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

MARCUS N. RHEM,1 ELIZABETH M. LECH,1 JOSEPH M. PATTI,2,† DAMIEN MCDVITT,2,‡ MAGNUS HÖÖK,2 DAN B. JONES,1 AND KIRK R. WILHELMUS1*  

Sid W. Richardson Ocular Microbiology Laboratory, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine,1 and Center for Extracellular Matrix Biology, Albert B. Alkek Institute of Biosciences and Technology, Texas A&M University,7 Houston, Texas  

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A collagen-binding strain of Staphylococcus aureus produced supplicative 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 deep epithelialized 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 bacterial contaminants increase the risk of eye infection. A breakdown in local defenses and a source of microbial contaminants increase the risk of eye infection.

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 adhesin and an antipodal domain that attaches to collagen. A breakdown in local defenses and a source of microbial contaminants increase the risk of eye infection.

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 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 106 CFU/ml were placed onto the deep epithelialized 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 106 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 × 106 CFU of the parental Cna+ strain adhered to each contact lens, which was not significantly dif-
different from the average of $3.6 \times 10^6$ 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’-GGTACC GGATCCACAGCTT CCGGTATATAGGTGTA 3’ (forward) and 5’-CCAGGTT CAGAACTAAGATAGGCTTATC 3’ (reverse). BamHI 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 BamHI-EcoRI, releasing a 4.2-kb fragment that was then ligated to BamHI-EcoRI-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 $\mu$g of chloramphenicol per ml. Chloramphenicol-resistant (Cm$^+$) 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$^+$) isogenic mutant. Transformants were plated on TSA containing gentamicin at 10 $\mu$g/ml and chloramphenicol at 5 $\mu$g/ml, yielding a Gm$^+$ Cm$^+$ 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 125I-labeled collagen type II. The cna-complemented strain bound levels of 125I-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, suppurrative 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 suppurrative 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 adhesion 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 $\mu$g/ml (8).
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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