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The Collagen-Binding Adhesin Is a Virulence Factor in *Staphylococcus aureus* Keratitis

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A collagen-binding strain of *Staphylococcus aureus* produced suppurative inflammation in a rabbit model of soft contact lens-associated bacterial keratitis more often than its collagen-binding-negative isogenic mutant. Reintroduction of the *cna* gene on a multicopy plasmid into the mutant helped it regain its corneal adherence and infectivity. The topical application of a collagen-binding peptide before bacterial challenge decreased *S. aureus* adherence to deepithelialized corneas. These data suggest that the collagen-binding adhesin is involved in the pathogenesis of *S. aureus* infection of the cornea.

Our understanding of the pathogenesis of infectious diseases of the eye is emerging. The intact ocular surface thwarts most microorganisms, but predisposing factors such as contact lens wear can expose tissue components conducive to bacterial adhesion. A breakdown in local defenses and a source of microbial contaminants increase the risk of eye infection.

Staphylococcus aureus is responsible for many types of human ocular infections (21) and accounts for 10% of culture-positive microbial keratitis at our institution. *S. aureus* adheres to the injured cornea (12) and releases proteins that disrupt corneal tissue (11).

S. aureus possesses a family of adhesins that are localized at the microbial surface and that interact with extracellular matrix components such as collagen, fibronectin, fibrinogen, laminin, and elastin with high affinity and specificity (4, 13). One staphylococcal adhesin is composed of an N-terminal domain with a collagen-binding site and an antipodal domain that attaches to the bacterial cell wall and projects into the cytoplasm (16). We sought to determine whether a rift in the corneal epithelial surface would increase the risk of corneal adherence and infection by collagen-binding *S. aureus*.

Previous animal models of staphylococcal keratitis used direct intrastromal injection to infect the cornea (1, 2, 5, 7, 9). However, direct inoculation into the corneal stroma does not allow the investigation of bacterial adherence to the corneal surface, a critical initial event in the pathogenesis of microbial keratitis. Because staphylococci attach to contact lenses (3), an animal model using soft contact lenses contaminated with *S. aureus* was developed to study the role of bacterial adherence in the initiation of keratitis. To enhance the risk of infection, we applied a high-inoculum challenge to surface-injured corneas. This model was then used to study the biological role of the collagen-binding adhesin in *S. aureus* keratitis by comparing the levels of virulence of a parental strain (Cna^+), its isogenic mutant (Cna^-), and the isogenic mutant complemented with an intact version of the gene (*cna*) encoding the collagen-binding adhesin. We also studied the protective effect of applying adhesin analogs before bacterial challenge.

Rabbit model of *S. aureus* keratitis. The animals used in this study were treated according to the criteria of the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. In the first set of experiments, a masked comparison was made between the Cna^+ strain and its Cna^- isogenic mutant (15). New Zealand White rabbits (eight in each group) were anesthetized by subcutaneous injection of ketamine (35 mg/kg of body weight) and xylazine (5 mg/kg of body weight). The corneal epithelium of the right eye of each rabbit was marked with a 9-mm trephine and then debrided within this area by using a Paton spatula. New etafilcon A contact lenses (Acuvue; Vistakon, Jacksonville, Fla.) that had been incubated at 35°C for 24 h in tryptic soy broth (TSB) containing 10^8 CFU/ml were placed onto the deepithelialized corneas. The nictitating membranes were removed to prevent dislocation of the contact lens, and the eyelids were sutured closed with 6-0 braided polyester. Eyelids were opened 48 h after contact lens placement, and slit-lamp biomicroscopy of the rabbit corneas was performed to determine the presence or absence of an epithelial defect with stromal suppuration. To confirm the presence of *S. aureus*, corneal scrapings were inoculated onto blood agar plates and incubated at 35°C. Corneas were then removed, embedded in paraffin, sectioned at 6 μm, stained with hematoxylin-eosin, and examined by light microscopy. In a second set of experiments, the virulence levels of the Cna^- mutant and its complemented derivative were compared by using the same rabbit model (five rabbits in each group).

Contact lens contamination. To determine whether rabbit corneas were exposed to similar amounts of bacteria, 10 new soft contact lenses (Acuvue) were incubated in TSB containing 10^8 bacteria for 24 h at 35°C. The contaminated contact lenses were washed with phosphate-buffered saline (PBS) to remove planktonic bacteria and then placed in tubes containing 2 ml of PBS and 10 glass beads that were vortexed for 2 min to dislodge adherent bacteria. A 0.5-ml aliquot was removed from the solution, and serial dilutions were plated on blood agar. Colony counts were quantified after 48 h of incubation at 35°C. An average of 2.4×10^6 CFU of the parental Cna^+ strain adhered to each contact lens, which was not significantly dif-

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ferent from the average of 3.6×10^6 CFU per lens for the Cna^- mutant ($P = 0.61$).

Construction of a *cna*-complemented *S. aureus* strain. To restore collagen-binding activity to the *S. aureus* isogenic mutant, the entire *cna* gene together with upstream DNA was amplified by PCR from *S. aureus* FDA 574 chromosomal DNA by using *Taq* polymerase (Life Technologies, Gaithersburg, Md.) and oligonucleotides 5′ GGTACCGGATCCACAGCTT CCGGTTTAATAGGTGTA 3′ (forward) and 5′ CGAGGTA CCAGAACTAAGAATAGCCTTATC 3′ (reverse). *Bam*HI and *Kpn*I restriction enzyme sites (underlined) were incorporated into the forward and reverse primers, respectively. The 4.2-kb PCR product was cloned into the *Escherichia coli* vector pGEM-3 (Promega, Madison, Wis.) and used to transform *E. coli* JM101 cells. A pGEM-3 derivative containing the *cna* gene was digested with *Bam*HI-*Eco*RI, releasing a 4.2-kb fragment that was then ligated to *Bam*HI-*Eco*RI-cleaved *E. coli-S. aureus* shuttle vector pL150 encoding chloramphenicol resistance and transformed into JM101 cells. Plasmid DNA, isolated from *E. coli* clones containing the proper plasmid construct, was then used to electrotransform *S. aureus* RN4220. To select for *S. aureus* RN4220 cells harboring the plasmid, cells were plated on tryptic soy agar (TSA) containing 5 μg of chloramphenicol per ml. Chloramphenicol-resistant (Cm^r) *S. aureus* colonies were screened by restriction digest analysis, and one transformant was selected (pCNA4.2). Finally, pCNA4.2 from *S. aureus* RN4220 was used to electrotransform the gentamicin-resistant (Gm^r) isogenic mutant. Transformants were plated on TSA containing gentamicin at 10 μg/ml and chloramphenicol at 5 μg/ml, yielding a Gm^r Cm^r transformant containing pCNA4.2.

Collagen-binding activity of *S. aureus* strains. The parental Cna^+ strain, its Cna^- isogenic mutant, and the *cna*-complemented strain (Cna^+) were analyzed for their collagen-binding activity. As shown in Fig. 1, *S. aureus* Cna^+ and Cna^- strains bound 75.3 and 3.7%, respectively, of the added ^{125}I-labeled bovine collagen type II. The *cna*-complemented strain bound levels of ^{125}I-labeled collagen similar to those of the wild-type strain.

Comparison of *S. aureus* strains in the rabbit model of keratitis. The corneas from six (75%) of the rabbits subjected to soft contact lenses contaminated with the parental Cna^+ *S. aureus* strain developed bacterial keratitis, as evidenced by dense, suppurative stromal infiltration. Cultures confirmed the presence of *S. aureus* in all inflamed corneas. None of the corneas exposed to the isogenic mutant developed suppurative keratitis, even though in all cases the induced epithelial defect was visible. The difference between the two groups was statistically significant ($P = 0.007$). Histopathological examination of corneas exposed to the parental Cna^+ *S. aureus* strain revealed bacteria attached to the corneal surface and within the corneal stroma, dense neutrophil infiltration, and a marked disruption of tissue integrity (Fig. 2).

In the second phase of the study, corneas from four (67%) of the rabbits exposed to contact lenses contaminated with the *cna*-complemented strain developed central suppurative keratitis, whereas two (40%) of the rabbits exposed to the Cna^- mutant developed bacterial keratitis. Corneal scrapings demonstrated the presence of *S. aureus* in all clinically infected corneas.

Effect of topical adhesin on *S. aureus* corneal adherence. A recombinant version of the collagen adhesin containing the entire A-domain (M55; amino acid residues 30 to 529) was produced by using the pOE vector (Qiagen, Chatsworth, Calif.) as described previously (14). The purified protein was dissolved in PBS to yield a solution containing 200 μg/ml (8).

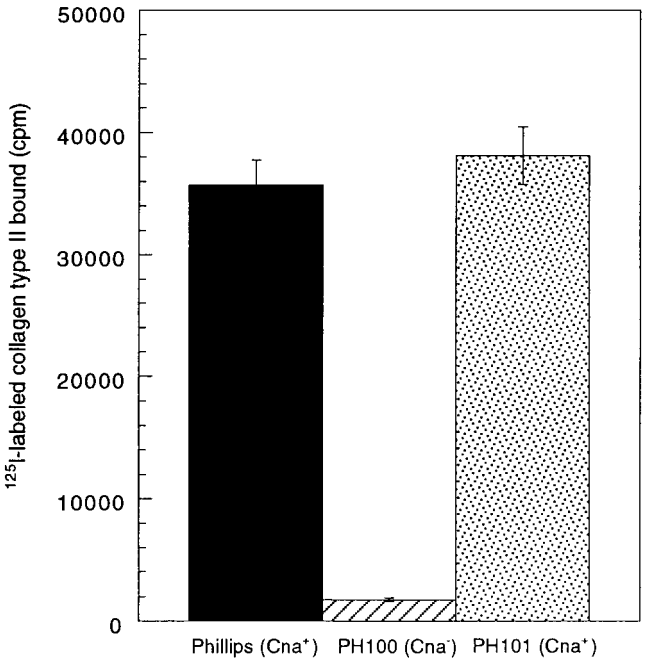

FIG. 1. Differential binding of ^{125}I-labeled collagen type II by *S. aureus* strains. The strains were incubated with 5×10^4 cpm of ^{125}I-labeled collagen type II for 1 h at room temperature. Bacterial binding was quantified as described previously (20). Each sample was done in duplicate, and results are expressed as the mean ± standard deviation for the Cna^+ parental strain (Phillips), the Cna^- isogenic mutant (PH100), and the *cna*-complemented mutant (PH101).

After debriding of the corneal epithelium, 9-mm-diameter central corneal buttons were excised and either were processed immediately or were first immersed in the adhesin solution for 15 min (two buttons in each group). Five microliters of TSB containing 10^8 CFU/ml was applied to the surface of each corneal button, which was then incubated at room temperature for 60 min. Scanning electron microscopy showed that the mean number of adherent bacteria in the nonpretreated control group was 7.5×10^4 per mm^2 of corneal surface area, while that of the pretreated corneas averaged 1.2×10^4 per mm^2.

Bacterial adherence involves surface-associated microbial proteins that bind to tissue ligands (6, 18). The collagen adhesin of *S. aureus* is a virulence factor in experimental bacterial arthritis and osteomyelitis (19). We now report that a contact lens-associated ulcerative keratitis model can be used to investigate the dynamics of bacterial adherence to the injured corneal surface. Significantly more eyes exposed to collagen-binding strains of *S. aureus* developed bacterial keratitis than eyes exposed to an isogenic mutant lacking a functional collagen-binding adhesin. Adhesin analogs decreased the amount of *S. aureus* adhering to the deepithelialized cornea. These findings implicate the role of adhesin-mediated bacterial adherence to the cornea's substrata in the pathogenesis of *S. aureus* keratitis.

Additional adhesion mechanisms may also be involved in initiating staphylococcal corneal infection (10, 17), since only one-third of our human *S. aureus* corneal isolates bind collagen (data not shown). Our *cna*-deficient mutant caused bacterial keratitis in a few animals, and preliminary experiments showed similar rates of corneal infection for both an *S. aureus* strain that does not bind collagen and its isogenic mutant into which the *cna* gene was introduced. Also, topical ophthalmic adhesins attenuated, but did not avert, bacterial keratitis following *S. aureus* challenge (data not shown).

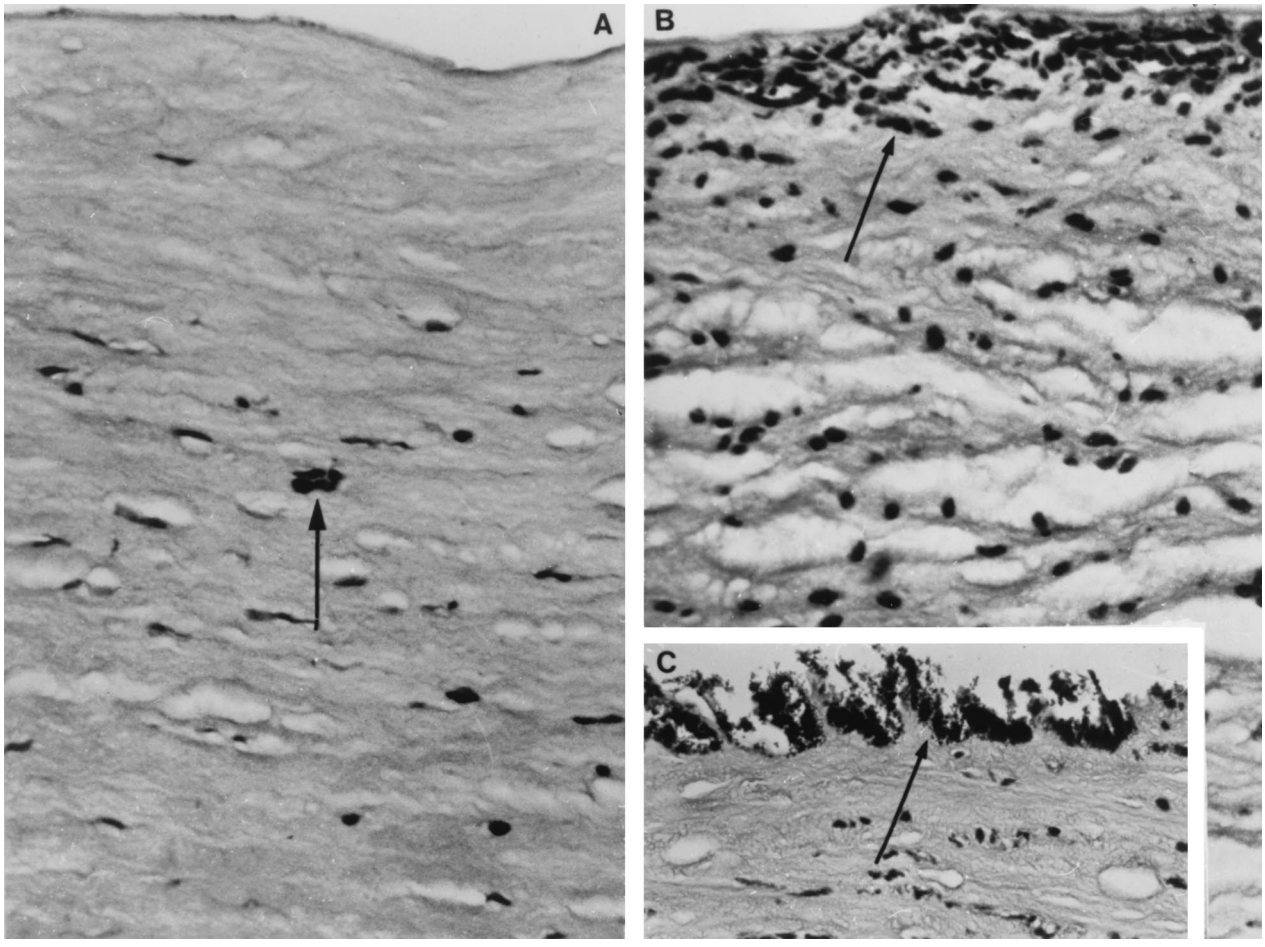

FIG. 2. Histopathological differences in experimental bacterial keratitis produced by different *S. aureus* strains. (A) Minimal polymorphonuclear infiltration (arrow) in a rabbit cornea exposed to the Cna^- mutant (hematoxylin-eosin; original magnification, ×240). (B) Rabbit cornea exposed to the parental Cna^+ strain with extensive neutrophil infiltration (arrow) with stromal necrosis and disruption of collagen lamellae (hematoxylin-eosin; original magnification, ×240). (C) Adherent cocci (arrow) on the surface of a cornea infected with the parental Cna^+ *S. aureus* strain (hematoxylin-eosin; original magnification, ×360).

This study demonstrates that the collagen-binding adhesin is a virulence factor for *S. aureus* keratitis. A better understanding of the early events that cause bacterial infection of the cornea may lead to the development of therapeutics that can inhibit bacterial binding and prevent microbial keratitis.

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