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Cell Surface O-Glycans Limit *Staphylococcus aureus* Adherence to Corneal Epithelial Cells[▽]

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The mucin-rich environment of the intact corneal epithelium is thought to contribute to the prevention of *Staphylococcus aureus* infection. This study examined whether O-glycans, which constitute the majority of the mucin mass of epithelial cell glycocalyces, prevented bacterial adhesion and growth. Abrogation of mucin O glycosylation using the chemical primer benzyl-α-GalNAc resulted in increased adherence of parental strain RN6390 to apical human corneal-limbal epithelial (HCLE) cells and to biotinylated cell surface protein in static and liquid phase adhesion assays, consistent with a role of mucin O-glycans in preventing bacterial adhesion. Comparable results were found with ALC135, an isogenic mutant strain defective in the accessory gene regulators *agr* and *sar*, indicating that the *agr*- and/or *sar*-regulated virulence factors did not play a major role in mediating adhesion to the corneal cell surface after mucin O-glycan truncation. In exoglycosidase digestion studies, treatment with sialidase from *Arthrobacter ureafaciens*—which hydrolyzed mucin-associated *O*-acetyl sialic acid—but not from *Clostridium perfringens* resulted in an increase in RN6390 and ALC135 adhesion. Abrogation of mucin O glycosylation in HCLE cell cultures did not affect bacterial growth. Overall, these data indicate that mucin O-glycans contribute to the prevention of bacterial adherence to the apical surface of corneal epithelial cells and suggest that alteration of cell surface glycosylation from disease or trauma, including that stemming from contact lens wear, could contribute to a higher risk of infection.

Staphylococcus aureus is one of the most frequent causes of bacterial keratitis. Infection can be severe, leading to corneal ulceration and perforation if not treated effectively. It is generally believed that an important factor in the initial interaction between *S. aureus* and its host is the ability of the bacterium to adhere to the host cell surface (1, 19, 24). Recent evidence has shown that cell surface-associated mucins, major components of apical membranes in wet-surfaced epithelia, are critical elements of the mucosal barrier to infection (7, 30, 31). Although mucin carbohydrates, or O-glycans, constitute up to 80% of the mucin mass of cell surface mucins (16), little is known about their contribution to a host's defense against bacterial adhesion and infection.

Membrane-anchored, cell surface-associated mucins are defined by the presence of long extracellular amino terminal domains containing hundreds of clustered O-linked glycans. These domains can extend 200 to 500 nm above the cell membrane—well beyond other glycoproteins on the glycocalyx—and, therefore, constitute the initial site of interaction between the cell and the extracellular milieu (21). The biosynthesis of O-glycans is enzymatically initiated by transfer of *N*-acetylgalactosamine to the side chain of a serine or threonine within the peptide core of the mucin molecule (45). Further elongation of this structure leads to various linear and branched extensions, which may bear different terminal carbohydrate structures. Some O-glycans are known to be targeted as ligands for carbohydrate binding by adhesins on the bacterial cell surface, thus facilitating attachment (13, 18). Nevertheless, the contribution of O-glycans on cell surface-associated mucins to *S. aureus* keratitis has not been elucidated.

Benzyl-*N*-acetyl-α-D-galactosaminide (benzyl-α-GalNAc) is a chemical primer commonly used to suppress the elongation of cell surface mucin-type O-linked glycans (34). The primer competes for elongation of the core GalNAc residue (GalNAc-*O*-Ser/Thr) found in mucin-type O-linked glycans. Benzyl-α-GalNAc has been used extensively to study physiological consequences of mucin-type O-glycans (23, 43) and does not interfere with N glycosylation (5) or with the quantity or expression pattern of glycolipids (46). In the present study, we used benzyl-α-GalNAc to determine the role of mucin-type O-glycans in preventing *S. aureus* adhesion to differentiated human corneal epithelial cells.

MATERIALS AND METHODS

Cell culture. Immortalized human corneal-limbal epithelial (HCLE) cells were plated at a seeding density of 5×10^4 cells/cm^2 and maintained at 37°C in 5% CO_2. Cultures were grown in keratinocyte serum-free medium (Gibco Invitrogen Corp., Carlsbad, CA) supplemented with 0.2 ng/ml epidermal growth factor, 25 μg/ml bovine pituitary extract, and 0.4 mM $CaCl_2$ until confluence was reached. HCLE cells were then switched into Dulbecco's modified Eagle's medium-Ham's nutrient mixture F12 (DMEM-F12) supplemented with 10% calf serum and 10 ng/ml epidermal growth factor and grown for 7 days to promote mucin and mucin O-glycan biosynthesis (17). To inhibit mucin O glycosylation, 2 mM benzyl-α-GalNAc (EMD Calbiochem, San Diego, CA) in dimethyl sulfoxide (DMSO) was added to HCLE cells grown for 7 days in serum-containing medium, as reported elsewhere (22, 44). For adhesion assays, cells were switched into antibiotic-free medium for the last 24 h.

Bacterial strains and growth conditions. Two *Staphylococcus aureus* strains were used in this study, RN6390 and ALC135. RN6390 is a toxigenic laboratory strain derivative of the clinical strain NCTC8325 (33). ALC135 is a nontoxigenic derivative of RN6390 carrying a mutation in two accessory gene regulators, *sar* and *agr*. Accessory gene regulator *sar* is involved in regulating the biosynthesis of cell surface adhesins as well as secreted virulence factors in conjunction with *agr*, which stimulates the expression of secreted proteins and toxins as a quorum-sensing system (8, 41). Both strains were grown aerobically at 37°C in brain heart

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infusion broth (Research Products International Corp., Mt. Prospect, IL) to early logarithmic and stationary phases (representing optical densities at 595 nm [OD_{595}] of 0.4 and 2.7, respectively, in a 10-mm polystyrene cuvette). After being pelleted, the cells were washed in phosphate-buffered saline (PBS) before they were used in the assay.

Biotinylation of cell surface proteins. Cell surface proteins on apical cell membranes of HCLE cultures were biotinylated and then isolated by chromatography through a NeutrAvidin-agarose affinity column, using the Pinpoint cell surface protein isolation kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Electrophoresis and Western blotting. Total protein was extracted from cell cultures in 2% sodium dodecyl sulfate with a protease inhibitor cocktail (Halt; Pierce Biotechnology). Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology). Proteins were separated in agarose gels (1% [wt/vol]) under reducing conditions with Laemmli loading buffer containing β-mercaptoethanol and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) by vacuum blotting. For immunoblotting, membranes were incubated with H185 antibody (hybridoma cell culture supernatant diluted 1:1 in 5% [wt/vol] nonfat dry milk in Tris-buffered saline), which recognizes an *O*-acetyl sialic acid residue on MUC16 (4) or OC125 antibody (diluted 1:2,000 in 5% [wt/vol] nonfat dry milk in Tris-buffered saline with 0.1% Tween 20) (Dako, Carpinteria, CA), which recognizes MUC16. Membranes were then probed with a horseradish peroxidase-linked goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and developed with chemiluminescent substrate (SuperSignal West Pico; Pierce Biotechnology).

Static adhesion assay. HCLE cells in one-well culture chamber slides (Laboratory-Tek, Naperville, IL) were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled bacteria for 1 h at 37°C in 5% CO_2. HCLE cultures were then washed four times in PBS to remove nonadherent bacteria and fixed in 4% paraformaldehyde. Coverslips were added to the slides with VectaShield mounting medium (Vector Laboratories, Burlingame, CA), and the slides were imaged under a fluorescence microscope. Images were analyzed using ImageJ software (NIH, Bethesda, MD). For FITC labeling, 5 ml (5×10^9 CFU/ml) of bacteria was incubated with 100 μg/ml FITC (Sigma Chemical Co., St. Louis, MO) for 30 min on ice, washed three times in PBS, and then resuspended in DMEM-F12.

Liquid phase adhesion assay. Cell surface proteins from HCLE cell cultures were biotinylated, as described above, and attached to NeutrAvidin agarose beads (Pierce Biotechnology). After being washed with PBS, beads were incubated with a bacterial suspension (1×10^6 CFU/ml) on a rocker at 37°C for 1 h. Unbound bacteria were removed by extensive washing with PBS. Bound bacteria were then quantified using the drop count method as previously described (11). NeutrAvidin beads alone (without cell surface proteins) were used as a negative control.

Exoglycosidase digestion. For static adhesion assays, HCLE cells were treated with 0.05 U/ml sialidase from *Arthrobacter ureafaciens* (Glyko Prozyme, San Leandro, CA) or *Clostridium perfringens* (EMD Calbiochem) in DMEM-F12, pH 6.0, for 2.5 h at 37°C. For Western blot assays, cell surface proteins on apical cell membranes of HCLE cultures were biotinylated and then isolated using the Pinpoint cell surface protein isolation kit (Pierce Biotechnology). Biotinylated HCLE protein was concentrated on Omega membranes using a NanoSep 10K spin column (Pall Corporation, East Hills, NY) and then treated with 0.05 U of sialidase from *Arthrobacter ureafaciens* or *Clostridium perfringens* in sodium phosphate buffer, pH 5.5, for 2 h at 37°C. Protein was recovered in Laemmli loading buffer containing β-mercaptoethanol and analyzed by Western blotting, as described above.

Bacterial growth assay. Mucin-rich fractions from HCLE cells treated with or without benzyl-α-GalNAc were obtained by size exclusion chromatography using a CL-4B Sepharose column coupled to a UA-5 absorbance/fluorescence detector (Teledyne Isco, Lincoln, NE), as previously described (2). The protein concentration of the fractions was determined using the Micro bicinchoninic acid kit (Pierce Biotechnology). Growth assays were carried out in microtiter wells (total volume, 100 μl) containing serial dilutions of mucin in PBS (starting at 12.5 μg) and bacterial densities of 0, 1×10^5, 1×10^6, and 1×10^7 CFU/ml. Bacterial growth under continuous shaking, at 37°C, was monitored every 30 min by OD_{595} with a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analyses. All statistical analyses were performed using InStat3 software (GraphPad Software, La Jolla, CA). *P* values were determined using the Student-Newman-Keuls test.

RESULTS

***S. aureus* adhesion increased after abrogation of O-glycan biosynthesis.** The adhesion of *S. aureus* to the apical surface of HCLE cells was evaluated using static adhesion assays. In these experiments, truncation of cell surface O glycosylation with benzyl-α-GalNAc was confirmed by Western blotting, using the H185 antibody to an *O*-acetyl sialic acid epitope on MUC16. As shown in biotinylation experiments, HCLE cells treated for 7 days with benzyl-α-GalNAc lacked the H185 carbohydrate epitope at the cell surface but not the MUC16 apomucin (Fig. 1A).

Abrogation of O glycosylation in HCLE cells increased adhesion of *S. aureus* RN6390 by sixfold for bacteria grown to early logarithmic phase and by sevenfold when grown to stationary phase (Fig. 1B). A comparable increase in bacterial binding to HCLE cells after O-glycan truncation was observed when analyzing the *agr sar* double mutant ALC135 grown in early logarithmic phase—10-fold compared to binding of HCLE cells exposed to medium alone (DMEM) or the vehicle (DMSO), in which the O glycosylation inhibitor benzyl-α-GalNAc (2 mM) had been dissolved (Fig. 1C). This indicates that the increase in bacterial binding to the underglycosylated cell surface is not facilitated by bacterial virulence factors under the control of the *agr* and *sar* global regulatory genes. In these experiments, bacteria bound to HCLE cells in a mosaic pattern, which could reflect the heterogeneous character of the cell surface glycocalyx after O-glycan truncation (Fig. 1D).

The role of cell surface O-glycans in limiting pathogen adherence to HCLE cells was further assessed by immobilizing biotinylated cell surface protein on NeutrAvidin agarose beads (Fig. 2). Under liquid phase, bacterial adhesion to beads coated with biotinylated surface protein from cells grown with benzyl-α-GalNAc was significantly higher than that of the controls (exposed to DMEM and DMSO), indicating that cell surface O-glycans contributed to limiting bacterial adhesion to components of the corneal epithelial glycocalyx.

Role of *O*-acetyl sialic acid in preventing *S. aureus* adhesion to corneal epithelial cells. The apical surface of the human corneal epithelium is heavily coated by MUC16-associated O-acetylated sialic acid, as shown by H185 antibody binding (3, 4, 47). *O*-acetyl sialic acid can decrease the rate of mucin oligosaccharide degradation and has been hypothesized to regulate bacterial access to mucosal surfaces (12). In this study, we evaluated the role of *O*-acetyl sialic acid on *S. aureus* adherence to corneal epithelial cells, taking advantage of the ability of two sialidases to differentially degrade the epitope recognized by the H185 antibody. *A. ureafaciens* sialidase degrades both O-acetylated and non-O-acetylated sialic acid, whereas *C. perfringens* sialidase degrades non-O-acetylated sialic acid (4). As shown in static adhesion assays, *S. aureus* RN6390 and ALC135 adherence to HCLE cells was significantly increased over that of controls after treatment with sialidase from *A. ureafaciens*, by 18-fold ($P < 0.001$) and 29-fold ($P < 0.05$), respectively (Fig. 3). Treatment with *C. perfringens* sialidase, however, did not increase adherence of either strain of bacteria, supporting the role of sialic acid *O*-acetyl modification in preventing bacterial adhesion to corneal epithelial cells.

Role of mucin-type O-glycans on bacterial growth. Previous data have shown that mucins extracted from human conjunc-

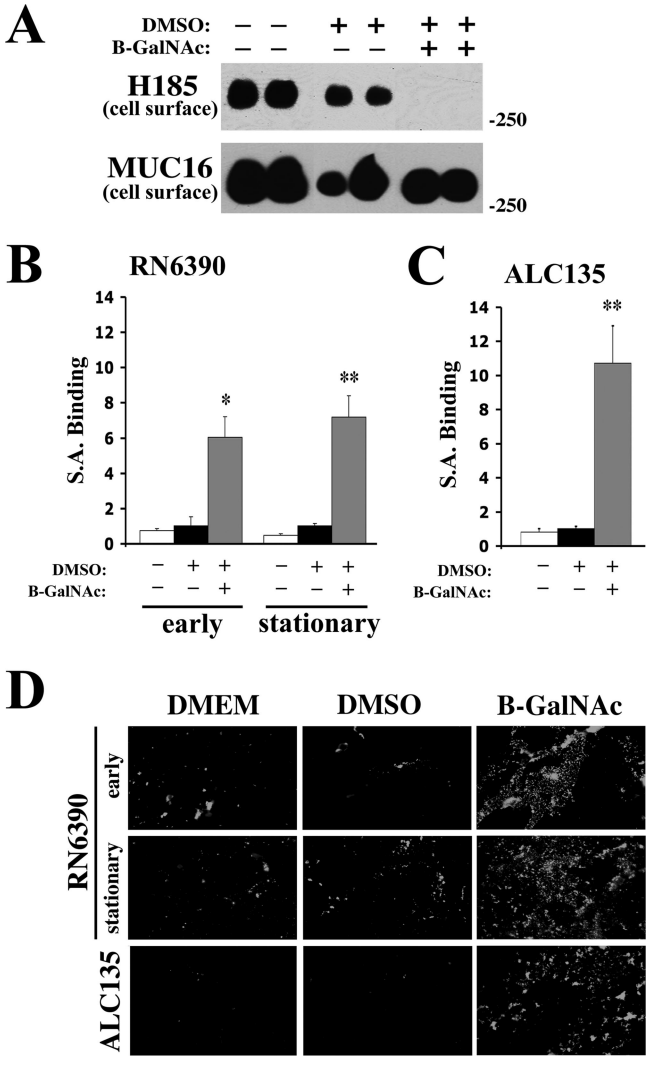

FIG. 1. Effect of benzyl-α-GalNAc (B-GalNAc) on *S. aureus* binding to corneal epithelial cells. (A) Cell surface protein from HCLE cells was biotinylated, isolated by chromatography, and analyzed by Western blotting. Treatment of HCLE cell cultures with benzyl-α-GalNAc abolished H185 antibody binding to an *O*-acetyl sialic acid epitope on cell surface MUC16, compared to vehicle control (DMSO) or medium alone. In comparison, levels of MUC16 apomucin at the cell surface of HCLE cells were not affected. Experiments were performed in duplicate. (B) Static adhesion assays showing that adhesion of FITC-labeled *S. aureus* (S.A.) RN6390 grown to both early logarithmic and stationary growth phases increased after incubation with benzyl-α-GalNAc. (C) Similarly, adhesion of the *agr sar* mutant strain ALC135 increased after abrogation of mucin-type O glycosylation. In these experiments, binding was normalized to a DMSO vehicle treatment control. Representative ×20 images are shown in panel D. Asterisk, $P < 0.01$; double asterisk, $P < 0.001$.

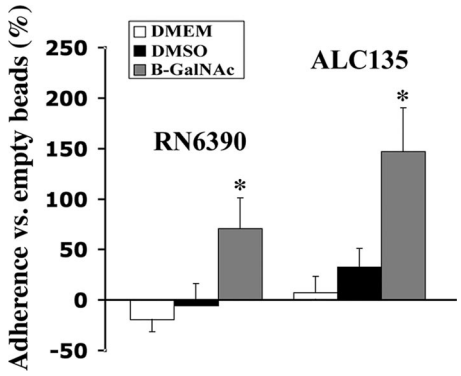

FIG. 2. Effect of benzyl-α-GalNAc on *S. aureus* binding to biotinylated cell surface protein. Cell surface protein from HCLE cells was biotinylated, attached to NeutrAvidin agarose beads, and incubated in liquid phase with a bacterial suspension (1×10^6 CFU/ml) for 1 h. Bound bacteria were quantified using the drop count method (11). Under these conditions, binding of *S. aureus* RN6390 to immobilized cell surface protein increased after culture under conditions that abrogate mucin O-glycan biosynthesis (B-GalNAc). Similar results were obtained with the *agr sar* double mutant strain ALC135. Adhesion in all experimental conditions was normalized to that obtained with uncoated beads. Asterisk, $P < 0.05$.

tiva or contact lenses can inhibit the growth of commensal bacteria and, therefore, participate in the mucosal control of microbiota (6). We evaluated whether cell surface-associated mucins or their O-glycans could also affect *S. aureus* growth in HCLE cultures. For these experiments, *S. aureus* was grown in the presence of mucin-rich fractions extracted from HCLE cells treated with either benzyl-α-GalNAc or the DMSO vehicle control. As shown in Fig. 4, no differences in growth were observed with increasing amounts (from 0 to 12.5 μg) of deglycosylated mucin and control at four different bacterial concentrations (represented are 10^6 and 10^7 CFU/ml), indicating that, under the conditions of our study, cell surface-associated mucins and their O-glycans do not interfere with *S. aureus* growth.

DISCUSSION

The ability of *Staphylococcus aureus* to adhere to the epithelial cell glycocalyx is thought to be one of the first steps in the colonization and infection of wet mucosal surfaces. In this study, we used static and liquid phase adhesion assays to show that cell surface O-glycans, which constitute up to 80% of the mucin mass, limit adherence of *S. aureus* to corneal epithelial cells. *S. aureus* RN6390 and its isogenic mutant ALC135 bound in a similar pattern to the apical surface of stratified corneal epithelial cells after inhibition of mucin O glycosylation. These observations indicate that, in the absence of injury, cell surface mucin O-glycans contribute to the prevention of bacterial adherence to the apical surface of the cornea and that this binding is independent of other changes in the epithelial cell or its surface that may be induced by *sar* and *agr* regulatory genes.

Secreted mucins have long been considered major contributors to the protection of mucosal surfaces, including the ocular surface, by binding and clearing pathogens (15, 37). In contrast, the role of cell surface-associated mucins, which heavily coat the apical surface of the epithelial cell, has received relatively little attention. Observational studies have shown that *Muc1*$^{-/-}$ mice housed under conventional conditions were more susceptible to ocular infection by environmental bacteria, suggesting that cell surface-associated mucins are critical components of the mucosal defense system (26). Mechanistically, it has been proposed that cell surface mucins contribute to host defense against *Campylobacter jejuni* infection in the gastrointestinal tract by expressing diverse and complex

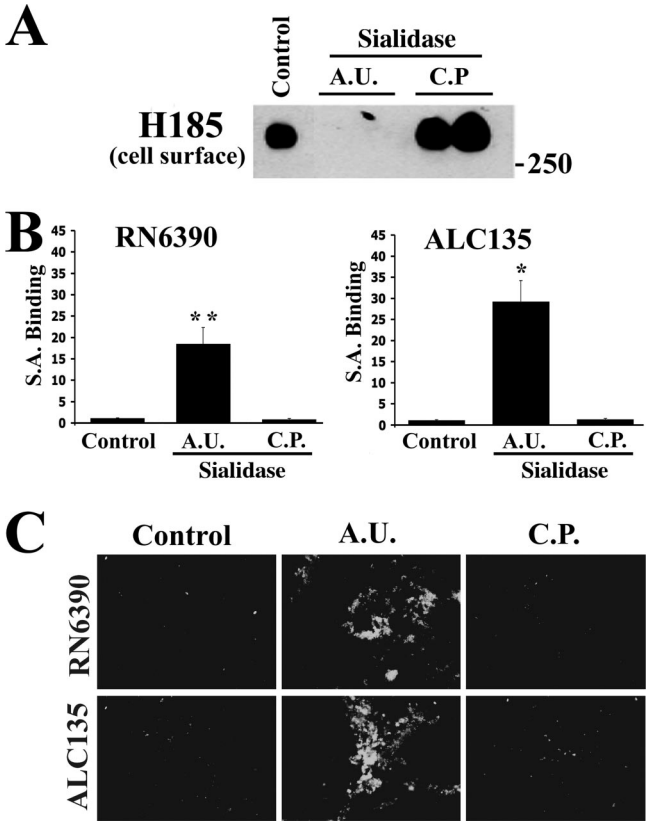

FIG. 3. *S. aureus* binding to HCLE cells after sialidase digestion. (A) Cell surface protein from HCLE cells was biotinylated, isolated by chromatography, and analyzed by Western blotting after sialidase treatment. Treatment of HCLE cell cultures with sialidase from *A. ureafaciens* (A.U.), but not from *C. perfringens* (C.P.), hydrolyzed *O*-acetyl sialic acids on cell surface mucin (as shown by the lack of H185 binding). (B) Static adhesion assays showing that RN6390 adhesion to cells treated with sialidase from *A. ureafaciens* increased 18-fold compared to *C. perfringens* or the vehicle control (DMEM-F12, pH 6.0). Similarly, adherence of the *agr sar* double mutant strain ALC135 increased significantly, by 29-fold, after treatment with sialidase from *A. ureafaciens*. In these experiments, binding was normalized to a no-sialidase treatment control. Representative ×25 images are shown in panel C. Asterisk, $P < 0.05$; double asterisk, $P < 0.001$.

oligosaccharides that act as releasable decoy ligands for bacterial adhesins, thereby limiting attachment of pathogens to other cell surface molecules and subsequent invasion (30). For *S. aureus*, no carbohydrate-specific lectins have been found that mediate binding to mucin O-glycans on the corneal glycocalyx under physiological conditions (10, 29, 40). At the ocular surface, several studies have shown that *S. aureus* binds to the apical surface of normal corneal epithelial cells (24, 25, 32, 35, 39). These studies, however, evaluated epithelial adhesion in rabbits—O-glycan structures of ocular surface mucins display species differences (36)—or in human corneal epithelial cell lines not optimized for mucin and mucin O-glycan production. In human conjunctival epithelial cells differentiated in a pattern similar to that observed in vivo (17), induction of mucin expression resulted in reduced adherence of nontypeable *Streptococcus pneumoniae* to the cell surface, whereas sialidase treatment enhanced adherence (49). Also, silencing MUC16 expression by small interfering RNA in human corneal epithelial cells optimized for mucin production resulted in increased adherence of *S. aureus* to the differentiated apical cell surface glycocalyx (7), suggesting that the biosynthesis of cell surface-associated mucins prevents adhesion to differentiated human corneal cells. These findings are in complete agreement with those of the present study, which uses differentiated human corneal epithelial cells, and indicate that in addition to MUC16, induction of mucin O glycosylation limits the binding of *S. aureus* to the corneal apical surface.

Increased binding of *S. aureus* to the corneal epithelial cell surface after truncation of mucin O glycosylation suggests the presence of cell surface adhesins that facilitate attachment. Surface proteins on nonmucoid strains of *S. aureus* have been shown to recognize sites on bronchial mucins located on the naked or poorly glycosylated regions of the mucin protein backbone (42). A multiple repertoire of surface adhesins expressed by *S. aureus*, known as MSCRAMMS (microbial surface components recognizing adhesive matrix molecules), has been implicated in the adhesion of this organism to its host. MSCRAMMS can promote selective adhesion to epithelial cells; for instance, Pls (plasmin-sensitive cell wall protein) has been shown to promote bacterial attachment to nasal epithelial cells but not to buccal epithelial cells or cultured keratinocytes (10). Similarly, attachment of *S. aureus* was significantly affected by absence of *sar* and *agr* global regulators in airway epithelial cell monolayers but not in corneal epithelial cell monolayers (20, 24). In agreement with the latter observations, we could not find differences in the pattern of adhesion when comparing the parental strain RN6390 to the isogenic mutant strain ALC135, suggesting that virulence factors and extracellular matrix adhesins under the control of the global regulatory genes *sar* and *agr* are not involved in the enhanced attachment of *S. aureus* to differentiated corneal epithelial cells that lack surface O-glycans. It is possible that other cell wall proteins under different regulatory control may account for the enhanced attachment of *S. aureus* to underglycosylated cells or be involved in biofilm formation. Alternatively, changes in the physiochemical character of the corneal epithelial surface after mucin O-glycan abrogation could also account for enhanced adhesion. Analysis of the regulation and mechanism of action of additional staphylococcal adhesins, as well as of the physiochemical character of the corneal cell surface, would prove valuable in understanding the interaction of *S. aureus* with the mucin-rich environment of the corneal glycocalyx.

The specific mechanisms by which *S. aureus* induces bacterial keratitis are still unclear. Extended contact lens wear and, to a lesser extent, dry eye are associated with an increased incidence of *S. aureus* keratitis (9, 38). It may be that microabrasions on the apical epithelial cell surface caused by contact lens shear stress, dryness, or hypoxia alter the mucin O-glycan composition of the epithelial glycocalyx, contributing to bacterial adhesion and invasion. In rabbits, contact lens wear has been shown to modify the corneal epithelial glycocalyx, which by electron microscopy was thinner and had altered glycan chains, as shown by lectin binding (27, 28). In humans, the apical ocular surface glycocalyx of patients with superior limbic keratoconjunctivitis (a disease commonly associated with contact lens wear) and dry eye has reduced levels of *O*-acetyl sialic acid in MUC16 (14, 48), which suggests that alteration of cell surface O-glycans may compromise the mucin barrier and in-

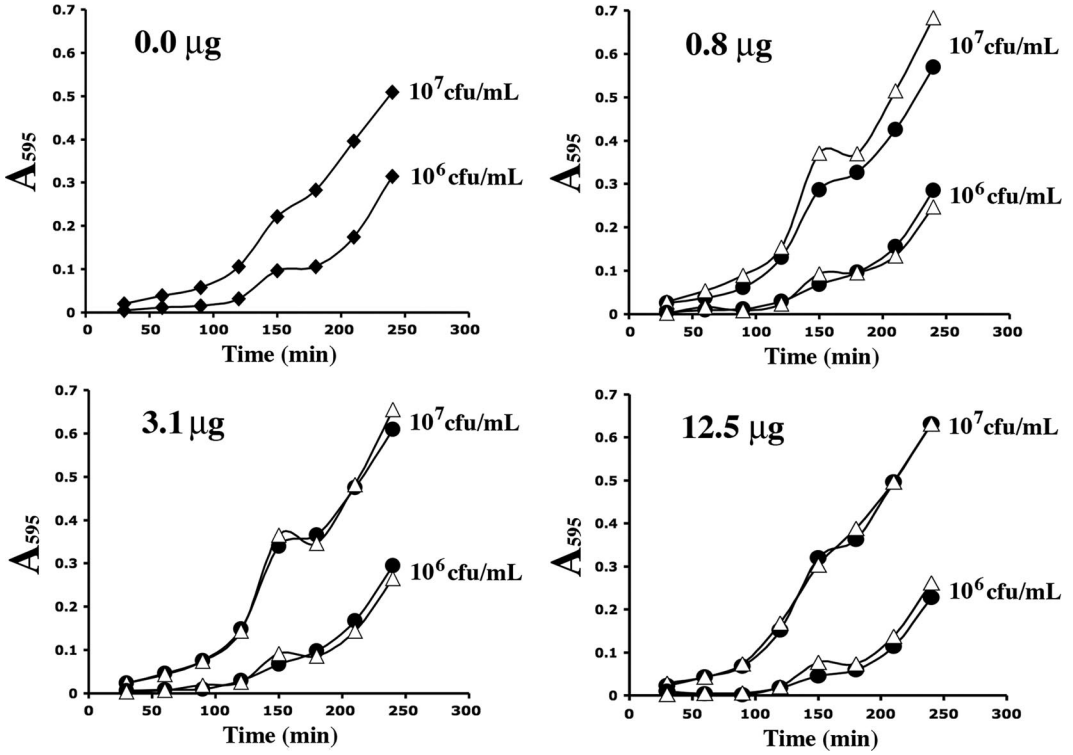

FIG. 4. Effect of mucin-type O-glycan inhibition on *S. aureus* growth. Four different concentrations of bacteria (10^6 and 10^7 CFU/ml are represented) were incubated with medium alone (◆) or with medium containing increasing amounts (0.0 to 12.5 μg) of CL-4B-purified mucin from HCLE cells treated with benzyl-α-GalNAc (△) or DMSO (●). Growth assays were carried out in microtiter wells, and bacterial growth was monitored every 30 min by OD_{595}. The growth curves of *S. aureus* RN6390 over time were not affected by cell surface mucins or by inhibition of O-glycan biosynthesis.

crease the risk of bacterial keratitis. Further characterization of the glycosylation changes on the epithelial cell surface in patients with higher risk of infection could, therefore, prove relevant to the development of pharmacological drugs aimed at restoring the normal composition of the glycocalyx and to the prophylactic inhibition of bacterial adhesion and invasion.

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