

# Chemical Inhibition of Alpha-Toxin, a Key Corneal Virulence Factor of *Staphylococcus aureus*

Clare C. McCormick, Armando R. Caballero, Charles L. Balzli, Aihua Tang, and Richard J. O'Callaghan

**PURPOSE.**  $\alpha$ -Toxin mediates extreme corneal damage during *Staphylococcus aureus* keratitis. Chemical inhibition of this toxin was sought to provide relief from toxin-mediated pathology.

**METHODS.** Inhibition of  $\alpha$ -toxin by phosphate-buffered saline (PBS), 0.1% methyl- $\beta$ -cyclodextrin (CD), or CD plus cholesterol (0.1%, CD-cholesterol) was assayed by hemolysis of rabbit erythrocytes. Pathologic changes in rabbit corneas injected with 12 hemolytic units of  $\alpha$ -toxin suspended in PBS, 1% CD, or 1% CD-cholesterol were compared over time. Rabbit corneas injected with  $10^2$  colony forming units (CFU) of *S. aureus* were treated from 7 to 13 hours postinfection (PI) with a total of 15 drops of CD-cholesterol, CD, or PBS. Slit lamp examination (SLE) and measurement of erosions were performed at 13 hours PI and bacteria were quantified at 14 hours PI.

**RESULTS.** Toxin-mediated lysis of erythrocytes was inhibited up to 16,000-fold in the presence of CD-cholesterol compared with CD or PBS. Eyes injected with  $\alpha$ -toxin mixed with CD-cholesterol had, at 7 hours postinjection, significantly smaller erosions than eyes injected with  $\alpha$ -toxin in PBS or  $\alpha$ -toxin mixed with CD ( $P = 0.0090$  and  $P = 0.0035$ , respectively). Eyes infected with *S. aureus* and treated with CD-cholesterol had significantly lower SLE scores than eyes treated with CD or PBS ( $P \leq 0.0103$  and  $P \leq 0.0017$ , respectively); however, there were no differences in the number of bacteria present ( $P \geq 0.0648$ ).

**CONCLUSIONS.** CD-cholesterol is a potent inhibitor of  $\alpha$ -toxin activity in vitro and an effective means to arrest corneal damage during *S. aureus* keratitis. (*Invest Ophthalmol Vis Sci*. 2009;50:2848-2854) DOI:10.1167/iov.08-3157

A gram-positive coccus, *Staphylococcus aureus*, is one of the most important human pathogens. *S. aureus* causes a variety of infections with high morbidity and mortality including osteomyelitis, soft tissue infections, pneumonia, endocarditis, and brain abscesses.<sup>1-4</sup> In the United States, there are approximately 292,000 hospital admissions of *S. aureus* infection annually.<sup>5</sup> A survey showed that 19,000 people died in 2006 from *S. aureus* infections.<sup>6</sup> To further intensify the scope of the problem, the rate of methicillin-resistant *S. aureus* iso-

lates found both in the community and in hospitals is continuing to increase.<sup>5,7-9</sup>

*S. aureus* is also a major cause of ocular infections, including blepharitis, conjunctivitis, keratitis, and endophthalmitis.<sup>10-12</sup> Patients with these infections have intense pain, redness, and photophobia, and the infection can result in an ulcer with numerous infiltrating polymorphonuclear leukocytes.<sup>10</sup> These infections can result in loss of visual acuity or blindness. Intensive antibiotic therapy can eradicate *S. aureus* from the infected site, but pathologic changes can continue due to action of toxic proteins that are secreted by *S. aureus*.<sup>13</sup> The appearance of an infected eye may not be a good measure of the success of antibiotic treatment because the activity of the secreted toxins is not directly affected by the antibiotic-mediated killing of the bacteria.

An extremely important *S. aureus* protein that mediates ocular tissue damage is  $\alpha$ -toxin.<sup>14-16</sup>  $\alpha$ -Toxin has also been proven to have an important role in brain and respiratory infections caused by *S. aureus* and is being proposed as a vaccine target for humans.<sup>17-20</sup>  $\alpha$ -Toxin is a 33 kDa protein that forms a ring of seven  $\alpha$ -toxin molecules that penetrate the host cell membrane forming a pore, causing cell lysis.<sup>21</sup>  $\alpha$ -Toxin has also been demonstrated to cause cellular changes in human cells, upregulate inflammatory cytokines, cause calcium disruptions reducing host defenses, and cause apoptosis.<sup>22-27</sup> Furthermore,  $\alpha$ -toxin has been shown to be an important virulence factor in both rabbit and murine models of keratitis.<sup>15,16,28,29</sup> When the  $\alpha$ -toxin gene was mutated, the pathology associated with the infection was significantly reduced compared to the parent strain.<sup>15</sup> However, when the mutated  $\alpha$ -toxin gene in the knockout strain was complemented, the pathology was restored.<sup>15</sup>

To date, there are no known inhibitors of  $\alpha$ -toxin available to treat *S. aureus* infection, including ocular infections. There have been efforts to develop inhibitors of this important virulence factor including the use of steroids or steroid-like molecules.<sup>30,31</sup> Pany et al.<sup>32</sup> demonstrated that  $\alpha$ -toxin binds to caveolin, a protein present in lipid rafts.  $\alpha$ -Toxin has a caveolin-1 binding motif that, when removed, results in a nonhemolytic form of  $\alpha$ -toxin.<sup>32</sup> Peptides that mimic the caveolin-binding domain of  $\alpha$ -toxin have been shown to limit the action of  $\alpha$ -toxin on cell membranes.<sup>33</sup> Gu et al.<sup>34</sup> showed that methyl- $\beta$ -cyclodextrin (CD) can bind weakly to the pore of  $\alpha$ -toxin heptamer. On introducing amino acid changes in the  $\alpha$ -toxin, Gu et al.<sup>34</sup> were able to increase the binding of CD. Furthermore, Vijayvargia et al.<sup>35</sup> observed that cholesterol depletion of cells arrested the action of  $\alpha$ -toxin and that chelation of cholesterol, using CD, helped retard the pore formation by  $\alpha$ -toxin. Karginov et al.<sup>36</sup> have demonstrated that  $\alpha$ -toxin action can be inhibited by positively charged side groups chemically attached to CD.

The studies presented herein test the hypothesis that a complex of cholesterol and CD (CD-cholesterol) can inhibit the action of  $\alpha$ -toxin. The results show that CD-cholesterol complex can inhibit  $\alpha$ -toxin action in vitro and in vivo.

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Supported by NEI Grant EY010974 and by donations for research from Alcon Laboratories, Inc.

Submitted for publication November 13, 2008; revised December 9, 2008; accepted March 31, 2009.

Disclosure: C.C. McCormick, None; A.R. Caballero, None; C.L. Balzli, None; A. Tang, None; R.J. O'Callaghan, None

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## MATERIALS AND METHODS

### Bacteria

*Staphylococcus aureus* strain 8325-4 is a well-characterized laboratory strain that has been used in numerous studies of experimental keratitis in both rabbit and murine models.<sup>14-16,28,29</sup> All *S. aureus* isolates (Table 1) were grown at 37°C on tryptic soy agar (TSA, Becton Dickinson and Co., Sparks, MD) and sub-cultured in tryptic soy broth (TSB, Becton Dickinson and Co.) at 37°C for 18 hours.

### Animals

New Zealand White rabbits ( $n \geq 4$  eyes per group) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). All animals were specific-pathogen free and were maintained according to institutional guidelines and tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Before any procedure was performed, each rabbit was anesthetized as previously described.<sup>14-16,37-39</sup>

### Hemolysis Assays

Freshly collected rabbit erythrocytes were centrifuged to a pellet and resuspended in 10 mM phosphate-buffered saline (PBS; pH 7.2) with 0.02% gelatin; this washing procedure was repeated three times. Washed erythrocytes were diluted to a concentration of  $10^8$  cells per mL. Purified  $\alpha$ -toxin (Sigma-Aldrich, St. Louis, MO) was serially diluted twofold in microtiter plates, rabbit erythrocytes ( $10^7$  per well) were added to the  $\alpha$ -toxin dilutions, and the plates were incubated at 37°C until the erythrocytes in the control lacking  $\alpha$ -toxin had settled. The highest dilution producing red cell lysis was considered the end point. One hemolytic unit was equivalent to an  $\alpha$ -toxin dilution that lysed erythrocytes but could not cause lysis on further dilution.

For inhibition of hemolysis, supernatants of overnight cultures or purified  $\alpha$ -toxin samples were serially diluted twofold in a fashion similar to that described above for assays performed using  $\alpha$ -toxin. Erythrocytes ( $10^7$  per well) were mixed at 4°C with PBS, 0.1% CD (Sigma), or 0.1% CD-cholesterol (Sigma) and then added to dilutions of the culture supernatant or purified toxin in microtiter plates. The dilutions producing lysis were observed after 30 minutes. All hemolysis inhibition assays were performed in duplicate or triplicate and were repeated twice.

### Intrastromal Injection of $\alpha$ -Toxin

$\alpha$ -Toxin (12 hemolytic units) was injected into the stroma of rabbit corneas ( $n \geq 4$  eyes) to evaluate the subsequent development of pathologic changes at 7 hours after toxin injection.  $\alpha$ -Toxin in PBS or  $\alpha$ -toxin in 1% CD, or 1% CD-cholesterol was incubated for 30 minutes at 4°C then injected into rabbit corneas ( $n \geq 4$  per group). The pathologic changes were observed and the diameter of erosions was measured at 2, 5, and 7 hours postinjection using fluorescein strips

(Fluorets, Aubenas, France). By 7 hours after injection, the epithelial erosions in eyes injected with  $\alpha$ -toxin in PBS reached their maximal diameter. Heat-inactivated  $\alpha$ -toxin ( $n = 4$  eyes) served as a negative control.

### Experimental Keratitis

Rabbits ( $n \geq 4$  eyes per group) were infected as previously described, injecting approximately 100 colony forming units (CFU) of strain 8325-4 or strain 60171, a clinical isolate, in 10  $\mu$ L of TSB into the stroma of the cornea.<sup>14,16,37</sup> Accuracy of the inoculum size was verified by plating aliquots (100  $\mu$ L) of serial dilutions in triplicate on TSA.

### Treatment of Keratitis

At 7 hours postinfection (PI), treatment began and consisted of a single topical drop of PBS, 1% CD, or 1% CD-cholesterol every 15 minutes from 7 to 8 hours PI. Then, beginning at 8.5 hours PI, a single drop was administered every 30 minutes until 13 hours PI; a total of 15 drops per eye. At 13 hours PI, corneal erosions were measured and all eyes underwent slit lamp examination (SLE) to quantify pathologic changes.

### Slit Lamp Examination

SLE of pathologic changes of rabbit eyes was performed by two masked observers as described previously.<sup>14-16,37-39</sup>

### Colony Forming Unit Determination

Corneas of rabbits infected with either *S. aureus* 8325-4 or 60171 were harvested at 14 hours PI and bacteria were quantified as described previously.<sup>14-16,37-39</sup>

### Statistics

Statistical analyses were performed using statistical analysis software (SAS [Cary, NC] or Microsoft Excel [Seattle, WA]) as previously described.<sup>14-16,28,29,37-39</sup>

## RESULTS

### In Vitro Inhibition of $\alpha$ -Toxin

To test whether CD or CD-cholesterol could inhibit the action of  $\alpha$ -toxin, aliquots of erythrocytes were mixed with PBS, 0.1% CD, or 0.1% CD-cholesterol and then added to serial dilutions of purified  $\alpha$ -toxin. The  $\alpha$ -toxin titer for the sample in PBS was approximately 4096 (Fig. 1A). The  $\alpha$ -toxin titer for the mixture with CD was reduced only twofold compared with the mixture with only PBS (Fig. 1B). In contrast, the hemolysis titer of the same amount of  $\alpha$ -toxin incubated with CD-cholesterol was approximately 4 (Fig. 1C), a reduction in titer of 500-fold.

A higher concentration of  $\alpha$ -toxin was tested for inhibition using 0.1% CD-cholesterol. The hemolytic titer of the  $\alpha$ -toxin in PBS was 65,536 (Fig. 1D), but when the  $\alpha$ -toxin preparation was incubated with CD-cholesterol the titer was reduced to 4, approximately a 16,000-fold decrease in hemolytic activity.

The ability of CD-cholesterol to inhibit the hemolytic activity of  $\alpha$ -toxin in culture supernatants of multiple *S. aureus* strains was then tested. Rabbit erythrocytes incubated with CD-cholesterol or PBS were added to serially diluted culture supernatants of six *S. aureus* strains. For all six strains tested, at least a fourfold inhibition of hemolytic activity was observed (Table 1).

### In Vivo Inhibition of $\alpha$ -Toxin

Because CD-cholesterol inhibits hemolysis of rabbit erythrocytes by  $\alpha$ -toxin, there was interest in determining if this inhibitor could limit  $\alpha$ -toxin effects on the rabbit cornea.  $\alpha$ -Toxin (12 hemolytic units) was incubated with PBS, 1% CD, or 1% CD-cholesterol for 30 minutes at 4°C. After incubation,

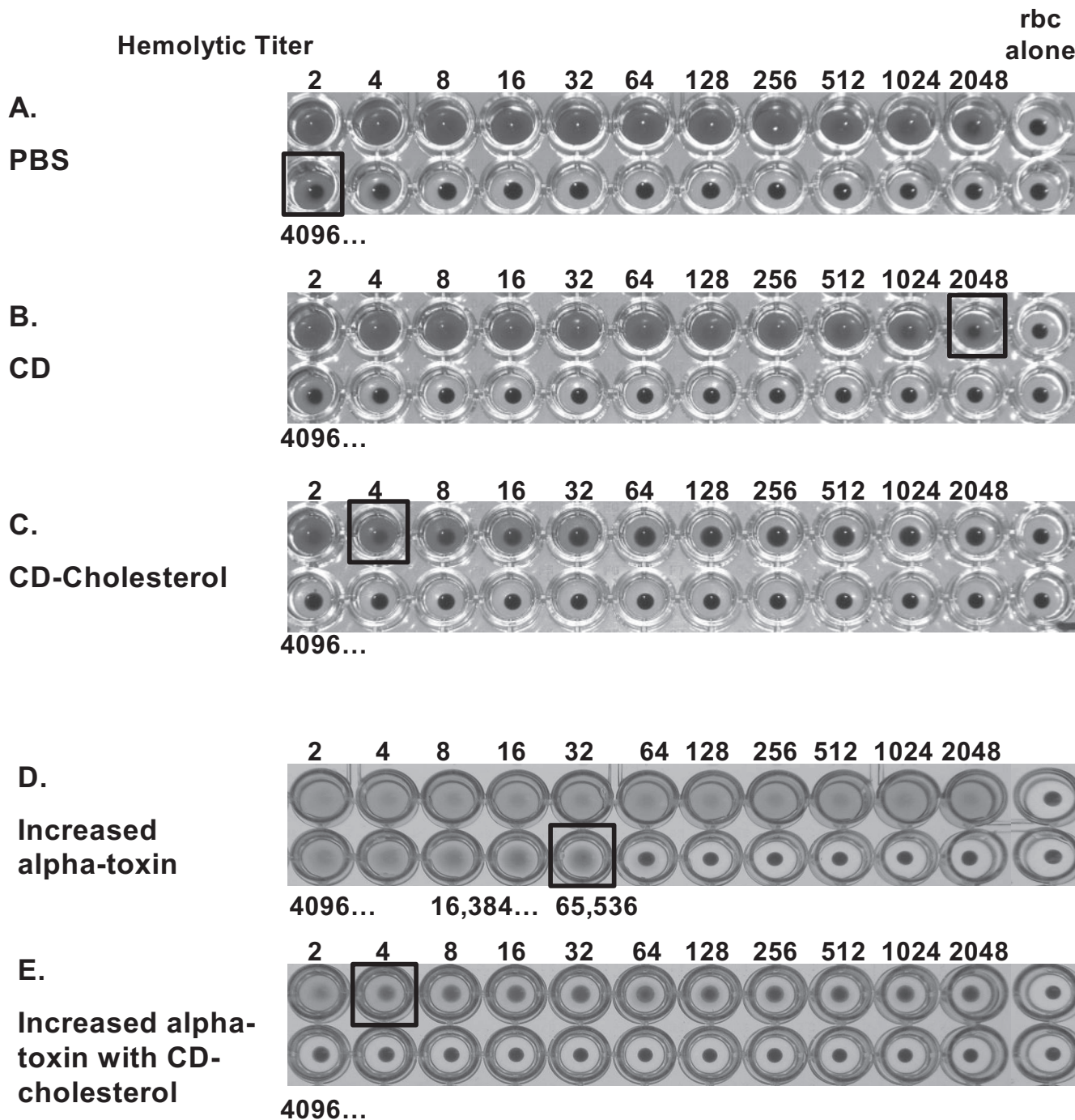
TABLE 1: In Vitro Analysis of CD-Cholesterol Inhibition of Alpha-Toxin Activity

Strain	Alpha-Toxin Titer	CD-Cholesterol Titer*	Fold Reduction
8325-4†	2048	0	2048
70490‡	32	8	4
60171‡	128	4	32
30084‡	16	0	16
30281‡	64	16	4
30155‡	128	16	8

\* Residual hemolysis can, to a limited degree, be mediated by a hemolysin other than alpha-toxin (e.g., gamma-toxin).

† Gift from Timothy Foster, University of Dublin, Dublin, Ireland.

‡ Gift from David Stroman, Alcon Laboratories, Fort Worth, TX.



**FIGURE 1.** In vitro analysis of  $\alpha$ -toxin inhibition with 0.1% CD-cholesterol, or 0.1% CD. Rabbit erythrocytes ( $10^8$  per mL) were either incubated with 0.1% CD-cholesterol, 0.1% CD, or untreated for 30 minutes at 4°C. Serial dilutions (1:2) of  $\alpha$ -toxin were made in microtiter plates. Aliquots of erythrocytes were added to each well ( $10^7$  per well). Untreated erythrocytes were suspended in PBS with 0.02% gelatin to serve as a negative control. (A) Untreated erythrocytes were incubated with  $\alpha$ -toxin. The hemolytic titer of the  $\alpha$ -toxin was 4096. (B) Erythrocytes treated with 0.1% CD did not demonstrate a significant reduction in  $\alpha$ -toxin titer compared with the negative control, a two-fold reduction. (C) The hemolytic titer of  $\alpha$ -toxin was reduced from 4096 to 4 when the red blood cells were incubated with 0.1% CD-cholesterol, a reduction of approximately 500-fold. (D) Erythrocytes were incubated with  $\alpha$ -toxin at an increased concentration. The hemolytic titer was 65,536. (E) Erythrocytes were incubated with 0.1% CD-cholesterol, but with an increased concentration of  $\alpha$ -toxin. The hemolytic titer was reduced from 65,536 to 4, a reduction of approximately 16,000.

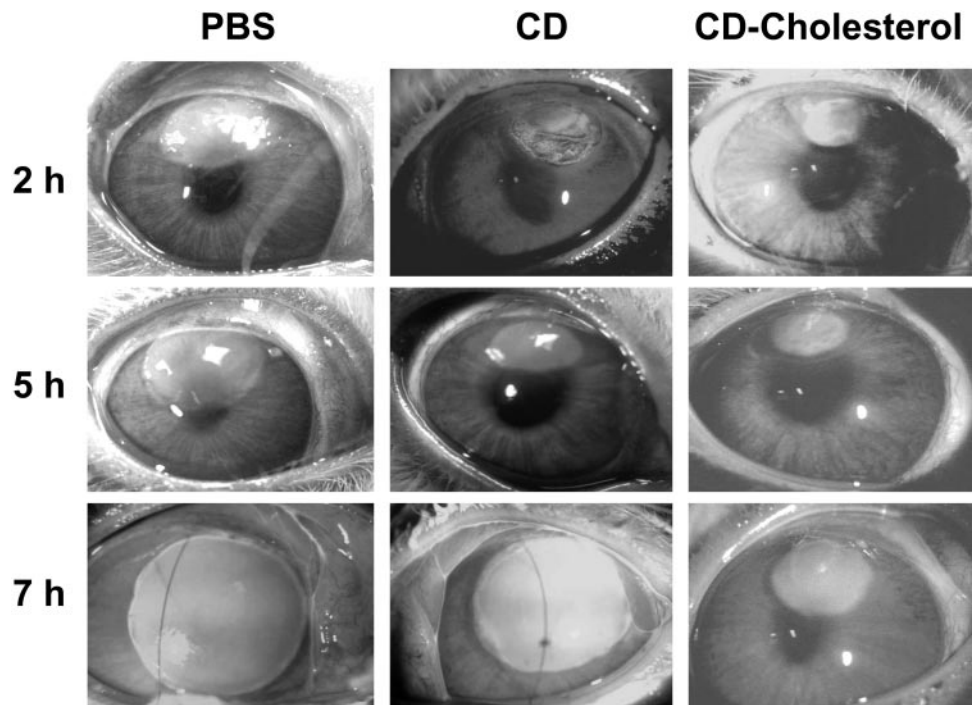
each mixture was injected intrastromally into rabbit corneas and the size of epithelial erosion was measured at 2, 5, and 7 hours, the latter being the time of maximal erosion diameter (Fig. 2).

The corneal erosion sizes at all times studied for the eyes receiving toxin mixed with CD-cholesterol were significantly

smaller than that of eyes receiving toxin mixed with PBS ( $P \leq 0.0165$ ; Table 2).

The erosion sizes of eyes injected with toxin mixed with CD alone were significantly smaller than that of eyes injected with toxin mixed with PBS at 2 hours postinjection ( $P = 0.0131$ ), but not at 5 or 7 hours postinjection ( $P \geq 0.1977$ ).





**FIGURE 2.** Photographs of rabbit corneas injected with  $\alpha$ -toxin. Rabbit corneas ( $n \geq 4$  corneas per group) were injected with 12 hemolytic units of  $\alpha$ -toxin suspended in 1% CD-cholesterol, 1% CD, or PBS. At 2, 5, and 7 hours postinjection, erosions were stained with fluorescein and measured. Eyes injected with  $\alpha$ -toxin suspended in CD-cholesterol had smaller erosions compared with  $\alpha$ -toxin-injected eyes. CD afforded some protection from large erosion formation for a short time, but by 5 hours postinjection the CD failed to protect the cornea and the erosion formation was similar to the erosions diameters of the eyes injected with  $\alpha$ -toxin mixed with PBS.

The corneal erosion sizes of eyes injected with toxin mixed with CD-cholesterol were significantly smaller than that of eyes injected with toxin mixed with CD alone at 7 hours postinjection ( $P = 0.0048$ ), but not at 2 or 5 hours postinjection ( $P \geq 0.1173$ ).

### In Vivo Treatment of *S. aureus* Keratitis

The studies described above demonstrate that the lytic effects of  $\alpha$ -toxin on erythrocytes and the effects of purified toxin on rabbit corneas can be inhibited by CD-cholesterol. These findings raised the possibility that CD-cholesterol could treat corneas infected with *S. aureus*. Rabbit corneas were injected with either *S. aureus* strain 8325-4 or *S. aureus* clinical isolate 60171. Rabbit eyes were treated from 7 to 13 hours PI with PBS, 1% CD, or 1% CD-cholesterol. At 13 hours PI, the rabbits were observed for pathologic changes, and erosion formation (Fig. 2B). At 14 hours PI, the rabbits were euthanized and the corneal tissue was harvested to determine the number of bacteria present.

At 13 hours PI, *S. aureus* strain 8325-4 infected eyes were evaluated by SLE to quantify pathologic changes (Fig. 3). Eyes treated with PBS had an average SLE score of  $9.94 \pm 0.37$  (Fig. 4A). The eyes treated with CD had an average SLE score of  $9.23 \pm 0.39$ , which was statistically similar to the PBS treated eyes ( $P = 0.2191$ ). In contrast, eyes treated with CD-cholesterol had a significantly lower SLE score of  $6.35 \pm 0.28$  ( $P < 0.0001$ ). The SLE score of the rabbits treated with CD-choles-

terol was significantly lower than the SLE score of the eyes treated with CD ( $P = 0.0002$ ).

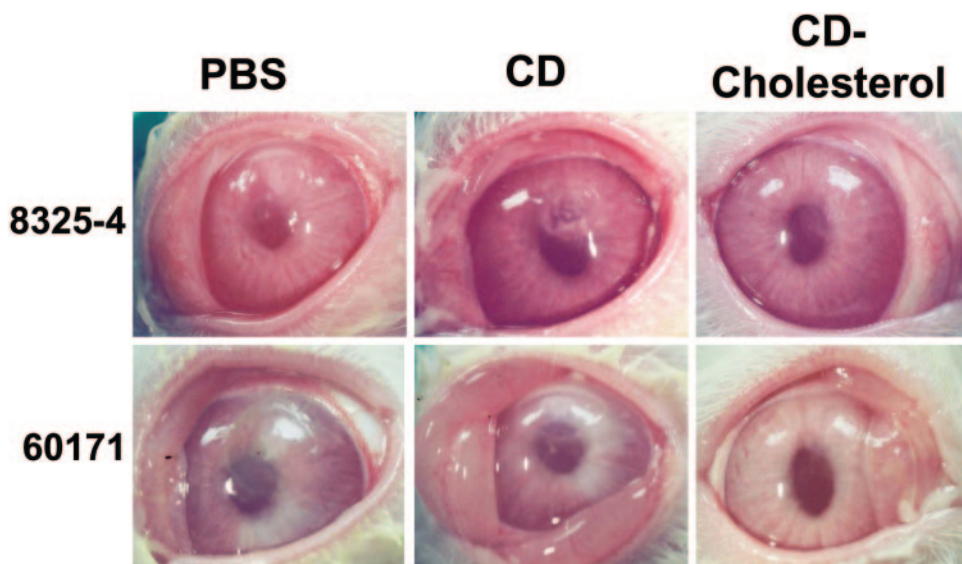
At 13 hours PI, eyes infected with *S. aureus* strain 8325-4 and treated with PBS developed erosions of  $5.89 \pm 0.36$  mm in diameter (Fig. 4B). Eyes treated with CD had corneal erosions of  $4.55 \pm 0.28$  mm, a size significantly smaller than that of eyes treated with PBS ( $P = 0.0103$ ). Eyes treated with CD-cholesterol had corneal erosions measuring  $4.25 \pm 0.20$  mm in diameter, a size also significantly smaller than the PBS-treated eyes ( $5.89 \pm 0.36$ ;  $P = 0.0017$ ). The eyes treated with CD-cholesterol or CD had similar erosion sizes ( $P = 0.4002$ ).

The number of surviving *S. aureus* 8325-4 was determined at 14 hours PI. The PBS treated eyes had  $1.12 \times 10^7 \pm 3.66 \times 10^6$  bacteria per cornea. The eyes treated with CD-cholesterol or CD had  $5.04 \times 10^6 \pm 1.56 \times 10^6$  or  $9.36 \times 10^6 \pm 4.22 \times 10^5$  bacteria per cornea, respectively, which was not significantly different from the number of bacteria per cornea in the eyes treated with PBS ( $P \geq 0.1941$ ). The eyes treated with CD-cholesterol and CD had statistically similar numbers of bacteria ( $P = 0.0648$ ).

Strain 60171, an ocular clinical isolate, was injected into the corneas of rabbits to determine the effectiveness of treatments with CD-cholesterol, CD, or PBS (Fig. 3). As described above, the pathologic changes and erosion development were observed via SLE at 13 hours PI (Fig. 2B) whereas the number of surviving bacteria was determined at 14 hours PI.

**TABLE 2:** Erosion Diameters of Rabbit Injected with Alpha-Toxin Mixed with 1% CD-cholesterol, 1% CD, or PBS

Time Postinjection	Erosion Diameter (mm $\pm$ SEM)			P-value		
	CD-cholesterol	CD	PBS	CD-cholesterol vs. CD	CD-cholesterol vs. PBS	CD vs. PBS
2 hours	$1.75 \pm 1.03$	$2.40 \pm 1.22$	$6.20 \pm 0.35$	0.6925	0.0165	0.0131
5 hours	$3.00 \pm 0.71$	$5.30 \pm 1.16$	$7.05 \pm 0.50$	0.1173	0.0028	0.1977
7 hours	$3.88 \pm 0.66$	$8.10 \pm 0.23$	$7.64 \pm 0.33$	0.0048	0.0041	0.2984

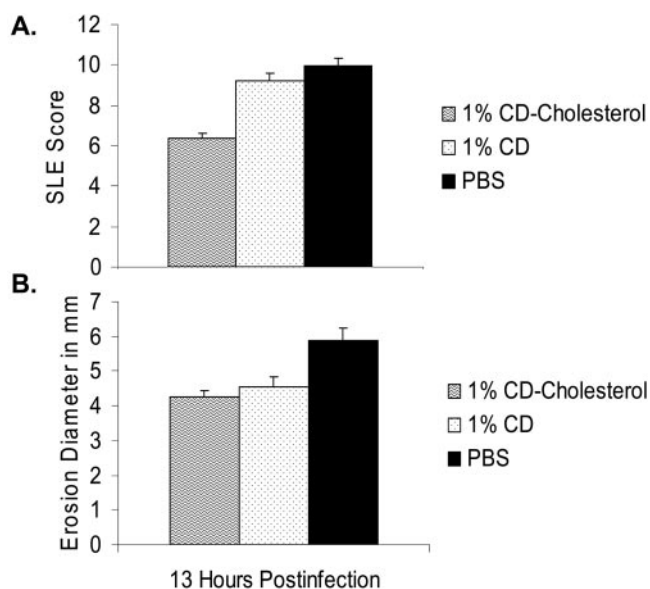


**FIGURE 3.** Photographs of rabbit corneas infected with *S. aureus* keratitis treated with 1% CD-cholesterol, 1% CD, or PBS. Rabbit corneas were injected with  $10^2$  CFU of *S. aureus* 8325-4 or 60171. Treatments began at 7 hours PI until 13 hours PI. A single topical drop was applied every 15 minutes between 7 and 8 hours PI. Starting at 8.5 hours, drops were applied every 30 minutes until 13 hours PI. At 13 hours PI, all eyes underwent SLE scoring and photographs were taken to record the degree of pathologic change.

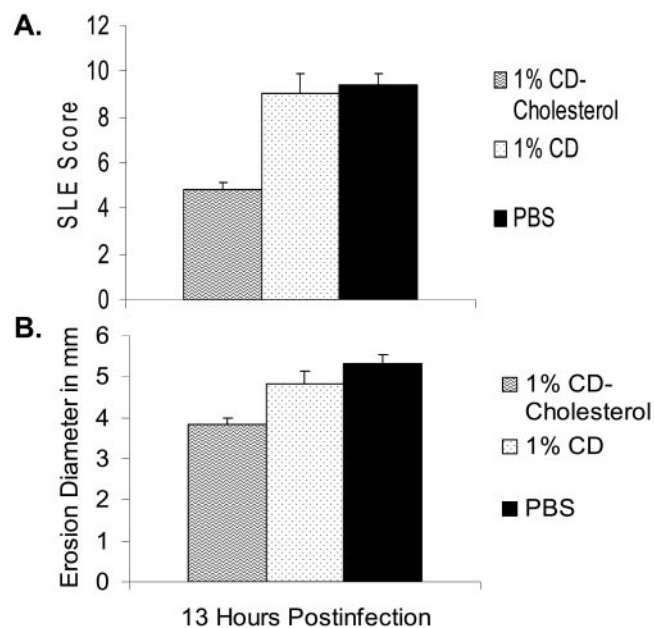
Eyes treated with PBS had an average SLE score of  $9.38 \pm 0.53$  (Fig. 5A). The eyes treated with CD had an average SLE score of  $9.02 \pm 0.90$ , a score which was statistically similar to

the eyes treated with PBS ( $P = 0.7403$ ). In contrast, eyes treated with CD-cholesterol had reduced pathology compared with PBS treated eyes; the average SLE score of CD-cholesterol treated eyes was  $4.83 \pm 0.30$ , a value which was significantly lower than the eyes treated with PBS ( $P < 0.0001$ ). Furthermore, the eyes treated with CD-cholesterol had statistically lower average SLE scores than the eyes treated with CD ( $P = 0.0013$ ).

The diameter of corneal epithelial erosions of eyes infected with 60171 and treated with PBS was  $5.33 \pm 0.21$  mm (Fig.



**FIGURE 4.** Effect of CD-cholesterol treatment of eyes infected with strain 8325-4. Rabbit corneas were injected with  $10^2$  CFU of *S. aureus* 8325-4. Eyes were randomized and treated with 1% CD-cholesterol, 1% CD, or PBS from 7 to 13 hours PI. At 7 hours PI, eyes were treated with a single topical drop every 15 minutes from 7 to 8 hours PI. At 8.5 hours PI, eyes were treated with a single topical drop every 30 minutes until 13 hours PI (a total of 15 drops). At 13 hours PI, the eyes were evaluated by SLE (A) and erosion diameters were measured using fluorescein strips (B). The pathologic changes were observed then averaged and statistical comparisons were performed using one-way ANOVA. The erosions were averaged and statistical comparisons using analysis of variance and Student's *t*-test were performed. Error bars are the SEM.  $P \leq 0.05$ , for either test, was considered significant. (A) Eyes treated with CD-cholesterol had a significantly lower SLE score than either the eyes treated with 1% CD or with PBS ( $P = 0.0002$  and  $P < 0.0001$ , respectively). The eyes treated with 1% CD or with PBS had statistically similar SLE scores ( $P = 0.2191$ ). (B) The eyes treated with 1% CD-cholesterol or 1% CD had significantly smaller erosions than the PBS treated eyes ( $P = 0.0017$  and  $P = 0.0103$ , respectively). The eyes treated with 1% CD-cholesterol or 1% CD had erosion diameters similar to one another ( $P = 0.4002$ ).



**FIGURE 5.** Effect of CD-cholesterol treatment of eyes infected with strain 60171. Eyes were treated and evaluated as described for Figure 3. Error bars are the SEM. (A) Eyes treated with CD-cholesterol had significantly lower SLE scores than either the eyes treated with 1% CD or PBS ( $P = 0.0013$ , or  $P < 0.0001$ , respectively). In contrast, the CD-treated eyes had similar SLE scores compared to the PBS-treated eyes ( $P = 0.7403$ ). (B) The eyes treated with 1% CD-cholesterol had significantly smaller erosions than either the eyes treated with 1% CD alone or the eyes treated with PBS ( $P = 0.0220$  and  $P = 0.0003$ , respectively). There was no statistical difference between the eyes treated with 1% CD and the eyes treated with PBS ( $P = 0.2131$ ).

5B). The diameter of corneal erosions in eyes treated with CD was  $4.83 \pm 0.31$  mm, a size statistically similar to eyes treated with PBS ( $P = 0.2131$ ). In contrast, eyes treated with CD-cholesterol had significantly smaller corneal erosions measuring  $3.83 \pm 0.17$  mm than either the PBS-treated or CD-treated eyes ( $P = 0.0003$  or  $P = 0.0220$ , respectively).

At 14 hours PI, the corneas were harvested to obtain the number of CFU present in the corneas. There were  $1.48 \times 10^7 \pm 2.55 \times 10^6$  bacteria in the corneas of the eyes treated with PBS. In the eyes treated with CD, there were  $2.42 \times 10^7 \pm 8.96 \times 10^6$  bacteria, which is not significantly different from the eyes treated with PBS ( $P = 0.4687$ ). There were  $8.78 \times 10^6 \pm 1.61 \times 10^6$  bacteria found in eyes treated with CD-cholesterol, which was similar to the numbers of bacteria found in the eyes treated with CD or with PBS ( $P \geq 0.1017$ ).

## DISCUSSION

The present study demonstrates that CD-cholesterol is an effective inhibitor of  $\alpha$ -toxin that can block the lytic action on erythrocytes and, more importantly, protect the cornea and surrounding tissues from toxin action during infection or when toxin is injected into the cornea. CD-cholesterol limited toxin action on infected eyes, but did not reduce bacterial growth or kill infecting bacteria. This implies that CD-cholesterol inhibition of  $\alpha$ -toxin achieved an effective therapy of the infected eyes despite the continued growth of bacteria and the continuing production of  $\alpha$ -toxin. CD-cholesterol inhibition of  $\alpha$ -toxin was not strain specific; that is, the inhibitor was active for commercial toxin as well as toxin produced by a variety of strains, including ocular clinical isolates (Table 1).

The mechanism by which CD-cholesterol inhibits  $\alpha$ -toxin has not yet been demonstrated. The effectiveness of cholesterol as an inhibitor of the toxin is not unexpected, because Raff et al.<sup>31</sup> previously demonstrated that  $\alpha$ -toxin activity was weakly inhibited by a high concentration of hydrocortisone or methylprednisolone. It has been determined that caveolin, as found in lipid rafts, is an important glucocorticosteroid receptor that could bind molecules like methylprednisolone, hydrocortisone, or, in the present study, cholesterol.<sup>40</sup> Pany et al.<sup>32</sup> and Vijayvargia et al.<sup>33,35</sup> have demonstrated that  $\alpha$ -toxin activity is dependent on caveolin-1. Vijayvargia et al.<sup>35</sup> have also demonstrated that  $\alpha$ -toxin activity on cells can be delayed by sequestering cholesterol, or inhibited altogether when cholesterol is depleted from cells. Therefore, a possible mechanism for the effectiveness of CD-cholesterol as an inhibitor of  $\alpha$ -toxin could be that CD-cholesterol competes for the same cellular target as  $\alpha$ -toxin. This competitive binding to the cellular target molecule would reduce the number of caveolin molecules available to  $\alpha$ -toxin resulting in fewer cells being lysed as a consequence of  $\alpha$ -toxin pore formation.

There have also been reports of  $\alpha$ -toxin inhibition by modified CD.<sup>36</sup> Karginov et al.<sup>36</sup> showed that hepta-6-substituted CD molecules can create a molecule that interferes with the lytic action of the toxin. These modified CD molecules are thought to have affinity for the  $\alpha$ -toxin heptamer. Specifically, the modified CD molecules are thought to occlude the central pore of the toxin. Relative to the present study, one could speculate that the cholesterol molecule in CD-cholesterol interacts with the central portion of the  $\alpha$ -toxin pore. Thus, the cholesterol in complex with CD is envisioned to occlude the toxin pore in a manner similar to modified-CD inhibitors of  $\alpha$ -toxin described by Karginov et al.<sup>36</sup>

The CD molecule alone had variable effects on the action of  $\alpha$ -toxin. In the in vitro hemolysis assay, CD alone failed to significantly inhibit the hemolytic action of  $\alpha$ -toxin. In contrast, when CD was mixed with  $\alpha$ -toxin then injected into

rabbit corneas, there were some protective effects mediated by CD; that is, at 2 hours postinjection the corneas showed staining with fluorescein, but the epithelium was still intact. The fluorescein had penetrated under the epithelial layer suggesting that the layer had been partially loosened by the toxin. However, at 5 hours postinjection, the eyes developed erosions comparable to eyes injected with toxin mixed with PBS. Furthermore, the application of CD alone to eyes infected with *S. aureus* 8325-4 reduced the size of corneal erosions, but CD alone did not reduce the overall SLE score. These findings can be understood if one considers that, in the eye, the CD molecule can react with cholesterol or other lipid present in the tear film or surrounding tissues to form a complex that has significant inhibitory action on  $\alpha$ -toxin. Because of the greater availability of lipids in the eye (e.g., tear film) than in the erythrocyte lysis assay, such spontaneous formation of an inhibitory complex could be more likely to occur in vivo than in the erythrocyte suspension used for the hemolysis assays in vitro.

$\alpha$ -Toxin, as has been demonstrated by virulence studies of bacterial mutants in rabbits and mice, can cause the majority of the pathologic changes observed during ocular infection.<sup>14-16,28,29</sup> Studies with purified  $\alpha$ -toxin injected into rabbit corneas show that nanogram quantities of  $\alpha$ -toxin are toxic.<sup>16</sup>  $\alpha$ -Toxin is produced in readily detectable quantities by most isolates. Strain Newman was reportedly deficient in  $\alpha$ -toxin production, but more recent studies showed that the very small amount of  $\alpha$ -toxin produced by this strain made a significant contribution to its virulence in the rabbit cornea.<sup>37,38</sup> Additional findings on the importance of  $\alpha$ -toxin to corneal virulence have been obtained through the study of  $\alpha$ -toxin neutralizing antibody.<sup>39</sup> Neutralizing antibody was capable of inhibiting the damage associated with  $\alpha$ -toxin during keratitis infection without affecting the growth of bacteria. Although these studies were performed in a rabbit model of keratitis, the action of  $\alpha$ -toxin on human cells is very similar to its action on rabbit cells indicating that the results could be relevant to humans.

Based on the demonstrated importance of  $\alpha$ -toxin to keratitis, the treatment of *Staphylococcus* keratitis with an inhibitor of  $\alpha$ -toxin could be a valuable adjunctive therapy that could potentially limit corneal damage while an antibiotic therapy kills the infecting bacteria. The inhibition of  $\alpha$ -toxin activity could be especially beneficial in those cases in which the antibiotic therapy is slow or ineffective at killing the infecting bacteria in the cornea.

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