

Internalization of *Staphylococcus aureus* by Human Corneal Epithelial Cells: Role of Bacterial Fibronectin-Binding Protein and Host Cell Factors

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Wild-type *Staphylococcus aureus* was observed to be capable of invading human corneal epithelial cells (HCEC) in vitro. Internalization of *S. aureus* required expression of fibronectin-binding proteins (FnBPs); the capacity of an FnBP-deficient isogenic strain to invade HCEC was reduced by more than 99%. The binding of *S. aureus* to HCEC did not require viable bacteria, since UV-killed cells were observed to adhere efficiently. Invasion of HCEC by *S. aureus* involved active host cell mechanisms; uptake was nearly completely eliminated by cytochalasin D and genistein. These data suggest that FnBPs play a key role in host-parasite interactions and may serve as an important adhesin or invasin in ulcerative keratitis caused by *S. aureus*.

Staphylococcus aureus is a leading cause of ulcerative keratitis, accounting for approximately one quarter of confirmed cases (10, 15, 21). Nevertheless, the specific mechanisms by which this organism colonizes and damages the ocular epithelium are unclear. Because of the importance of *S. aureus* at other anatomical sites and its role as a leading nosocomial pathogen, a great deal of effort has been focused on investigating its virulence factors and their regulation. Multiple surface adhesins expressed by this organism, including the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), have been implicated in the pathogenesis of disease at a number of anatomical sites (5, 17); however, comparatively little is known of their role in the pathogenesis of keratitis caused by *S. aureus*.

It is generally believed that bacterial adhesion to the corneal surface is a prerequisite for ulcerative keratitis. In previous studies, *S. aureus* was observed to bind ocular epithelial cells (8, 14, 18). Two of these earlier studies employed rabbit corneal epithelial cells, and none specifically addressed the underlying mechanisms of adhesion. A recent study assessed the role of *S. aureus* collagen-binding adhesin in a rabbit model of contact lens-associated keratitis. While this model may provide a useful tool for studying bacterial surface adhesins in keratitis, it remained somewhat unclear whether collagen-binding adhesin played a direct causal role and the results suggested the involvement of other *S. aureus* factors (19).

Recent research has revealed that *S. aureus* fibronectin-binding protein (FnBP) mediates bacterial adhesion and invasion in many cell types, including mammary epithelial cells (4), embryonic kidney cells (22), osteoblasts (1), and endothelial cells (11). The current model for FnBP-mediated adhesion and

invasion proposes a fibronectin-dependent bridging between *S. aureus* FnBPs and host cell $\alpha_5\beta_1$ integrin (11). Because of the importance of *S. aureus* as a cause of ulcerative keratitis, the availability of isogenic strains, and the demonstrated utility of in vitro epithelial-cell culture systems for the study of *S. aureus* adhesion and invasion, we were interested in determining whether the FnBP-mediated invasive properties of *S. aureus* extended to human corneal epithelial cells (HCEC).

Invasion assay. HCEC were kindly provided by James Chodosh (Dean A. McGee Eye Institute, Oklahoma City, Okla.) and have been extensively characterized by Araki-Sasaki et al. (2). HCEC were cultivated in serum-free defined keratinocyte medium (DK-SFM; Gibco BRL) in 24-well plates to a confluence of >95%. All incubations were carried out at 37°C in an atmosphere of 5% CO₂. Bacterial invasion assays were conducted as previously described (4, 22, 25), with the use of mid-logarithmic-phase cultures to maximize the expression of *S. aureus* surface proteins (5, 13, 17). Briefly, *S. aureus* strains were propagated to an optical density at 650 nm of approximately 0.5 in brain heart infusion broth, washed twice with DK-SFM, and applied to HCEC monolayers (approximately 10⁸ CFU in 1 ml of DK-SFM). The actual number of bacteria applied to monolayers was determined by serial dilution and plate counting (7). Following incubation for 1 h, bacteria were removed and monolayers were washed twice with DK-SFM. In order to measure bacterial invasion, monolayers were then covered with 1.0 ml of DK-SFM containing 100 µg of gentamicin/ml and incubated for 1 h to kill residual extracellular bacteria. Monolayers were washed twice with antibiotic-free DK-SFM, lysed, and homogenized in 1 ml of lysis solution (0.25% trypsin–0.025% Triton X-100). Lysates were serially diluted and plated on brain heart infusion agar, and colonies were enumerated following overnight incubation (7).

Role of *S. aureus* FnBP. Invasion of HCEC was assessed by use of wild-type *S. aureus* strain 8325-4 (12) and its isogenic mutant, DU5883, which is defective in FnBP-AB (6). The contribution of global regulatory genes was assessed by use of

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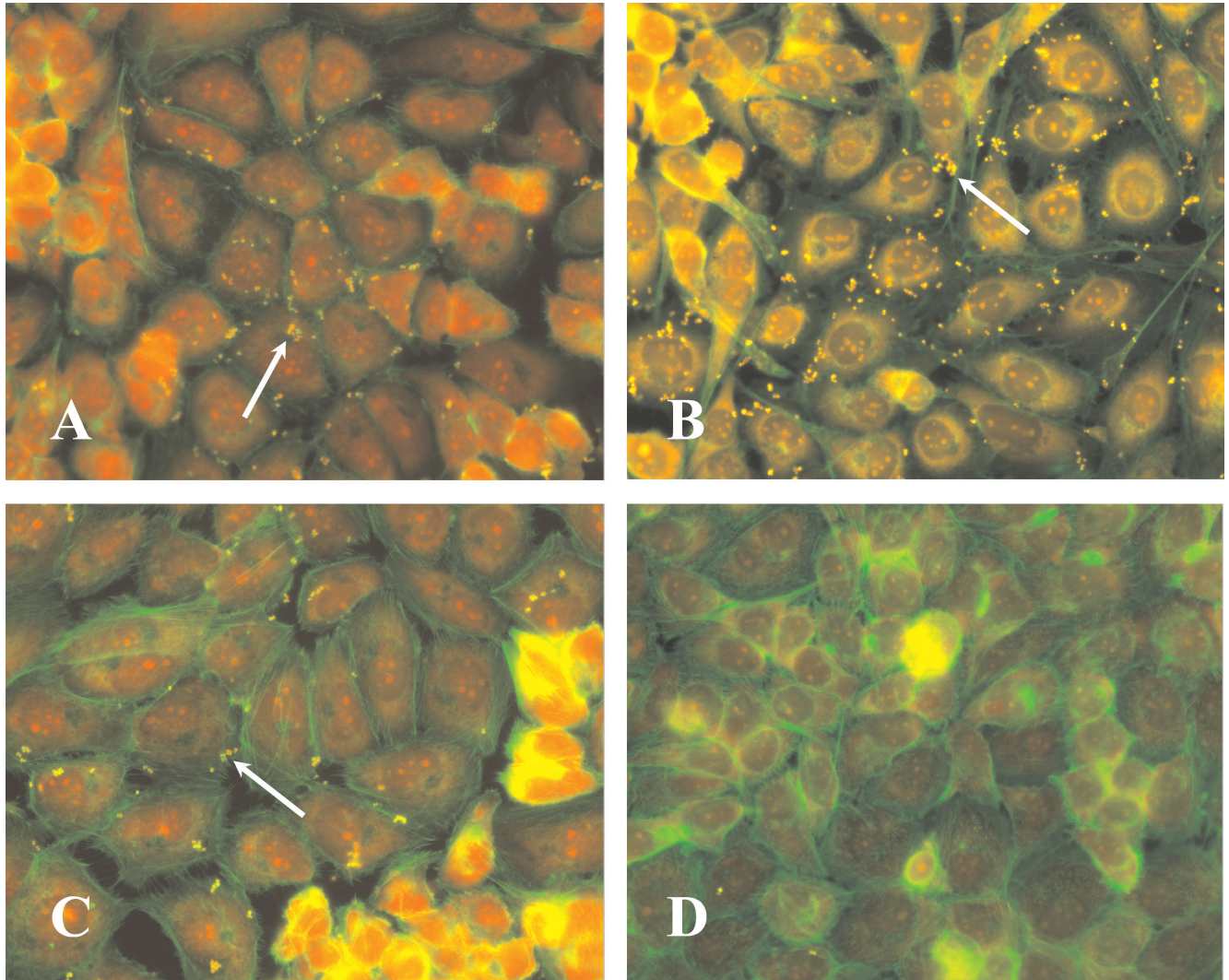


FIG. 1. Adhesion of *S. aureus* to HCEC. HCEC monolayers were incubated for 1 h in the presence of *S. aureus* 8325-4 (wild type) (A), UV-killed 8325-4 (B), ALC135 (*agr sar*) (C), or DU5883 (*fnbAB*) (D). Bacteria (arrows) were labeled with FITC prior to inoculation. Monolayers were stained with Alexa-Fluor 488 phalloidin and ethidium bromide. Magnification, $\times 20$.

the *agr sar* double mutant ALC135 (kindly provided by Ambrose Cheung, Dartmouth Medical School, Hanover, N.H.). HCEC monolayers stained with Alexa-Fluor 488 phalloidin (Molecular Probes, Eugene Oreg.) and ethidium bromide were examined by fluorescence microscopy with fluorescein isothiocyanate (FITC)-labeled (1 mg of FITC/ml in phosphate-buffered saline for 2 h) *S. aureus*. Qualitatively, FnBP mutants exhibited dramatically reduced binding ability compared to what was exhibited by the wild-type and *agr sar* double mutant strains (Fig. 1). Binding did not require living bacteria, since UV-killed and live *S. aureus* (wild type) demonstrated similar adhesion patterns (Fig. 1).

Quantitative invasion assays confirmed the results of fluorescence microscopy. Internalization of the FnBP-defective strain was reduced approximately 100-fold compared to the wild-type levels (Fig. 2). Mutations in the global regulatory loci *agr* and *sar* did not significantly affect adhesion and internalization ($P > 0.05$, Student's *t* test), a finding similar to that

previously reported for the adhesion of *S. aureus* to human endothelial cells (16). Although the penetration of epithelial surfaces by *S. aureus* has generally been considered a multifactorial phenomenon, the results of the present study suggest that FnBP serves as the predominant invasion factor for corneal cells.

Role of HCEC factors. Because of our initial observations that metabolically active *S. aureus* was not required for adhesion and the results of previous studies using epithelial cell lines from other species and tissues (4, 9) that identified the importance of active processes in mediating uptake, we were interested in assessing whether the uptake of *S. aureus* by corneal cells involved similar host cell pathways. Prior to the addition of *S. aureus* to HCEC, monolayers were incubated for 1 h in DK-SFM containing one of several metabolic inhibitors. Inhibitors were also included in the bacterial inoculum, prepared as described above. Preincubation of HCEC in the presence of colchicine (40.0 $\mu\text{g/ml}$; Sigma) reduced the invasion of

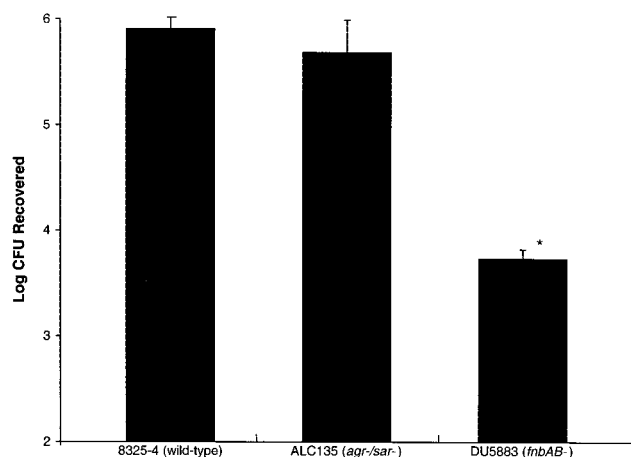


FIG. 2. Invasion of HCEC by *S. aureus*. HCEC monolayers were incubated for 1 h in the presence of *S. aureus*. Monolayers were washed, and extracellular bacteria were killed by the addition of gentamicin. Monolayers were homogenized, serially diluted, and plated for enumeration of viable intracellular bacteria. Data represent mean values + standard errors of the means for duplicate experiments, each performed in triplicate. Asterisk indicates statistically significant reduction in internalization ($P < 0.05$, Student's *t* test) compared to wild-type internalization.

wild-type *S. aureus* by a measurable, yet statistically insignificant, amount ($P > 0.05$, Student's *t* test), suggesting a minimal role for microtubule polymerization during bacterial uptake. In sharp contrast, preincubation with either the actin microfilament inhibitor cytochalasin D (0.5 $\mu\text{g/ml}$; Sigma) or the tyrosine kinase inhibitor genistein (250 $\mu\text{g/ml}$; Calbiochem) reduced the internalization of *S. aureus* by >99.9% ($P < 0.001$) compared to wild-type levels (Fig. 3). These findings indicate

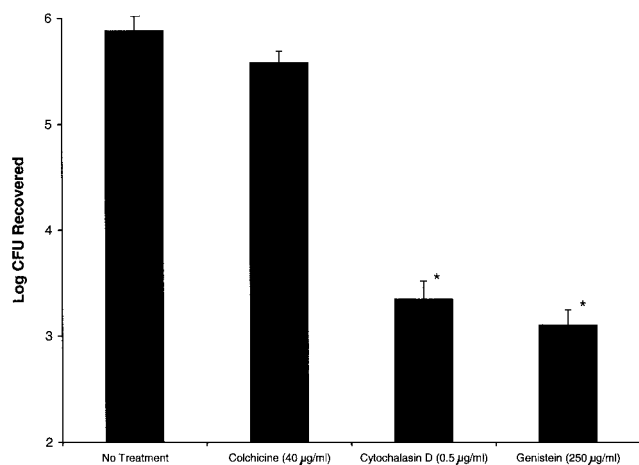


FIG. 3. Inhibition of *S. aureus* invasion. HCEC monolayers were incubated for 1 h in the presence of inhibitors. Monolayers were incubated for an additional 1 h in the presence of inhibitors and wild-type *S. aureus*. Monolayers were washed, and extracellular bacteria were killed by the addition of gentamicin. Monolayers were homogenized, serially diluted, and plated for enumeration of viable intracellular bacteria. Data represent mean values + standard errors of the means for duplicate experiments, each performed in triplicate. Asterisks indicate statistically significant reductions in internalization ($P < 0.05$, Student's *t* test) compared to wild-type internalization.

that invasion of HCEC by *S. aureus* is an active process involving host cell cytoskeletal rearrangements and one or more intracellular signaling cascades.

Taken together, the results of these studies provide a framework for a model of *S. aureus*-corneal epithelium interaction. In this model, *S. aureus* may bind to corneal epithelial cells via its cell surface FnBPs. Following ligation of the appropriate corneal epithelial cell receptor, intracellular signaling cascades become activated, leading to actin polymerization and formation of endocytic vesicles.

Similar models have been proposed for *S. aureus* adhesion to and invasion of bovine mammary epithelial cells (3), human embryonic kidney cells (22), and endothelial cells (11). Sinha et al. (23) described the entry of *S. aureus* into epithelial cells in terms of a "zipper-type" mechanism resembling professional phagocytosis and involving F-actin, as demonstrated by complete inhibition by cytochalasin D (22). They mapped the interaction between the host cell and the fibronectin-binding domain of FnBP and suggested that fibronectin acts as a bridging molecule between *S. aureus* FnBPs and host cell $\alpha_5\beta_1$ integrin, an integrin which is known to occur in the human corneal epithelium (24). The cumulative results of multiple similar studies led Sinha et al. to exclude an efficient second *S. aureus* invasin, highlighting FnBP as the dominant *S. aureus* MSCRAMM in epithelial cell adhesion and invasion (22, 23). The findings of the present study are in good agreement with this model, as the invasion of HCEC by *S. aureus* was markedly reduced in the absence of FnBP or active host cell uptake processes.

To the best of our knowledge, this study represents the first identification of an *S. aureus* surface ligand for cells of the human cornea and the first demonstration of its importance in triggering invasion. Nevertheless, a number of questions remain unanswered. Further experiments are required to precisely quantify adhesion and elucidate the mechanism for the internalization and subcellular localization of internalized bacteria (i.e., confocal microscopy studies). Competitive inhibition studies using anti-FnBP antibodies or FnBP-derived peptides might be useful in characterizing the receptor-ligand interactions at the corneal cell surface. Since, in this study, *S. aureus* invasion was substantially but not completely eliminated by the absence of FnBP, there appears to be a role, albeit a minor one, for other factors. Finally, it remains to be determined to what extent these in vitro results can be extrapolated to actual cases of ulcerative keratitis. The corneal surface is bathed in a mucinous tear film, which contains multiple antimicrobial factors that collectively present a formidable barrier to bacterial adhesion and invasion. Murine models have proven useful for the investigation of keratitis caused by *Pseudomonas aeruginosa* (20), and rabbit models of *S. aureus* adhesion to the corneal surface are in the early stages of development (19). Establishment of a reliable in vivo model that incorporates quantifiable initial-adherence processes will be required to elucidate the molecular pathogenesis of ulcerative keratitis caused by *S. aureus* and to assess the effectiveness of therapeutic agents aimed at blocking bacterial adhesion and/or invasion.

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