Staphylococcus aureus Binding to Human Nasal Mucin

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Colonization of human nasal mucosa with *Staphylococcus aureus* sets the stage for subsequent systemic infection. This study characterizes *S. aureus* adhesion to nasal mucosa in vitro and investigates the interaction of *S. aureus* with human nasal mucin. *S. aureus* binding to cell-associated and cell-free mucus was greater than to nonmucin-coated epithelial cells. Scanning electron microscopy of *S. aureus* incubated with human nasal mucosal tissue showed minimal binding to ciliated respiratory epithelium. In a solid-phase assay, *S. aureus* bound to purified human nasal mucin-coated wells significantly more than to bovine serum albumin-coated microtiter wells. Binding to mucin was saturable in a dose- and time-dependent fashion. Staphylococcal adherence to human nasal mucin was inhibited by bovine submaxillary mucin but not by fibrinogen. Pretreatment of mucin with periodate but not with pronase reduced adherence. Trypsin treatment of the bacteria significantly reduced adherence to mucin. ¹²⁵I-labelled nasal mucin bound to two surface proteins (138 and 127 kDa) of lysostaphin-solubilized *S. aureus*. Binding to human nasal mucin occurs in part via specific adhesin-receptor interactions involving bacterial proteins and the carbohydrate moiety in mucin. These experiments suggest that *S. aureus* binding to mucin may be critical for colonization of the nasopharyngeal mucosa.

Staphylococcus aureus remains one of the most frequently occurring community- as well as hospital-acquired pathogens (42, 44). The consequences of these infections, especially when bacteremic, are often devastating (26, 44). Nasal colonization with *S. aureus* is common, occurs in 15 to 44% of the general population, and is usually without ill effects (29, 57). Certain groups, however, have higher rates of carriage, including dialysis patients (21, 58), diabetics (51), parenteral drug users (52), and AIDS patients (55). In these high-risk groups, there are strong epidemiologic data linking nasal carriage with an increased risk of subsequent infection. Phage typing and antibiotic susceptibility profiles suggest that the colonizing and invading strains are identical (21, 53, 58). Colonized individuals at risk of subsequent infection are often treated with topical antimicrobial agents in an attempt to eliminate carriage. This approach has had only limited success (56). The recent emergence of multiply antibiotic-resistant staphylococci has raised concern that effective antimicrobial regimens may not be available in the near future (25, 28, 47). As a result, new approaches to both the therapy and prevention of these infections must be developed.

In contrast to the substantial body of epidemiologic information that exists regarding the risks of *S. aureus* carriage, relatively little is known of the basic host surface components or mechanisms that mediate attachment of staphylococci to the nasal mucosa. Investigators have demonstrated binding to desquamated nasal epithelial cells (3, 6), to ferret airway mucus (41), bovine submaxillary mucin (41, 46), as well as immunoglobulin A (IgA)-salivary mucin complexes (7) and respiratory tract glycolipids (14). The purpose of the present investigation was to characterize the nature of the *S. aureus*-nasopharyngeal interaction and to define the bacterial and host surface components that mediate this process.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The primary *S. aureus* strain used in this study, Wb, is a blood culture isolate that has been used in previous studies (48, 49). 8325-4 is a standard laboratory strain used in *S. aureus* genetic studies, and DU5723 is the protein A-deficient part of this isogenic pair (kindly provided by T. Foster) (33). WOOD is also a protein A-deficient strain (15). COL is a methicillin-resistant clinical isolate, and RUSA4 is a transposon mutant of COL with a Tn551 insertion in the *mecA* gene locus (kindly provided by A. Tomasz) (24, 32). RN4220 is a laboratory strain derived from 8325-4 (13). Strain 502A (ATCC 27217) is a strain previously used in bacterial interference assays (2). The remaining strains of *S. aureus* are isolates from nasal swab cultures of patients in the Montefiore Medical Center clinic system. SUT is a strain of *Staphylococcus epidermidis* obtained from a bacteremic patient that has also been used in a previous study (20).

Bacterial strains were stored in THB (Difco Laboratories, Detroit, Mich.) with 20% glycerol (Sigma Chemical Co., St. Louis, Mo.) at –70°C. In preparation for an experiment, isolates were streaked onto a blood agar plate. A single colony was inoculated into THB and placed into a gyrosaker at 37°C.

Chemicals and reagents. Chemicals and reagents were obtained from the Sigma Chemical Co., unless otherwise indicated. Phosphate-buffered saline (PBS) solutions were pH adjusted to 7.2 unless stated otherwise. Tris-buffered saline (TBS) consisted of 10 mM Tris and 0.9% NaCl adjusted to pH 7.5.

Light microscopic study of *S. aureus* adherence to desquamated nasal epithelial cells. A modification of the method of Aly et al. (3) was used. Nasal epithelial cells from the anterior nares were scraped from a single investigator (J.S.) with a Cytosoft cytology brush (Medical Packaging Corp., Camarillo, Calif.). The brush was immersed in 2 ml of PBS that contained penicillin (5,000 U/ml; Marsham Pharmaceuticals, Cherry Hill, N.J.) and gentamicin (0.5 mg/ml; Lyphomed, Deerfield, Ill.) and vortexed for 60 s. The solution was refrigerated at 4°C for 0.5 h and then centrifuged (Sorvall GLC 2B) at 3,000 rpm for 10 min. The pellet was washed twice with PBS and resuspended in 1 ml of PBS.

A suspension of Wb in the logarithmic growth phase was centrifuged (Sorvall GLC 2B) at 1,500 × g for 10 min. The pellet was washed, and the bacteria were decumped by passage through a 25-G needle. A bacterial suspension (1.8 × 10⁸ CFU/ml) in 1 ml of PBS was combined with the 1-ml suspension of nasal epithelial cells. This mixture was incubated at 37°C in a gyrosaker for 1 h. Thereafter, the suspension was passed through a 12-μm-pore-size Nucleopore filter (Costar Corp., Cambridge, Mass.) and washed with PBS to remove non-adherent bacteria. The filter was immersed in PBS and vortexed for 60 s. The final suspension was centrifuged at 15,000 rpm for 5 min. The supernatant was discarded, and the pellet was dried down on microscope slides. These slides were stained with the Diff-Quick stain set (Baxter Corp., Miami, Fla.) or with mucicarmine (Sigma) according to the manufacturer's instructions.

A semiquantitative assay was performed in order to compare binding of *S. aureus* to mucin alone or mucin-coated epithelial cells versus binding to nonmucin-coated epithelial cells. The material examined was graded on a scale of 0 to 4 as follows: 0, no bacteria; 1, 1 to 10 bacteria; 2, 11 to 50 bacteria; 3, 51 to 100 bacteria; and 4, >100 bacteria.

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Electron microscopic studies. Human nasal mucosal tissue samples obtained from surgical specimens were immersed in M199 medium (Gibco BRL, Grand Island, N.Y.) containing penicillin (5,000 U/ml) and gentamicin (0.5 mg/ml) and kept at room temperature for 1 h. The pellet from an overnight culture of *S. aureus* Wb was suspended in PBS, yielding a bacterial density of 10^9 CFU/ml. The mucosal tissue was washed with PBS and then was incubated in the bacterial suspension for 0.5 h at 37°C. After five more washes with PBS, the tissue was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h, rinsed in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in cacodylate buffer, and then prepared for scanning electron microscopy as previously described (19).

Purification of human nasal mucin. Human nasal mucus was collected from healthy volunteers who were exposed to the cold for 0.5 h. Nasal mucus was collected and stored at -20°C. Mucin was purified by two cesium chloride (CsCl) density ultracentrifugations via the protocol of Carlstedt et al. (8). Fractions from the gradients were assayed for sugar content via the anthrone method (36). The final solution of purified mucin was dialyzed against PBS and stored at 4°C.

Analysis of purified nasal mucin. (i) Protein and carbohydrate determinations. Protein content in the mucin was measured by the bicinchoninic acid technique (Micro BCA kit; Pierce Chemical Co., Rockford, Ill.). Carbohydrate content was determined with a quantitative anthrone assay with values normalized to a standard glucose curve (36).

(ii) Analysis of crude mucus and purified mucin for contaminating proteins. Crude human nasal mucus, purified human nasal mucin, and commercially acquired bovine submaxillary mucin (Sigma) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Bio-Rad Protean II system in a 7.5% (wt/vol) acrylamide gel (16). The gel was silver stained according to a previously published method (39).

(iii) Electrophoretic analysis of mucin. Mucin specimens were electrophoresed on a 0.7% agarose (Gibco-BRL)-1% acrylamide gel in a Mini-Sub DNA cell apparatus (Bio-Rad Laboratories, Richmond, Calif.), by an adaptation of the method of Holden et al. (12). The glycoprotein in the gel was then blotted to nitrocellulose (Schleicher & Schuell, Keene, N.H.) according to a method commonly employed for DNA transfer (40). The blot was washed with 0.9 M sodium chloride-0.09 M sodium citrate (pH 7.0) and then placed into 0.2% periodic acid for 1 h. Next, the acid solution was poured off, and Schiff reagent, prepared as previously described (12), was applied to the blot. Periodic acid-Schiff staining was allowed to proceed until an appropriate color change was observed, and the reaction was stopped with 7.5% acetic acid (Fisher Scientific, Newark, Del.).

(iv) Assay for the presence of Ig. Crude and purified samples were run on an SDS-PAGE gel (7.5% polyacrylamide) and then were blotted to Immobilon-P polyvinylidene difluoride (Millipore Corp., Bedford, Mass.) with a Bio-Rad Trans-Blot cell (50). The blot was blocked with 10% milk (Carnation Co., Los Angeles, Calif.) in TBS for 1 h at room temperature and then washed with TBS. For overall Ig content, the blot was probed with goat-derived anti-human Ig-alkaline phosphatase conjugate (Sigma). For IgA content, an identical blot was probed with rabbit-derived anti-human IgA (Boehringer Mannheim) followed by three washes with TBS and then goat-derived anti-rabbit Ig-alkaline phosphatase conjugate (Sigma). The blots were then developed with the appropriate chromogenic substrates.

Binding assay. (i) Quantitation of *S. aureus* adherence to mucin by colony counts. Staphylococcal adherence to mucin immobilized in microtiter wells was performed by a modification of previously described techniques (49). A 96-well microtiter plate (Nunc Inc., Roskilde, Denmark) was inoculated with 0.15 µg of purified nasal mucin (by protein content) in 100 µl of PBS and was kept overnight at 37°C (mucin-coated wells). Wells were then washed four times with PBS and were subsequently blocked, in parallel with empty control wells, with 2% bovine serum albumin (BSA) in PBS for 1 h. All wells were again washed four times with PBS prior to inoculation of bacteria.

An aliquot of an overnight bacterial suspension was diluted 1:50 in fresh THB and brought into logarithmic growth phase. Bacteria were then pelleted and resuspended in sterile PBS. The bacteria were declumped by repeated passages through a 25-G needle. The optical density at 620 nm (OD_{620}) of the bacterial suspension was adjusted to 0.250 with sterile PBS. Serial dilutions of the bacterial suspension were made, and 100 µl of each dilution was inoculated into microtiter wells in triplicate. The plates were incubated for 1.5 h at 37°C. Thereafter, the wells were emptied and washed four times with PBS. Adherent bacteria were lifted with 200 µl of 0.05% trypsin-0.5 mM EDTA in Hanks' balanced salt solution without calcium or magnesium (Gibco BRL) for 5 min at 37°C. After the initial volume of trypsin was collected, the trypsinization was repeated in each well. Serial 10-fold dilutions of the 400 µl of trypsin solution lifted from each well were made in sterile PBS. Aliquots of the appropriate dilutions from each well were inoculated, in duplicate, into heart infusion agar (Difco Laboratories), and pour plates were made. Colony counts were performed after incubation at 37°C for 36 to 48 h.

(ii) Colorimetric assay of *S. aureus* binding to mucin. The colorimetric assay was a modification of solid-phase binding assays done in prior studies with tritium-labelled bacteria (37, 41). Bacteria in logarithmic growth phase were labelled with biotin-NHS (Calbiochem, San Diego, Calif.) as previously described (48). The biotinylated *S. aureus* preparation was adjusted to an OD_{620} of 0.250 by dilution with PBS. A further 1:1 dilution of this solution with PBS approximated the inoculum that achieved saturation in our dose-response ex-

periments and was therefore used as the standard concentration in most experiments. A microtiter plate was inoculated with mucin and blocked with BSA in a manner identical to that of the pour plate assay described in the preceding section. One hundred microliters of the bacterial suspension was introduced into the wells. In most experiments, the bacteria were left in the wells for 1.5 h, because this was the point at which adherence was found to plateau. Wells were washed four times with PBS to remove nonadherent bacteria. One hundred microliters of a 1:4,000 solution of streptavidin-horseradish peroxidase conjugate (Gibco BRL) diluted in PBS was added to the wells. The plate was incubated for 1 h at 37°C and then washed four times with PBS. One hundred microliters of McIlvanie's buffer (pH 4.8) with 4.4 mM *o*-phenylenediamine and 0.5 µl of 30% hydrogen peroxide (Mallinckrodt Inc., Paris, Ky.) per ml was added to each well. The OD_{490} of the wells was read and recorded by the Biotek Automated Microplate Reader EL 311S (Biotek Instruments, Winooski, Vt.).

(iii) Determination of inoculum size in the colorimetric assay. We were unable to directly measure the inoculum size by colony count in the colorimetric assay because biotinylation rendered the *S. aureus* nonviable. In order to indirectly validate this parameter, bacteria were labelled with water-soluble sulfo-biotin-NHS (Calbiochem). The curves of OD_{620} versus CFU for labelled (sulfo-biotin-NHS) and unlabelled Wb were identical (data not shown). The binding pattern of the sulfo-biotin-NHS-labelled bacteria was also similar to that of the biotin-NHS-labelled specimens in the microtiter assay (data not shown). Bacterial morphology was also assessed microscopically after biotin-NHS labelling. The bacteria retained the crystal violet on gram staining and appeared morphologically normal after biotinylation.

(iv) Binding of staphylococci to crude mucus versus pure mucin. An aliquot of crude mucus was removed prior to the first CsCl density gradient ultracentrifugation. It was dialyzed against PBS and stored at 4°C. This sample was compared with mucin purified from the same specimen for bacterial binding in the colorimetric assay. Because mucus contains nonmucin proteins, quantitative anthrone and protein assays were performed with the crude and pure preparations. The pure mucin was introduced into microtiter wells in the usual quantity, and an equivalent amount of mucus (by carbohydrate content) was introduced into parallel wells for the bacterial binding assay.

Inhibition assays. (i) Bovine submaxillary mucin. The bacterial suspension was preincubated with bovine submaxillary mucin (Sigma; 10 to 500 µg/ml) for 1 h at 37°C prior to addition to the microtiter wells. As a control, bacteria were preincubated with identical concentrations of fibrinogen.

(ii) Carbohydrate inhibition. Prior to inoculation of bacteria into microtiter wells, a panel of potential inhibitors, including glucose, galactose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *N*-acetylneuraminic acid type VI (sialic acid) were added individually to yield a final concentration of 20 mM (37, 38). The suspensions were incubated in a gyrosaker at 37°C for 1 h. The wells were then inoculated with bacteria from these solutions, and the assay was performed as previously described.

(iii) TMU. Immediately prior to inoculation of bacteria into the wells, tetramethyleurea (TMU) was added to a final concentration of 0.5 M. Suspensions with and without TMU were directly compared in the binding assay.

Assays with modified mucin. (i) Periodate-treated mucin. After microtiter wells were coated with mucin, 100 µl of 25 mM sodium metaperiodate solution was added for 1 h at 37°C in the dark. Thereafter, the wells were washed four times with PBS and blocked with BSA, and the assay was completed in the usual fashion. In order to confirm that periodate treatment did not dislodge mucin from the wells, protein concentrations were determined prior to and after periodate treatment of the wells (Micro BCA kit; Pierce). These assays were performed prior to the addition of BSA. The protein content of periodate-treated wells was equivalent to that of untreated wells (data not shown).

(ii) Pronase-treated mucin. Purified nasal mucin (3.25 µg by protein content) in 1.3 ml of PBS was incubated in the presence of 6.5 µg of protease type XIV for 2 h at 37°C by an adaptation of the method of Sajjan et al. (38). After incubation, the pronase was inactivated with phenylmethylsulfonyl fluoride (2 mM). The solution was dialyzed against PBS at 4°C overnight. Aliquots of this sample were inoculated into microtiter wells for direct comparison with untreated purified mucin. In order to determine whether pronase itself could function as a binding ligand in the assay, control wells were run with the same quantity of pronase, but without any mucin.

(iii) Trypsin-treated *S. aureus*. *S. aureus* cells were preincubated with trypsin (100 µg/ml; Sigma) in PBS or with PBS alone for 1 h at 37°C in 1% BSA-coated Eppendorf tubes. The bacteria were then washed in PBS three times prior to adjustment of the OD and performance of the mucin binding assay (colony count method) as outlined above.

(iv) Preparation of solubilized bacterial proteins. Logarithmic-growth-phase bacteria were centrifuged at 3,000 rpm for 0.25 h, and the pellet was washed twice with 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-0.15 M NaCl (pH 7.2). The bacteria were then resuspended in the same buffer, supplemented with phenylmethylsulfonyl fluoride (1 mM), iodoacetamide (1 mM), and benzamidine (10 mM). Lysozyme (30 µg/ml; Applied Microbiology Inc., Brooklyn, N.Y.) was added, and the solution was incubated at 37°C for 0.5 h in a gyrosaker. DNase (10 µg/ml) and RNase (50 µg/ml) (both from Boehringer Mannheim) were added for a 1-h incubation. Finally, the solution was centrifuged at 16,000 rpm for 0.5 h. The supernatant, containing a crude

FIG. 1. Mucin analysis. (A) Silver stain of gels after SDS-PAGE of crude mucus (lane 1), purified mucin (lane 2), and commercial bovine submaxillary mucin (lane 3). (B) Periodic acid-Schiff stain of crude mucus (lane 1) and purified mucin transferred to nitrocellulose (lane 2).

preparation of *S. aureus* proteins plus protease inhibitors, was stored at 4°C for further use.

(v) **Labelling of surface proteins with sulfo-biotin-NHS.** Staphylococci were labelled with sulfo-biotin-NHS as previously described (48). The staphylococci were solubilized as described above. The final preparation represented a crude lysostaphin digest of *S. aureus* with sulfo-biotin-NHS-labelled surface proteins.

(vi) **Iodination of mucin.** Purified nasal mucin was iodinated (^{125}I ; Dupont-NEN Research, Boston, Mass.) by the *N*-chlorobenzenesulfonamide technique (18) with Iodobeads (Pierce) with 2 mCi of ^{125}I . Free iodine was removed by extensive dialysis against PBS at 4°C.

(vii) **Identification of *S. aureus* proteins that bind purified human nasal mucin.** A lysostaphin digest of *S. aureus* proteins was separated by SDS-PAGE on a 7.5% (wt/vol) acrylamide gel and then was blotted to polyvinylidene difluoride. Excess binding sites were blocked with 10% milk in TBS for 1 h at room temperature. After three washes with TBS, the iodinated mucin, diluted in TBS, was added to the blot. The blot and mucin were agitated for 2 h at room temperature, followed by an overnight incubation at 4°C. After the ^{125}I -labelled mucin solution was removed, the blot was washed extensively with PBS. Autoradiography of bound ^{125}I -mucin was performed with Kodak X-Omat AR film (Kodak, Rochester, N.Y.).

Statistical analysis. In the dose- and time-response experiments, the dose and time points yielding the highest mean OD were designated as 100% adherence. All other wells on the plate were normalized to this value. In all other colorimetric assays, the mean OD recorded from wells coated with untreated purified human nasal mucin and inoculated with the standard bacterial suspension of 9.1×10^6 bacteria in PBS was designated 100% adherence. All other wells on the plate were again normalized to this value. Each datum point represents the mean of at least two independent experiments, each done in triplicate, unless otherwise specified. Comparison of two means was performed with an unpaired Student's *t* test (two tailed) with the assistance of the Primer of Biostatistics: The Program computer package (McGraw-Hill Publishing, New York, N.Y.). The effect of mucin on bacterial adherence over a range of inocula was evaluated by an analysis of variance with the S-plus computer program (Math Soft, Inc.).

RESULTS

Analysis of mucin. The mucin preparations were subjected to several analyses in order to assure their purity. Analysis of the crude human nasal mucus by silver stain after SDS-PAGE demonstrated an abundance of protein components with discrete bands not visible (Fig. 1A, lane 1). Purified human nasal mucin was free of demonstrable protein bands (Fig. 1A, lane 2). Bovine submaxillary mucin (Sigma) contained five protein bands (Fig. 1A, lane 3). Periodic acid-Schiff staining of the pre- and postpurification steps also demonstrated a more homogeneous mucin product characteristic of the electrophoretic pattern of mucin glycoproteins (12) (Fig. 1B). The crude nasal mucus was also markedly positive for both IgA and Ig, while the purified specimen was negative (data not shown).

Microscopic studies. Giemsa-stained slides of desquamated anterior nasal epithelial cells incubated with *S. aureus* showed marked heterogeneity in bacterial binding to the cells. Some cells showed little or no binding, while others were heavily coated. Bacterial adhesion to acellular material was also noted. Mucicarmine staining was performed to determine whether binding correlated with the presence of mucin. These slides demonstrated avid *S. aureus* binding to cells and acellular material that stained positively by mucicarmine. Few bacteria bound to mucicarmine-negative surfaces (Fig. 2). A semiquantitative assay was performed to compare the binding capacity of *S. aureus* to mucicarmine-stained material or cells versus that to mucicarmine-negative cells. Staphylococcal binding to the mucin-positive material was significantly greater than to the unstained cells, with a mean ($\pm$ standard error) score of 3.6 ± 0.6 versus 2.1 ± 0.1 respectively ($P < 0.001$).

Scanning electron microscopy of nasal mucosal tissue incubated with *S. aureus* showed minimal bacterial adherence to ciliated mucosal cells after 0.5 h of incubation (Fig. 3A). Bacteria did bind to the collagen-rich basement membrane (Fig. 3B). These microscopic studies suggested that staphylococcal binding to mucus was critical. As a result, subsequent studies focused on this interaction.

Standardization of the in vitro binding assays. Two different adherence assays were utilized; a viability assay with colony counts and a colorimetric assay with biotinylated *S. aureus*. Results with the two assays were comparable. Initial experiments demonstrated that the number of *S. aureus* cells bound correlated directly with the quantity of pure human nasal mucin immobilized in the microtiter well (data not shown). A concentration of 0.15 μg of mucin per well was chosen for subsequent studies, because significant differences in binding over control wells could be demonstrated, and the use of this quantity allowed multiple experiments to be performed from a single mucin preparation.

Quantitative colony count assay. *S. aureus* binding to immobilized nasal mucin was significantly increased over binding to BSA-coated control wells. The mean percentage of the bacterial inoculum bound per mucin-coated well ranged from 2.1% (initial inoculum, 4.1×10^5 CFU per well) to 3.6% (initial inoculum, 1.4×10^7 CFU per well) in contrast with 0.03 to 0.44% for the BSA-coated wells (Fig. 4).

Colorimetric assay with biotinylated *S. aureus*. Further studies of *S. aureus* binding to immobilized mucin were done with biotinylated bacteria in a colorimetric assay. Bacterial inocula (calculated from OD measurements) ranging from 1.2×10^5 CFU per well to 1.8×10^7 CFU per well were introduced into mucin- and BSA-coated wells. The biotinylated *S. aureus* bound significantly better to the mucin-coated wells ($P < 0.001$ by analysis of variance). As with the previous assay, the difference in binding compared with control wells increased with increasing inocula until saturation, which occurred at 7.6×10^6 CFU per well or 5.1×10^7 CFU/ μg of mucin added (Fig. 5). Time-response assays with an initial bacterial inoculum of 9.1×10^6 CFU per well showed saturation at 1.5 h. Increased incubation time had no effect on the low-level binding to control wells (Fig. 6).

Binding of *S. aureus* to crude mucus and modified mucin. The purpose of this experiment was to assess the role of mucin versus other nonmucin components in mucus as mediators of staphylococcal adherence. The amounts of mucin glycoprotein coating each well were equal as measured by a quantitative anthrone assay. Levels of *S. aureus* binding to purified mucin and crude mucus were equivalent ($100\% \pm 3.0\%$ for pure mucin, $102\% \pm 13.7\%$ for crude mucus, and $44.5\% \pm 4.0\%$ for control wells) (Fig. 7). The minimal difference in binding to the

FIG. 2. Mucicarmine stain after incubation of *S. aureus* with scraped nasal epithelial cells. Scraped human nasal epithelial cells were coincubated with *S. aureus* at a final concentration of 9×10^7 CFU/ml for 1 h. The epithelial cells and adherent bacteria were collected thereafter on a 12- μ m-pore-size Nuclepore filter and then were resuspended in PBS. The pellet from this suspension was dried down on slides and subjected to mucicarmine staining. The staphylococci are shown adherent to the mucicarmine-stained (arrow) mucin in contrast with the unstained epithelial cells. Magnification, $\times 1,000$.

crude versus purified mucus, despite the demonstrated large quantities of nonmucin proteins, suggests that the role of these proteins in staphylococcal binding was of minor importance.

S. aureus cells adhered better to untreated than to periodate-treated human nasal mucin ($P < 0.001$). Periodate pretreatment of human nasal mucin resulted in an 86% reduction in binding (Fig. 7). Pronase treatment of human nasal mucin did not interfere with *S. aureus* attachment in the colorimetric assay. In fact, binding was increased in pronase-treated wells, as shown in Fig. 7. Pronase, by itself, did not function as an effective ligand for *S. aureus* adhesion (data not shown).

Adherence of different staphylococcal strains to mucin. The levels of adherence of 11 laboratory and clinical strains of *S. aureus* and 1 strain of *S. epidermidis* were compared by the colorimetric assay (Fig. 8). All bacteria were biotinylated and adjusted to an OD₆₂₀ of 0.250 and then were diluted 1:1 with PBS to achieve the usual working concentration. Nine strains of *S. aureus* as well as the lone strain of *S. epidermidis* (SUT) bound preferentially to mucin over BSA-coated wells (Fig. 8). Binding was variable, with some strains such as 502A, the strain used in bacterial interference studies (2), demonstrating a high level of adherence and other strains, such as the clinical isolate LS, being relatively nonadherent. The protein A-deficient WOOD strain was not an avid binder. To further evaluate the contribution of protein A to binding, the ability of the isogenic pair 8325-4 (protein A positive) and DU5723 (protein A negative) to bind mucin was compared. Binding to mucin

was 2.3 ± 0.4 versus 2.0 ± 0.6 for 8325-4 versus DU5-723, respectively ($P > 0.05$).

Characterization of the staphylococcus-mucin binding interaction. The specificity of the binding interaction was further examined by comparing the ability of a related mucin versus fibrinogen, an unrelated glycoprotein, to block binding. Preincubation of staphylococci (9.1×10^6 CFU per well) with various concentrations of bovine submaxillary mucin (0 to 50 μ g/ml) resulted in a dose-related decrease in *S. aureus* binding to mucin-coated wells that did not occur with identical concentrations of fibrinogen (data not shown).

TMU, a potent disruptor of hydrophobic interactions (27), was introduced into the assay in order to evaluate the importance of hydrophobic interactions in *S. aureus*-mucin adherence. TMU (0.5 M) was added at the time of bacterial inoculation into the microtiter wells. TMU significantly increased binding, causing a 3.7-fold increase in adherence at a bacterial inoculum of 9.1×10^6 CFU per well ($P = 0.003$). These findings suggest that hydrophobic bonds are not responsible for *S. aureus* adherence to mucin.

A panel of six monosaccharides and disaccharide derivatives were preincubated with biotinylated bacteria prior to inoculation into microtiter wells. None of these haptens significantly inhibited *S. aureus* binding to mucin (Table 1). Preincubation with sialic acid did not alter bacterial adhesion to mucin-coated wells but did increase binding threefold in the control wells.

FIG. 3. Scanning electron micrograph of *S. aureus* incubated with human nasopharyngeal tissue. A surgical specimen of human nasal mucosal tissue was coincubated in a suspension containing *S. aureus* cells at a concentration of 10^9 CFU/ml for 1 h. After a washing to remove nonadherent bacteria, scanning electron microscopy was performed. (A) *S. aureus* did not bind to the ciliated epithelial side of the tissue. Magnification, $\times 3,000$. (B) Abundant staphylococci bound to the submucosal surface. Magnification, $\times 5,000$.

Effect of trypsin treatment on *S. aureus* adherence to mucin. Pretreatment of Wb with trypsin (100 μ g/ml for 1 h at 37°C) resulted in a significant decrease in adherence to mucin. The levels of adherence to mucin were 2.6% versus 0.25% for untreated versus trypsin-treated bacteria, respectively ($P < 0.001$). Binding to BSA was also reduced, 0.7% versus 0.2%.

TABLE 1. Carbohydrate inhibition study results^a

Sugar	% Binding to ^b :	
	Mucin-coated wells	BSA-coated wells
Control	100 $\pm$ 11.2	41.2 $\pm$ 20.6
Glucose	92.7 $\pm$ 25.8	41.7 $\pm$ 11.7
Galactose	86.1 $\pm$ 18.3	55.5 $\pm$ 22.5
Mannose	73.6 $\pm$ 23.3	54.6 $\pm$ 38.7
N-Acetylgalactosamine	94.6 $\pm$ 13.5	39.7 $\pm$ 16.7
N-Acetylglucosamine	91.6 $\pm$ 7.5	28.3 $\pm$ 20.1
N-Acetylneuraminic acid (sialic acid)	100 $\pm$ 9.3	120 $\pm$ 80.8

^a Biotin-labelled *S. aureus* Wb cells (9.1×10^6 CFU per well) were preincubated with six different sugars (20 mM) for 1 h prior to performance of the colorimetric adherence assay. Binding in the presence of sugar haptens was normalized against the wells containing *S. aureus* without additional sugars.

^b Each value represents the mean $\pm$ standard error of two independent experiments, each performed in triplicate.

Identification of staphylococcal proteins that bound ¹²⁵I-labelled nasal mucin on Western blotting (immunoblotting). Staphylococci were solubilized with lysostaphin, separated by SDS-PAGE, and blotted to polyvinylidene difluoride. The separated proteins were then probed with ¹²⁵I-mucin. The mucin bound to two proteins with molecular masses of 138 and 127 kDa (Fig. 9). Of these, the 138-kDa protein was the dominant band. Similar banding patterns were observed with the strains Wb, LS, and 502A. A surface-biotinylated Wb protein preparation was run in parallel with the nonbiotinylated protein to determine if these proteins were on the bacterial surface. The iodinated mucin bound to two protein bands with similar sizes that were biotin labelled (Fig. 9). These findings suggest that the two mucin binding proteins reside on the bacterial cell surface.

DISCUSSION

The present study provides evidence that a specific interaction between *S. aureus* and purified human nasal mucin is a critical part of nasal colonization. Three modalities were used to demonstrate and define the properties of microbial adherence. First, bacterial suspensions incubated with nasal epithelial cells and crude nasal mucus demonstrated avid binding of *S. aureus* to both cell-bound and cell-free mucus. Electron microscopy demonstrated the failure of *S. aureus* cells to bind to human nasal ciliated respiratory epithelium. Second, two

FIG. 4. Dose-response study of staphylococcal adherence to human nasal mucin by the pour plate assay. Microtiter wells were coated with 0.15 μ g of pure human nasal mucin and then blocked with 2% BSA. Control wells were blocked with 2% BSA. *S. aureus* Wb cells were introduced into the wells at various concentrations and incubated in the wells for 1.5 h. After extensive washing, adherent bacteria were removed with trypsin. Pour plates were made, and adherence was measured by colony counts. Each point represents the mean of three wells $\pm$ standard error. ●, mucin-coated wells; ○, BSA-coated wells. The lines reflect a logarithmic transformation of the data.

solid-phase adherence assays were used to define the dynamics of the interaction between *S. aureus* and mucin, the major component of mucus. Third, a Western blot overlay assay with radioiodinated nasal mucin was used to identify the *S. aureus* surface proteins that bind mucin.

In our binding assays, *S. aureus* adherence to immobilized, purified human nasal mucin was saturable in a dose- and time-dependent manner. Binding to crude nasal mucus was not increased compared with purified mucin. TMU, an agent that interferes with nonspecific hydrophobic bonds, increased bacterial attachment to mucin, perhaps as a consequence of conformational changes. Enzymatic pretreatment of bacteria with

FIG. 5. Dose-response study of staphylococcal adherence to human nasal mucin by colorimetric assay. Microtiter wells were coated with 0.15 μ g of pure human nasal mucin and then blocked with 2% BSA. The control wells were blocked with 2% BSA. Various concentrations of biotinylated *S. aureus* Wb cells were introduced into the wells and were incubated for 1.5 h. After extensive washing, the wells were probed with streptavidin-horseradish peroxidase conjugate, followed by the appropriate chromogens. Adherence was measured by OD in an enzyme-linked immunosorbent assay reader. Each point represents the mean $\pm$ standard error of two independent experiments performed in duplicate. ●, mucin-coated wells; ○, BSA-coated wells.

FIG. 6. Time-response assay of staphylococcal adherence to human nasal mucin. The bacterial inoculum was 9.1×10^6 CFU per well. Adherence was measured with the colorimetric assay. Each point represents the mean $\pm$ standard error of two independent experiments performed in triplicate. ●, mucin-coated wells; ○, BSA-coated wells.

trypsin significantly reduced adherence. Bovine submaxillary mucin, but not fibrinogen, functioned as a specific inhibitor of bacterial adherence in a dose-dependent fashion. All of these observations support the view that *S. aureus* adherence to human nasal mucin is important in the colonization process and that it is mediated by a specific protein adhesin-receptor interaction.

Eleven different laboratory and clinical isolates of *S. aureus* were tested for their adherence potential and showed variable rates of adhesion. Western blots of three strains, including LS, a nonadherent strain, showed similar banding patterns of proteins that bound 125 I-labelled nasal mucin. The differential expression and/or surface presentation of an adhesin among strains may dictate each strain's binding potential. The presence of a staphylococcal collagen-binding protein has been

FIG. 7. Binding of *S. aureus* to crude mucus and to modified pure mucin. Pure nasal mucin (0.15 μ g) was inoculated into microtiter wells. Crude nasal mucus was likewise inoculated in equivalent quantities as determined by the quantitative anthrone assay. The wells received 0.15 μ g of pure mucin per well. Modified mucins consisted of pure nasal mucins that were pretreated with pronase (5 μ g/ml) or periodate (25 mM) as described in Materials and Methods. Control wells were blocked with 2% BSA. Each bar represents the mean $\pm$ standard error of four independent experiments done in triplicate, except the pronase bar, which represents two independent experiments. Binding is compared with that of the purified mucin in the colorimetric assay.

FIG. 8. Binding of staphylococcal strains as determined with the colorimetric binding assay. Adherence of 11 different clinical and laboratory isolates of *S. aureus* and 1 strain of *S. epidermidis* to human nasal mucin (●) versus adherence to BSA (○) is shown. Adherence of the strains was compared with that of Wb as determined by repeated measurements in the colorimetric assay. Each point represents the mean $\pm$ standard error of two independent experiments done in triplicate.

positively correlated with a propensity to cause bone and joint infections (45). de Lencastre et al. (9) demonstrated that the ability of a strain to colonize patients led to the replacement of a diverse *S. aureus* population by a single clone of methicillin-resistant *S. aureus* in a Portuguese hospital. This clone was subsequently responsible for an outbreak of wound infections at this institution (9). The variable expression and presentation of adhesins among different isolates may help explain differences in clinical behavior that have been observed among isolates of staphylococci.

In evaluating binding, we found that periodate pretreatment of the mucin decreased binding. Pronase, which digests the glycoprotein at locations at which the polypeptide backbone is exposed (22, 38), increased attachment, suggesting that conformational changes in the structure of the mucin glycoprotein may affect binding. These findings suggest that the binding site on mucin involves carbohydrate moieties (38).

The nasal mucosa is a biologically complex environment. The epithelium includes squamous cells with various degrees of keratinization, transitional cells, ciliated respiratory epithelium, and mucus-secreting goblet cells (1). Overlying the cells is a layer of mucus which serves numerous biological roles. The respiratory mucus layer protects against bacteria by trapping them and exporting them out of the body through mucociliary clearance. Respiratory mucins may, however, become a sanctuary for the carriage and growth of pathogens, as has been suggested for *Pseudomonas* species in cystic fibrosis patients (35, 37, 38). Differences in the content of the nasal mucin may play a role in the colonization process. Thomas et al. (46) demonstrated calcium-dependent and -independent binding of *S. aureus* to bovine submaxillary mucin and identified three *S. aureus* proteins (40, 35, and 29 kDa) that bound mucin. The molecular masses of the proteins and the pronase sensitivity of the mucin ligand suggest that this interaction is distinct from

the *S. aureus*-human nasal mucin interaction described in the present study.

It is clear from epidemiologic studies that *S. aureus* can establish an ecological niche in the human nares. Aly et al. proposed that *S. aureus* binds to squamous nasal epithelial cells and implicated teichoic acid as a potential adhesin (3, 4, 6). Schwab et al. (43) showed that *S. aureus* binding to airway epithelium, including nasal cells (ciliated and squamous), was sensitive to protease treatment of the bacteria. Sanford et al. (41) studied *S. aureus* nasal colonization in a ferret model and found minimal adherence to respiratory epithelial cells but avid binding to the overlying mucus layer. A specific interaction of *S. aureus* with crude ferret mucus, as well as commercially available bovine submaxillary mucin, was demonstrated previously (41).

Other substances found in the respiratory tract, including secretory IgA (7) and respiratory glycolipids (14), have been implicated as potential ligands for *S. aureus*. If IgA or lipids were the receptor, one would expect higher levels of binding to crude mucus than to purified mucin, since the purification process removes all detectable Ig and glycolipid (38). *S. aureus* adherence to the human nasal mucosa is a complex process mediated by multiple bacterial adhesins and host receptors. Our finding of equivalent binding of *S. aureus* to purified mucin and crude mucus suggests that the bacterium-mucin interaction is of primary importance.

The recognition that nasal colonization with *S. aureus* is a prelude to invasive infection has led to numerous attempts at eradication of the carrier state with trials of topical, oral, and parenteral antibiotics (10, 58). These attempts have met with limited success secondary to the persistence of the organism

FIG. 9. Identification of staphylococcal proteins that bind 125 I-labelled pure nasal mucin. Lanes: 1, lysostaphin-solubilized staphylococcal proteins (Wb strain) separated in an SDS-PAGE gel (7.5% polyacrylamide) and stained with Coomassie blue; 2, biotin-labelled surface staphylococcal proteins separated on the same SDS-PAGE gel, transferred to polyvinylidene difluoride, probed with streptavidin-alkaline phosphatase conjugate, and developed with the appropriate chromogens; 3, autoradiogram of the Western blot of staphylococcal proteins from the same gel probed with 125 I-nasal mucin, demonstrating adherence of the 127- and 138-kDa staphylococcal proteins to pure nasal mucin.

despite in vitro susceptibility to the antibiotic being used (56), recurrence soon after the discontinuation of therapy (23), or the emergence of resistant strains (17, 58). Researchers have investigated other means of combatting this problem. Repopulation studies with the "avirulent" strain 502A were stopped when the strain caused invasive disease (11). Intranasal instillation of Ig preparations has proven effective in preventing pneumonia in animal models of *S. aureus* (34) and *Streptococcus pyogenes* (5).

Multidrug resistance in *S. aureus* adds urgency to the quest for novel approaches for the prevention and treatment of staphylococcal infections (30, 47). The demonstration of a specific interaction between *S. aureus* and human nasal mucin and the identification of a putative bacterial adhesin or adhesins provide another potential target for intervention in the colonization process.

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