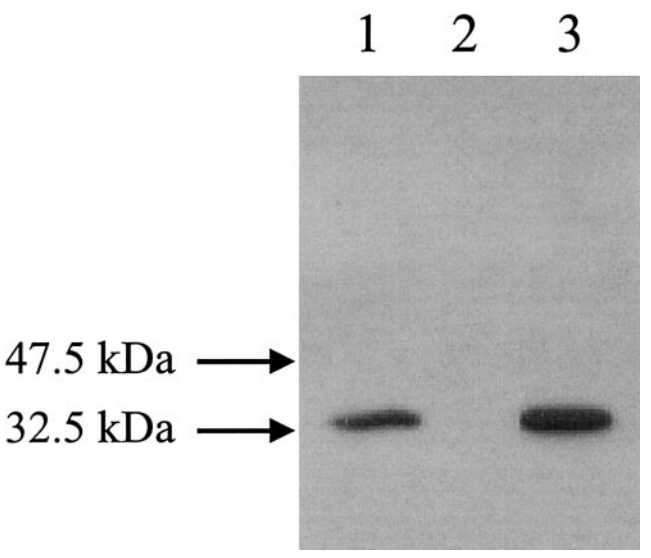

FIGURE 4. Western blot analysis of culture supernatants. Bacterial culture supernatants (10 μL) were assayed for α-toxin by Western blot analysis using polyclonal antibody to α-toxin. *Lane 1*: Newman culture supernatant; *lane 2*: Newman Δ*hla* culture supernatant; and *lane 3*: Newman Δ*hlg* culture supernatant.

The Newman parent strain, its γ-toxin–deficient mutant strain, the γ-toxin rescued strain, and the α-toxin–deficient Newman strain grew at equivalent rates in the cornea, demonstrating that differences in virulence among these strains were not due to differences in bacterial growth in the cornea. This similarity in growth rates facilitated direct comparison of virulence produced by each of these strains.

The Newman strains deficient in either α- or γ-toxin were less virulent than the parent strain; however, each retained considerable virulence. These findings suggest that an additional virulence factor or factors could be active in infections caused by strain Newman. Supersac et al.[14] and Gravet et al.[23] suggested that an unidentified toxin could be produced by strain Newman. Accordingly, it can be concluded that tissue-damaging reactions mediated by *S. aureus* remain ill-defined, including those reactions that play a role in ocular virulence.

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