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Specific Roles of Alpha-Toxin and Beta-Toxin during *Staphylococcus aureus* Corneal Infection

RICHARD J. O'CALLAGHAN,[1,2]* MICHELLE C. CALLEGAN,[1]† JUDY M. MOREAU,[1] LINDA C. GREEN,[1,2] TIMOTHY J. FOSTER,[3] ORLA M. HARTFORD,[3] LEE S. ENGEL,[1] AND JAMES M. HILL[1,2]

Department of Microbiology, Immunology, and Parasitology, Louisiana State University Medical Center,[1] and Department of Ophthalmology, Louisiana State University Eye Center,[2] New Orleans, Louisiana, and Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland[3]

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Staphylococcus aureus **corneal infection results in extensive inflammation and tissue damage. Our previous studies of bacterial mutants have demonstrated a role for alpha-toxin in corneal virulence. This study analyzes, by genetic rescue experiments, the virulence of mutants affecting alpha-toxin and beta-toxin activity and demonstrates the ocular toxicity of these purified staphylococcal proteins. Three types of isogenic mutants were analyzed: (i) mutants specifically deficient in alpha-toxin (Hla) or beta-toxin (Hlb), (ii) a mutant deficient in both Hla and Hlb, and (iii) a regulatory mutant, deficient in the accessory gene regulator (*agr*), that produces reduced quantities of multiple exoproteins, including alpha- and beta-toxins. Plasmids coding for Hla and Hlb (pDU1212 and pCU1hlb, respectively) were used to restore toxin activity to mutants specifically deficient in each of these toxins. Either corneas were injected intrastromally with logarithmic-phase *S. aureus* or purified alpha- or beta-toxins were administered to normal eyes. Ocular pathology was evaluated by slit lamp examination and myeloperoxidase activity of infiltrating polymorphonuclear leukocytes. Corneal homogenates were cultured to determine the CFU per cornea. Eyes infected with the wild-type strain developed significantly greater corneal damage than eyes infected with Agr$^-$, Hlb$^-$, or Hla$^-$ strains. Epithelial erosions produced by parent strains were not produced by Agr$^-$ or Hla$^-$ strains. Hlb$^+$ strains, unlike Hlb$^-$ strains, caused scleral edema. Plasmid pDU1212 restored corneal virulence to strain DU1090 (Hla$^-$), and plasmid pCU1hlb restored corneal virulence to strain DU5719 (Hlb$^-$). Application of purified alpha-toxin produced corneal epithelial erosions and iritis, while application of beta-toxin caused scleral inflammation. These studies confirm the role of alpha-toxin as a major virulence factor during *S. aureus* keratitis and implicate beta-toxin, a mediator of edema, as a lesser contributor to ocular damage.**

Staphylococcus aureus is the leading cause of bacterial keratitis in adults, including those who have sustained penetrating corneal injuries or are compromised by immunodeficiencies (3, 31). Tissue damage during bacterial keratitis results from the action of bacterial products on ocular tissues and from the host inflammatory response to the infection (13, 26). *Staphylococcus* keratitis can result in irreversible corneal scarring, resulting in a loss of visual acuity. Multidrug-resistant strains of *S. aureus* further complicate the therapy of these infections (34).

Previous studies from this laboratory examined the roles of specific staphylococcal proteins (alpha-toxin and protein A) in corneal virulence in an experimental rabbit model of keratitis (13). Alpha-toxin-producing (Hla$^+$) strains caused significantly greater ocular inflammation and corneal damage than did alpha-toxin-deficient (Hla$^-$) strains. Hla$^-$ strains produced significantly less inflammation of the conjunctiva and iris and almost no corneal epithelial erosion or stromal ulceration. Strains deficient in protein A production (Spa$^-$) were as virulent as their protein A-producing parent strains (Spa$^+$), suggesting the relative unimportance of protein A for virulence in the corneal stroma during *S. aureus* keratitis (13).

* Corresponding author. Mailing address: Department of Microbiology, Immunology, and Parasitology, Louisiana State University Medical Center, 1901 Perdido St., New Orleans, LA 70112. Phone: (504) 568-4072. Fax: (504) 568-2918. E-mail: rocall@pop3.lsumc.edu.

† Present address: Molecular Pathogenesis of Eye Infection Research Center, Department of Ophthalmology, Dean A. McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

Proteins other than alpha-toxin could contribute to ocular virulence during *S. aureus* keratitis. The expression of multiple proteins potentially involved in virulence is controlled by the accessory gene regulator (Agr) system. Mutants defective in Agr demonstrate reduced expression of some proteins normally induced in stationary phase (e.g., beta-toxin) and do not express many other such proteins, including several hemolytic toxins and enzymes (29). Agr-defective mutants produce increased quantities of coagulase and the cell wall-associated proteins, including protein A, clumping factor, and fibronectin-binding protein (23). Correlations between a mutation in the Agr system and decreased virulence have been described in nonocular models of infection (1, 18, 19, 25, 37) and in a model of endophthalmitis (8).

Several studies have correlated the biological activity of specific staphylococcal proteins with their toxicity in nonocular models of infection (10, 13, 24, 28, 32, 33, 36). The pore-forming alpha-toxin damages cell membranes, an activity suggested to contribute to tissue damage in several models of infection (10, 13, 24, 28, 33, 36). Beta-toxin, a sphingomyelinase, is a possible virulence factor responsible for tissue necrosis during experimental murine mastitis (10). Beta-toxin induces mild inflammatory changes in the bovine mammary gland (17) and lyses bovine epithelial cells in vitro (20). The lytic action of beta-toxin is known to be limited by the sphingomyelin content of cell membranes (6, 7). Corneal and scleral epithelial cell membranes have a high sphingomyelin content (11); therefore, both tissues could be targets for beta-toxin during ocular infection.

The staphylococcal proteins responsible for ocular tissue

TABLE 1. Characteristics of *S. aureus* strains used for experimental keratitis

Strain	Relative phenotype[b]	Hemolytic toxin titer[a]		Epithelial erosions (diam [mm])[c]
		Rabbit RBC	Sheep RBC	
8325-4	Parent	256	16	6.0 ± 0.5
DU1090[d]	Hla^-	0	16	None visible
DU5719[d]	Hlb^-	256	0	5.7 ± 0.5
DU5720[d]	Hla^- Hlb^-	0	0	None visible
ISP546[d]	Agr^-	0	2	None visible
DU1090/pDU1212[e]	Hla^-/Hla^+	256	64	4.7 ± 0.9
DU5719/pCU1hlb[f]	Hlb^-/Hlb^+	256	8	6.3 ± 0.6

[a] Filtered culture supernatants of *S. aureus* grown in TSB for 18 h were assayed as described in Materials and Methods. Rabbit RBC titers reflect the action of alpha-toxin, while sheep RBC titers after incubation at 37 and 4°C (hot-cold lysis) reflect the action of beta-toxin.

[b] The reported phenotype of the strain as described in the text.

[c] Measured with the slit lamp in four to eight eyes. The sizes of epithelial erosions of strains 8325-4, DU5719, DU1090/pDU1212, and DU5719/pCU1hlb were not significantly different ($P \geq 0.0716$).

[d] Isogenic mutant of strain 8325-4.

[e] Isogenic mutant of strain 8325-4 bearing a plasmid expressing alpha-toxin (pDU1212).

[f] Isogenic mutant of strain 8325-4 bearing a plasmid expressing beta-toxin (pCU1hlb).

damage and the tissues that they each target are in the process of being identified. In this study, we analyzed the virulence of mutants deficient in Agr, beta-toxin, alpha-toxin, or both alpha-toxin and beta-toxin by comparing the ocular virulence of the parent strain or the genetically rescued strain with that of the isogenic mutant. We also examined the effects of purified forms of alpha-toxin and beta-toxin on normal eyes to determine their specific toxicity for ocular tissues. Identification of specific proteins responsible for ocular damage is necessary for the development of inhibitors that could be used in adjunct chemotherapies.

(This work was performed in part as a partial fulfillment of the requirements for the doctoral degree [M.C.C.] and master's degree [J.M.M.] at the School of Graduate Studies of LSU Medical Center.)

MATERIALS AND METHODS

Strains used. The phenotypes of *S. aureus* strains used for experimental keratitis and the titers of alpha-toxin and beta-toxin expressed in culture supernatants are summarized in Table 1. The parent strain 8325-4 and the alpha-toxin-deficient mutant of 8325-4 (DU1090 [*hla*::Em^r] [35]) have been used previously to produce experimental keratitis (13). The beta-toxin-deficient strain (DU5719 [*hlb*::Φ42E]) was generated by negative lysogenic conversion of 8325-4 with phage Φ42E (10). The strain deficient in beta-toxin and alpha-toxin (DU5720 [Hla^- Hlb^-]) was generated by negative lysogenic conversion of strain DU1090 (Hla^-) with phage Φ42E (*hlb*::Φ42E [10]). The Agr-defective mutant of 8325-4 (strain ISP546, supplied by J. Iandolo) was generated by the insertion of transposon Tn*551* (21, 37). The Agr-defective mutant produced approximately 32-fold more protein A and 64-fold more coagulase than its parent 8325-4 (data not shown). The construction of plasmid pDU1212 (Hla^+) and its introduction into strain DU1090 (Hla^-) have been described previously (22, 35). The *hlb* gene of strain M60 (2, 10) was cloned into the shuttle vector pCU1 (4) and transformed into *Escherichia coli* DH5α, selecting for transformants on L agar with ampicillin (100 μg/ml). Plasmid pCU1hlb was then transformed into *S. aureus* RN4220 by electroporation, selecting for chloramphenicol (5 μg/ml) resistance (30, 35, 36). The plasmid was then transduced by using bacteriophage 85 from strain RN4220 to strain DU5719 (35, 36).

Strains were assayed by hemolytic titration of culture supernatants prior to intrastromal injection. Colonies (40 or more per cornea) obtained from homogenates of infected rabbit corneas were individually assayed by hemolytic titrations and antibiotic resistances to ensure that the isolated bacteria had retained the expected phenotype.

Growth and hemolysin production in vitro. Growth and hemolysin production of each *S. aureus* strain were assessed by inoculating tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) with 100 CFU of *S. aureus* per ml and removing aliquots every 2 h. Aliquots (0.1 ml) were serially diluted 10-fold in sterile TSB and plated in triplicate onto tryptic soy agar (Difco) to determine CFU per milliliter. Samples to be tested for hemolytic activity were centrifuged ($10,000 \times g$), and the supernatants were filter (0.22-μm pore size; Millipore, Bedford, Mass.) sterilized.

Purification of staphylococcal proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, silver staining, Western blotting, and bioassay of commercial preparations (Sigma, St. Louis, Mo.) of alpha-toxin and beta-toxin indicated that these preparations contained additional contaminating proteins (data not shown). Alpha-toxin and beta-toxin were purified to homogeneity by isoelectric focusing (Rotofor; Bio-Rad, Hercules, Calif.). Each protein solution (2 mg/ml) was isoelectric focused (using 3/10 ampholytes [Bio-Rad]), and fractions were tested for pH and protein activity as described below. Active fractions were refocused, and the pH and biological activity were determined. Alpha-toxin yielded a single hemolytic peak at pI 8.2 and a specific activity of 1.2×10^7 hemolytic units/mg. Beta-toxin yielded a single hemolytic peak at pI 9.5 and a specific activity of 5×10^5 hemolytic units/mg. Active fractions were found to produce single bands of appropriate molecular weights on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Alpha-toxin solutions were dialyzed against phosphate-buffered saline (PBS). Beta-toxin solutions were dialyzed against PBS, and then gelatin (0.2% wt/vol) was added as a stabilizing agent. Protein solutions were frozen (−70°C) for later use. Both alpha-toxin and beta-toxin were tested in normal rabbit eyes.

Protein activity assays. Alpha-toxin and beta-toxin activities were determined by lysis of rabbit and sheep erythrocytes (RBC; Becton Dickinson Microbiological Systems, Cockeysville, Md.), respectively, as previously described (6, 7).

Experimental *S. aureus* keratitis. New Zealand White rabbits (2.0 to 3.0 kg) were maintained according to institutional guidelines and the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. All rabbits were anesthetized by subcutaneous injection of a 1:5 mixture of xylazine (100 mg/ml; Rompun; Miles Laboratories, Shawnee, Kans.) and ketamine hydrochloride (100 mg/ml; Ketaset; Bristol Laboratories, Syracuse, N.Y.). Proparacaine hydrochloride (0.5% Alcaine; Alcon Laboratories, Fort Worth, Tex.) was topically applied to each eye before intrastromal injection.

Strains DU1090/pDU1212 and DU5719/pCU1hlb were used for genetic rescue experiments of alpha-toxin and beta-toxin, respectively. DU1090/pDU1212 was grown to log phase in TSB containing 10 μg of chloramphenicol per ml and diluted in TSB without antibiotic to approximately 10,000 CFU/ml for injection into corneas. DU5719/pCU1hlb was grown overnight on sheep blood agar containing chloramphenicol (10 μg/ml) and antibody to alpha-toxin. Antibody to alpha-toxin was included in the medium to minimize alpha-toxin hemolysis of sheep RBC. Hemolytic colonies were grown overnight in TSB containing chloramphenicol (10 μg/ml) and subcultured in the same medium. The log-phase culture was diluted to approximately 10,000 CFU/ml in TSB with chloramphenicol (10 μg/ml) and injected into rabbit corneas. Eyes infected with DU5719/pCU1hlb were treated with a single drop of chloramphenicol (30 μg/ml) at 2.5, 5, 7.5, and 10 h postinfection. Similar administration of chloramphenicol to normal eyes induced no ocular changes. Based on the expression of chloramphenicol resistance or production of hemolysins from 40 colonies per infected eye, the plasmids of both DU1090/pDU1212 and DU5719/pCU1hlb were retained in vivo by >90% of the bacteria present at 25 h postinfection.

Each eye was intrastromally injected as previously described (12–16) with the following strains (in CFU per cornea; mean ± standard error of the mean [SEM]): 8325-4, 106 ± 26; DU1090 (Hla^-), 94 ± 13; DU5719 (Hlb^-), 95 ± 25; DU5720 (Hla^- Hlb^-), 98 ± 15; ISP546 (Agr^-), 98 ± 13; DU1090/pDU1212, 47 ± 2; and DU5719/pCU1hlb, 53 ± 4. Injection of sterile TSB (10 μl) was performed as a negative control.

Ocular toxicity assessment of purified proteins. Purified staphylococcal proteins were assessed for ocular toxicity in normal rabbit eyes. Proteins in buffer (PBS for alpha-toxin and PBS with 0.2% gelatin for beta-toxin) were tested at the following concentrations: alpha-toxin, 0.002, 0.02, 0.2, 2.0, and 10.0 μg per eye; and beta-toxin, 0.02, 0.2, 2.0, 5.0, and 10.0 μg per eye. Controls consisted of bovine serum albumin (BSA; 10 μg; Sigma), buffer solutions (PBS or PBS with 0.2% gelatin), and heat-inactivated proteins at a concentration equivalent to the highest concentration tested for native proteins. Proteins were inactivated by heating at 60°C until biological activity was reduced by at least 95%. Protein concentrations were quantitated by bicinchoninic acid assay (Bio-Rad) and confirmed by the Bradford Coomassie protein assay (Bio-Rad).

Protein solutions were applied to normal rabbit eyes topically, by intrastromal injection, or by injection into the anterior chamber. For topical administration, aliquots (20 μl) of protein solutions were applied to rabbit eyes with a calibrated pipette. For intrastromal injections, rabbits were anesthetized with ketamine-xylazine and the eyes were treated topically with proparacaine prior to injection of a protein solution (20 μl), using a 30-gauge needle attached to a 100-μl syringe. Rabbits received general anesthesia and topical ocular anesthesia prior to paracentesis of aqueous humor (20 μl) and injection into the anterior chamber. Protein solutions (20 μl) were injected by using a 27-gauge needle attached to a 100-μl syringe. Eyes were examined by biomicroscopy every 30 to 60 min for 6 h and every 12 to 24 h thereafter until 72 h after toxin administration.

Slit lamp examinations. Slit lamp examinations of infected rabbit eyes were scored on a scale ranging from 0 (normal eye) to a theoretical maximum of 28 as

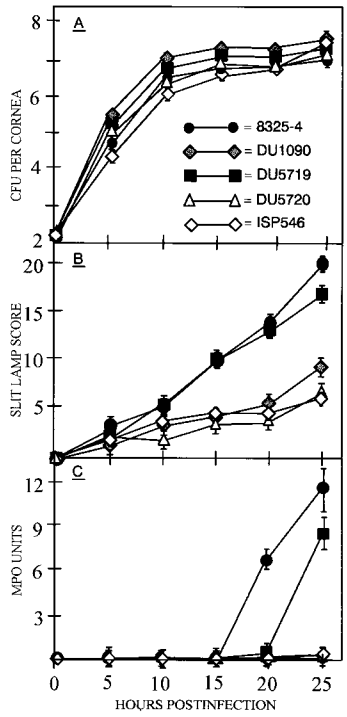

FIG. 1. Effect of alpha-toxin or beta-toxin deficiency on *S. aureus* corneal virulence. CFU ($\log_{10}$ values) per cornea (A), slit lamp examination scores (B), and MPO activities (C) of eyes infected with *S. aureus* 8325-4 (parent), DU1090 (Hla^-), DU5719 (Hlb^-), DU5720 (Hla^- Hlb^-), or ISP546 (Agr^-) were determined at various times postinfection following intrastromal injection of approximately 100 CFU per cornea. Values represent the mean ± SEM (four to eight corneas per time point). (Some SEM error bars are obscured by the data points.)

previously described (13). No infected eyes were allowed to progress beyond a score of 20.

For toxicity studies, eyes were examined by slit lamp biomicroscopy and scored as previously described (13). Two additional parameters were also assayed. Epithelial erosions, when present, were measured (diameter in millimeters) during slit lamp examination using a calibrated light band; erosions were then graded from 0 to 4 based on the percentage of coverage of the total corneal area. Scleral edema was graded from 0 to 4 based on the percentage of affected edematous area compared with the total observable scleral area.

Quantification of viable staphylococci per cornea. Quantification of viable *S. aureus* per cornea was determined by culturing corneal homogenates as previously described (12–15). CFU were expressed as base 10 logarithms.

MPO assay. Myeloperoxidase (MPO) activities of infiltrated polymorphonuclear leukocytes in corneal homogenates were assayed colorimetrically to estimate the extent of corneal inflammation, using *o*-dianisidine dihydrochloride (16.7 mg/100 ml; Sigma) and H_2O_2 (0.0005%) as previously described (9, 13, 27).

Statistical analysis. Statistical analysis of data was performed with the Statistical Analysis Systems program for personal computers (38). For CFU determinations, analysis of variance and Student's *t* tests between least-square means from each treatment group showing statistical variances were performed. For slit lamp examination scores and MPO activities, nonparametric one-way analysis of variance (Kruskal-Wallis test) and Wilcoxon's test were used for comparison among treatment groups. *P* values of ≤0.05 were considered significant.

RESULTS

Role of beta-toxin in ocular virulence. The virulence of the mutant deficient in beta-toxin (DU5719) was compared with that of its isogenic parent (8325-4). Infection with strain 8325-4 or DU5719 was similar in inducing increases in the slit lamp examination score (Fig. 1). Slit lamp examinations revealed that infections with either strain induced epithelial erosions and pus-filled intrastromal ulcers at the injection site. Although both strains caused a steady increase in ocular inflammation from 0 to 20 h postinfection, the slit lamp examination scores of eyes infected with DU5719 were significantly lower than those of eyes infected with 8325-4 at 25 h ($P \leq 0.0001$) (Fig. 1). Eyes infected with 8325-4 demonstrated a scleral inflammation that increased in severity from 15 to 25 h postinfection; however, this phenomenon was significantly less apparent in eyes infected with DU5719, suggesting that scleral edema is mediated by beta-toxin.

Strains 8325-4 and DU5719 grew logarithmically in the cornea until 10 h postinfection; the CFU per cornea from 10 to 25 h postinfection for these strains were not significantly different ($P \geq 0.0519$) (Fig. 1). The MPO activities from infiltrating polymorphonuclear leukocytes of corneas infected with 8325-4 were significantly greater than those of eyes infected with DU5719 (Hlb^-) at 25 h postinfection ($P = 0.0408$; Fig. 1).

Ocular virulence of *S. aureus* deficient in both alpha-toxin and beta-toxin. The virulence of the Hla^- Hlb^- strain (DU5720) was compared with those of its isogenic parent (8325-4) and strains deficient in *agr* (ISP546), alpha-toxin (DU1090), or beta-toxin (DU5719). The slit lamp scores for strain DU5720 from 10 to 25 h postinfection were significantly lower than those of eyes infected with the parent strain (8325-4) or the beta-toxin-deficient strain (DU5719) ($P \leq 0.0014$) (Fig. 1). Eyes infected for 20 h with DU5720 showed neither epithelial erosions nor scleral edema. These eyes demonstrated trace to mild conjunctival injection, chemosis, and iritis. Stromal edema and infiltrate were minimal, and small, punctate infiltrates were present along the needle tract. By 25 h postinfection, the infiltrates coalesced within the stroma to form small abscesses (2.0 mm); however, extensive stromal ulceration was not observed. The slit lamp examination scores at 25 h postinfection of eyes infected with DU5720 (Hla^- Hlb^-) were similar to that of ISP546 (Agr^-) ($P = 0.4026$) but significantly less than that of DU1090 (Hla^-) ($P \leq 0.0030$).

Strain 8325-4 (parent) and its isogenic mutants DU5720 (Hla^- Hlb^-), ISP546 (Agr^-), DU1090 (Hla^-), and DU5719 (Hlb^-) grew logarithmically in the cornea until 10 h postinfection. The CFU per cornea of these strains at 25 h postinfection were not significantly different ($P \geq 0.0511$) (Fig. 1). The MPO activities of corneas infected with either 8325-4 or DU5719 were significantly greater than those of eyes infected with ISP546, DU1090, or DU5720 at 25 h postinfection ($P \leq 0.0001$) (Fig. 1).

Comparison of strains with reduced virulence during extended infection. Infections with Hla^+ *S. aureus* strains induced extensive inflammation and were terminated by 25 h postinfection. Three mutants deficient in alpha-toxin (ISP546, DU1090, and DU5720) had significantly reduced virulence at 25 h postinfection (Fig. 1); therefore, infections with these strains were extended to 35 h postinfection.

Over an extended period of infection (35 h), the strains with reduced virulence induced different changes in the sclera but not in the cornea, conjunctiva, or iris. Scleral inflammation was present from 25 to 35 h postinfection in eyes infected with the strain producing substantial beta-toxin activity (DU1090) but not in eyes infected with beta-toxin-deficient strain ISP546 or DU5720. All other aspects of the infections were similar for these strains through 35 h of infection. In the cornea, small abscesses present at 25 h postinfection continued to produce pus, although the stromal infiltrate and edema remained mild. In the conjunctiva, inflammation remained mild to moderate. In the anterior chamber, fibrin continued to accumulate and iritis remained mild to moderate. During the extended times of infection (30 and 35 h postinfection), the overall slit lamp examination scores at only 30 h postinfection (Fig. 2) were significantly lower for the beta-toxin-deficient strains (ISP546 and DU5720) than for the strain producing substantial beta-toxin activity (DU1090) ($P \leq 0.0014$). At 30 and 35 h, the CFU

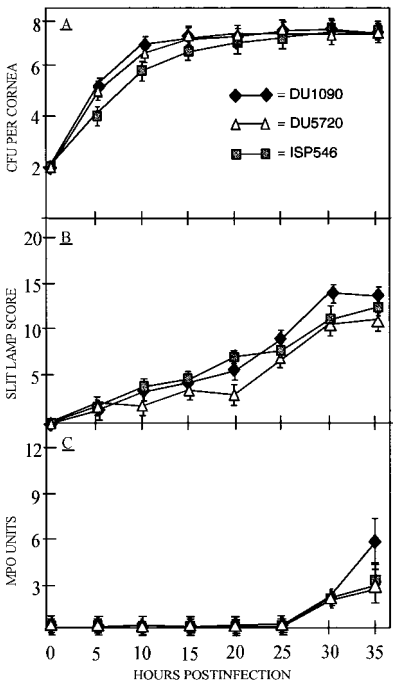

FIG. 2. Comparison of alpha-toxin-deficient strains during extended infection. Growth (log_{10} CFU per cornea) (A), slit lamp examination scores (B), and MPO activities (C) of eyes infected with *S. aureus* DU1090 (Hla^-), DU5720 (Hla^- Hlb^-), or ISP546 (Agr^-) were determined for infections extended to 35 h postinfection. Values represent the mean ± SEM (four to eight corneas per time point). (Some SEM error bars are obscured by the data points.)

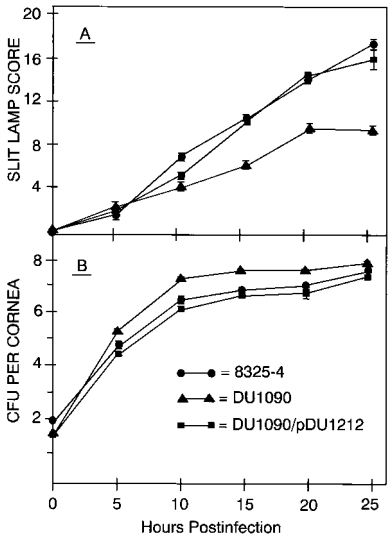

FIG. 3. Plasmid coding for alpha-toxin restores the virulence of the alpha-toxin-deficient mutant. Shown are the slit lamp examination scores (A) and growth (log_{10} CFU per cornea) (B) for eyes infected with the parent (8325-4), alpha-toxin-deficient (DU1090), or plasmid-rescued (DU1090/pDU1212) strain. Values represent the mean ± SEM (4 to 12 corneas per time point). (SEM error bars are obscured by the data points.)

per cornea (Fig. 2) and MPO activities (Fig. 2) of corneas infected with all three strains were similar ($P \geq 0.3354$ and $P \geq 0.0552$, respectively).

Restoration of corneal virulence by complementation of alpha-toxin. Infection of eyes with DU1090/pDU1212 produced slit lamp scores that were not significantly different at 25 h postinfection from those of eyes infected with parent strain 8325-4 ($P = 0.0902$) but were significantly higher than those of eyes infected with the alpha-toxin-deficient mutant DU1090 ($P \leq 0.0001$) (Fig. 3). The CFU per cornea for eyes infected with 8325-4, DU1090, or DU1090/pDU1212 were not significantly different at 25 h postinfection ($P \geq 0.2497$) (Fig. 3).

Restoration of corneal virulence by complementation of beta-toxin. Eyes infected with strains 8325-4 (parent) and DU5719/pCU1hlb had equivalent slit lamp examination scores ($P = 0.7060$) that were significantly higher than those of eyes infected with DU5719 (Hlb^-) ($P \leq 0.0090$) (Fig. 4). The CFU per cornea for eyes infected with 8325-4, DU5719, or DU5719/pCU1hlb were not significantly different at 25 h postinfection ($P \geq 0.3635$).

Relationship of ocular virulence to alpha-toxin activity. We have previously shown that the parent strain (8325-4) was significantly more virulent than its isogenic alpha-toxin-deficient mutant (DU1090), especially in terms of producing corneal epithelial erosions (13). A correlation between alpha-toxin activity and epithelial erosions was noted for all of the strains tested in this study; only strains producing alpha-toxin had epithelial erosions (Table 1).

(i) Toxicity of *S. aureus* proteins. As negative controls, heat-inactivated proteins, PBS with or without 0.2% gelatin, and BSA (10 μg) were administered to rabbit eyes topically or by intrastromal or anterior chamber injection. Application of heat-inactivated alpha-toxin, BSA, or PBS did not cause ocular inflammation. However, intrastromal injection of PBS plus 0.2% gelatin caused a diffuse cloudy precipitate at the injection site that diminished within 6 h.

The range of toxin concentrations (10 μg to 20 ng) chosen for testing in normal rabbit eyes extended from concentrations greater than that produced in culture to concentrations many-fold less than that produced in culture. The effects of purified staphylococcal proteins applied to normal rabbit eyes are summarized in Table 2.

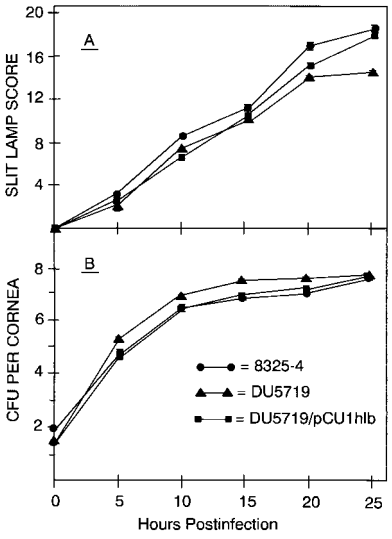

FIG. 4. Plasmid coding for beta-toxin restores the virulence of the beta-toxin-deficient mutant. Shown are the slit lamp examination scores (A) and growth (log_{10} CFU per cornea) (B) for eyes infected with the parent (8325-4), beta-toxin-deficient (DU5719), or plasmid-rescued (DU5719/pCU1hlb) strain. Values represent the mean ± SEM (6 to 18 corneas per time point). (Some SEM error bars obscured by the data points.)

TABLE 2. Effects of purified alpha-toxin and beta-toxin on normal rabbit eyes as measured by slit lamp examination[a]

Route of application	Effect(s) of protein tested[b] (dose[c] [μg])	
	Beta-toxin	Alpha-toxin
Topical drop	Mild conjunctival inflammation (2)	Moderate conjunctival inflammation and iritis (2)
Intrastromal injection	Severe scleral inflammation, moderate iritis, and conjunctival inflammation (2)	Corneal edema and epithelial sloughing, severe iritis, and moderate conjunctival inflammation (0.02)
Anterior chamber injection	Severe scleral inflammation (2)	Trace conjunctival inflammation (2); severe iritis (0.2)

[a] Slit lamp examination was used to grade seven ocular parameters, each on a scale of 0 to 4. A parameter score of 1 is described as trace, while scores of 2, 3, and 4 are described as mild, moderate, and severe, respectively.

[b] Proteins at various concentrations were prepared and diluted in PBS (for alpha-toxin) or PBS plus 0.2% gelatin (for beta-toxin). Heat-inactivated staphylococcal proteins, BSA, and diluents served as negative controls. Controls caused no ocular effect, except for PBS with 0.2% gelatin, which caused a diffuse cloudy precipitate that diminished by 6 h. The ocular changes indicated appeared within 6 h of protein administration.

[c] The lowest concentration of protein administered that caused the stated effect(s).

(ii) Alpha-toxin. Topical application of alpha-toxin resulted in significant dose-dependent increases in conjunctival and iris inflammation by 6 h; eyes receiving ≥2 μg of alpha-toxin in PBS showed mild but significant increases in conjunctival inflammation and blanching of the iris ($P \leq 0.0001$) by 6 h. Corneas intrastromally injected with purified alpha-toxin at concentrations from 0.002 to 10 μg demonstrated significant dose-dependent increases in corneal and iris inflammation from 0 to 6 h. While limited corneal epithelial sloughing was induced within 6 h by injection of 0.002 μg of alpha-toxin, corneas injected with 10 μg of alpha-toxin demonstrated sloughing of the epithelium at the injection site by 30 min, with progressive sloughing of the epithelium over the entire corneal surface by 2 h. Blanching of the iris and impairment of the light constriction reflex were evident by 2 h (Fig. 5A). These eyes also presented with mild conjunctival injection, which significantly worsened with time (from 0 to 6 h; $P < 0.0014$), and mild conjunctival edema, which remained constant over time ($P \geq 0.0604$). Anterior chamber injection of alpha-toxin resulted in significant dose-dependent increases in conjunctival and iris inflammation from 0 to 6 h. Eyes receiving ≥0.2 μg of alpha-toxin showed significant conjunctival inflammation as well as marked inflammation and subsequent blanching of the iris at 6 h ($P \leq 0.0001$). Anterior chamber injection of alpha-toxin did not induce scleral edema, corneal epithelial sloughing, or corneal edema.

(iii) Beta-toxin. Topical application of beta-toxin (≥2 μg in PBS with 0.2% gelatin) caused significant conjunctival inflammation from 0 to 6 h postinfection ($P < 0.0001$). Beta-toxin at concentrations below 2 μg did not cause inflammation. Corneas intrastromally injected with beta-toxin demonstrated significant dose-dependent increases in scleral inflammation from 0 to 6 h. Corneas injected with ≥2 μg of beta-toxin resulted in significant scleral edema by 2 h (slit lamp examination scleral edema score = 2.5 ± 0.3; $P < 0.0014$) that increased in severity with time, causing protrusion of the sclera beyond the boundary of the corneoscleral limbus and gross enlargement of the globe by 6 h (Fig. 5B). These eyes presented with mild to moderate conjunctival injection and a mild iritis, both of which remained constant over time ($P \geq 0.5000$). Also visible were mild to moderate corneal edema and diffuse cloudiness at the injection site, each of which decreased in size by 6 h and was probably a response to the gelatin used in the vehicle. Anterior chamber injection of purified beta-toxin also resulted in rapid and significant dose-dependent increases in scleral inflammation from 0 to 6 h. Eyes receiving a 10-μg injection showed by 2 h a rapid and significant increase in scleral edema, demonstrating a severe inflammation (slit lamp examination scleral edema score = 4.0 ± 0.5; $P \leq 0.0001$) and gross enlargement of the globe. Beta-toxin, when injected at concentrations below 10 μg, caused slower but significant increases in scleral inflammation, culminating in moderate to severe scleral edema by 4 h (2.0 μg) and 6 h (0.2 μg).

DISCUSSION

We have previously demonstrated the significant contribution of alpha-toxin to corneal virulence (13); however, the relative importance of other staphylococcal proteins to corneal damage required further investigation. The current study of isogenic mutants and purified toxins was designed to document the contribution of alpha-toxin to ocular damage and to identify the role of beta-toxin in keratitis. Identification of the ocular tissues subject to direct toxic action by these proteins was also sought. The staphylococcal proteins chosen for analysis have been frequently studied as virulence factors in nonocular models of infection (1, 10, 18, 19, 25, 35–37).

We have shown by the study of isogenic mutants deficient in alpha-toxin (13), by genetic rescue experiments (Fig. 3), and by the administration of purified protein (Table 2) that alpha-toxin has detrimental effects on the cornea, iris, and conjunctiva. Alpha-toxin is required during infection for destruction of the corneal epithelium (Table 1), and the administration of purified alpha-toxin has now been shown to directly destroy the epithelium (Table 2). The cornea is subject to edema as a result of alpha-toxin administration; mutants deficient in alpha-toxin cause less corneal edema than their isogenic parent strains.

Administration of alpha-toxin to the eye resulted in damage to the iris that was dose dependent and severe at high concentrations. Alpha-toxin caused the iris to lose the light constriction reflex reaction and to blanch to such a degree that the tissue resembled that seen previously only in rabbits after death. Infection with a mutant deficient in alpha-toxin resulted in less iritis and conjunctival edema than infection with an isogenic strain producing alpha-toxin (13). The action on multiple ocular tissues is consistent with the finding that alpha-toxin targets several types of cells, forming pores that cause osmotic lysis and cell death (39). Alpha-toxin injection into the cornea was more efficient in causing damage to the iris than alpha-toxin injection into the anterior chamber. After intrastromal injection, alpha-toxin could have leaked gradually into the anterior chamber, supplying toxin to iris tissue for an extended time and inducing progressive iritis and corneal damage. In contrast, a single injection of alpha-toxin into the anterior chamber could result in toxin being removed quickly via aqueous humor exchange. This finding suggests that prolonged exposure to alpha-toxin is necessary to cause an observable effect on the iris. Overall, these results illustrate the potency of

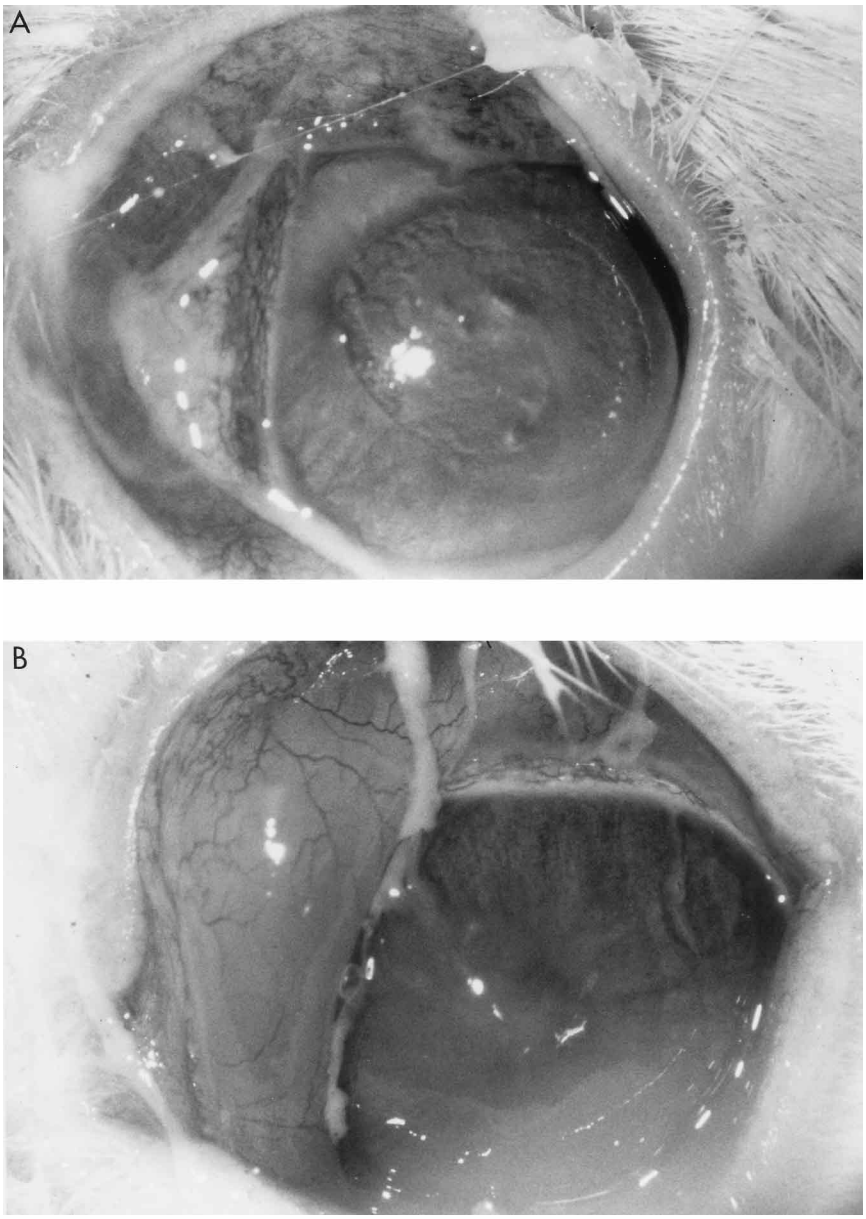

FIG. 5. Toxicity of purified beta-toxin and alpha-toxin in the rabbit eye at 6 h. Purified alpha-toxin (1 μg) was injected intrastromally (A), causing epithelial sloughing over the injection site as early as 30 min. Epithelial damage progressed over time, resulting in sloughing of epithelial cells over the entire corneal surface by 2 h and blanching of the iris by 4 h. Purified beta-toxin (10 μg) was injected intrastromally (B), causing significant scleral edema by 2 h and enlargement of the globe by 6 h.

alpha-toxin and demonstrate both its cytocidal and inflammatory activities.

The analysis of beta-toxin-deficient mutants (Fig. 1 and 4) and the effects of this protein following its administration to the eye (Table 2) demonstrate that beta-toxin can mediate edema in the sclera and conjunctiva. The introduction of a plasmid coding for beta-toxin restored the ocular virulence of the beta-toxin-deficient mutant (Fig. 4), confirming the role of beta-toxin in these infections. Infections with Hlb^- mutants did not cause the scleral edema that was observed late in infections caused by the isogenic parent strain. Consistent with this observation was the rapid and dramatic edematous reaction of the sclera induced by injection of beta-toxin at high concentrations (≥2 μg) into the cornea and anterior chamber (Fig. 5). This finding was also consistent with the known sphingomyelinase activity of beta-toxin (6, 7) and the high sphingomyelin content of the scleral epithelial cell membrane (11).

The extent of scleral inflammation was far greater after beta-toxin injection into the cornea or anterior chamber than after topical administration or infection with beta-toxin-producing strains. This difference could reflect both the rapid removal of solutes by the tear film and the ability of solutes to penetrate the sclera from its internal surface (40). The injection of beta-toxin demonstrated the ability of this toxin to cause severe scleral edema (Fig. 5) and confirmed the findings obtained by the analysis of mutants which showed a relationship between scleral edema and beta-toxin production (Table 2). The difference in the extent of scleral edema noted during

infection and that seen following the injection of purified toxin is probably due to the higher concentrations of toxin in the purified preparations which induced massive edema.

The reduced virulence of the Agr$^-$ mutant (ISP546) is consistent with the finding that alpha-toxin is a major virulence factor and a prime mediator of damage. The comparison of the alpha-toxin-deficient strain and the Agr$^-$ mutant during an extended period of infection indicated that the Agr$^-$ mutant was less virulent than the alpha-toxin-deficient strain. This finding suggests that one or more proteins, other than alpha-toxin and regulated by the Agr system, also contributed to virulence. The similarity between the Agr$^-$ mutant and the mutant deficient in both alpha-toxin and beta-toxin suggests that beta-toxin also contributes to corneal virulence. Beta-toxin could account for a significant portion of the virulence difference noted between the Agr$^-$ mutant and the alpha-toxin-deficient mutant. Beta-toxin expression is known to be significantly reduced, although not totally eliminated, by loss of a functional Agr$^-$ system (29).

The concept emerging from the current study of *S. aureus* in the intrastromal rabbit model of infection is one of a bacterium growing to stationary phase and then releasing potent exotoxins. The studies in this model indicate that alpha-toxin and, to a lesser degree, beta-toxin mediate a significant portion of the ocular changes through tissue-specific interactions. Alpha-toxin affected primarily the cornea and iris, while beta-toxin affected primarily scleral tissue. The roles of many potentially damaging proteins, including proteases, lipases, leukocidin, and exfolatin, have not yet been tested in an ocular model. Most staphylococcal products, including alpha-toxin and beta-toxin, are regulated by the Agr system, a proposed virulence determinant in itself. Our results have identified two bacterial proteins (alpha-toxin and beta-toxin) which could be targeted for a new type of chemotherapy designed to limit the ocular damage caused by these toxins. Such therapy, administered in conjunction with antibiotics, could act either by directly inhibiting these toxins or by preventing the expression of the toxins by inhibiting the Agr system, as proposed by Balaban and Novick (5). This combined therapy could reduce the major tissue damage and scarring reactions associated with *Staphylococcus* keratitis.

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