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Group A Streptococci Bind to Mucin and Human Pharyngeal Cells through Sialic Acid-Containing Receptors

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The first step in the colonization of group A streptococci (*Streptococcus pyogenes*) is adherence to pharyngeal epithelial cells. Prior to adherence to their target tissue, the first barrier that the streptococci encounter is the mucous layer of the respiratory tract. The present study was undertaken to characterize the interaction between mucin, the major glycoprotein component of mucus, and streptococci. We report here that *S. pyogenes* is able to bind to bovine submaxillary mucin in solid-phase microtiter plate assays. Western blots probed with ^{125}I-labeled mucin and a panel of monoclonal antibodies revealed that the streptococcal M protein is one of two cell wall-associated proteins responsible for this binding. The binding was further localized to the N-terminal portion of the M molecule. Further analysis revealed that the M protein binds to the sialic acid moieties on mucin, and this interaction seems to be based on M-protein conformation rather than specific amino acid sequences. We found that sialic acid also plays a critical role in the adherence of an M6 streptococcal strain to the Detroit 562 human pharyngeal cell line and have identified α2-6-linked sialic acid as an important sialylated linkage for M-protein recognition. Western blot analysis of extracted pharyngeal cell membrane proteins identified three potential sialic acid-containing receptors for the M protein. The results are the first to show that sialic acid not only is involved in the binding of the streptococci to mucin but also plays an important role in adherence of group A streptococci to the pharyngeal cell surface.

Group A streptococci (*Streptococcus pyogenes*) are the causative agents of numerous infections, such as acute pharyngitis and impetigo, and are associated with the poststreptococcal sequelae, rheumatic fever and glomerulonephritis. The upper respiratory pharyngeal mucosa is the primary site of adherence and colonization by these organisms and a number of their surface proteins have been shown to be important in this process (5, 7, 8, 21, 22). One of these proteins, the M protein (for a review, see reference 16) is a fibrillar molecule that is considered to be the major virulence factor of *S. pyogenes* because it renders these organisms resistant to phagocytosis (16, 27, 36) and is involved in the adherence to pharyngeal tissue (14, 51). The epitopes with which the M protein interacts on the pharyngeal cell surface and the details of the complex processes involved in M-protein-mediated adherence have yet to be fully elucidated. There is a large body of work on the interaction of group A streptococci with pharyngeal epithelial cells and the virulence factors, other than the M protein, which are necessary to initiate infection (1, 46, 49, 51). Many of these reports have focused on the adherence of streptococci to various glycoproteins, including fibronectin (30, 31, 40, 49), plasminogen (34), and collagen (9) and on the interaction of the streptococcal capsular polysaccharide to hyaluronic acid binding proteins on epithelial cells (43). Although much is known about the streptococcal adhesins involved in these interactions, the identity of the epitope(s) on the glycoproteins that is responsible for binding has also not been fully defined.

Despite numerous reports on streptococcal adherence to various glycoproteins, no information is available on the interaction of these organisms with mucin, the major glycoprotein component of respiratory tract mucus. By virtue of its anatomical location, the mucous layer coating all mucous membranes is the first major barrier encountered by nearly all pathogens, including group A streptococci, and how they deal with the mucous layer has not been fully explained.

The function of respiratory tract mucus is the entrapment of invading microorganisms and particulate matter to prevent pulmonary infection. Mucus consists of a major glycoprotein, mucin, in addition to several other components such as serum glycoproteins, lipids, and immunoglobulins (47, 56). Mucins are complex, carbohydrate-rich glycoproteins secreted by mucosal and submucosal glands and are generally subdivided into two major types, a secretory soluble type and a membrane-bound type (see references 47 and 48 for reviews). Structurally, the secretory mucin that coats the upper respiratory mucosa has been shown to be similar to the structure of cell surface glycoproteins on the underlying tissue (2).

Numerous bacterial pathogens such as *Pseudomonas aeruginosa* (37, 38, 54, 55), *Pseudomonas cepacia* (42), *Staphylococcus aureus* (44), and *Haemophilus influenzae* (11) have been shown to bind to mucin; however, the adhesins involved have not been well characterized and the actual mucin binding epitopes of these proteins have not been identified. Although *S. pyogenes* is a common upper respiratory tract pathogen, the details of its interaction with mucins have not been previously investigated.

The aim of this work was to understand the interaction between group A streptococci and mucus and, in particular, mucin. Because secretory mucin is similar in structure to cell surface glycoproteins, we hoped that by understanding the interaction between streptococci and mucin we could use this

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model system to elucidate some of the receptors on pharyngeal epithelial cells that are involved in adherence. We report here that the streptococcal M protein binds to bovine mucin and have identified sialic acid as the monosaccharide ligand on mucin. Additionally, through this work we found that sialic acid plays a critical role not just in the binding of streptococci to mucin but in the adherence of this organism to cultured pharyngeal cells.

MATERIALS AND METHODS

Bacterial strains and cell lines. Group A streptococcal strains D471 (M type 6) from the Rockefeller University culture collection and the isogenic M-negative mutant JRS75 (29) were grown at 37°C for 16 h in Todd-Hewitt broth (Difco Labs, Detroit, Mich.) supplemented with 1% yeast extract. These strains do not produce measurable capsule under these growth conditions. The bacterial concentration was determined spectrophotometrically, and viability counts were performed by plating on proteose peptone agar supplemented with 5% sheep's blood. An optical density (OD) of 1.0 at 660 nm corresponds to approximately 10^8 bacterial CFU/ml.

For studies examining the binding of various M-protein types to mucin, strains representing the class I and class II M protein serotypes (3, 4) were obtained from the Rockefeller collection and grown under the conditions described above. The following strains were used: representing class I, D710 (M1), 2RP19 (M5), 22RSS72 (M24), and 10R5101 (M32); representing class II, IRP256 (M2), D339 (M9), D691 (M11), and A945 (M49).

The Detroit 562 (ATCC CCL-138) human pharyngeal cell line obtained from the American Type Culture Collection (Manassas, Va.) was grown and maintained at 37°C in 5% CO_2 in minimal essential medium (MEM) (Gibco-BRL, Life Technologies, Grand Island, N.Y.) supplemented with 10% (vol/vol) fetal bovine serum and 1 mM sodium pyruvate as described before (32).

Chemicals and reagents. Chemicals and reagents were obtained from the Sigma Chemical Co. (St. Louis, Mo.) unless otherwise mentioned.

Proteins and antisera. The monoclonal antibodies (MAbs) 10A11 and 10B6, which are specific for the N- and C-terminal regions of the M protein, respectively, were available in our laboratory (26). The recombinant M6 protein was purified from *Escherichia coli* as described by Fischetti et al. (18). The N- and C-terminal portions of the recombinant M protein were isolated after pepsin digestion of the molecule as described previously (18). A specific sialic acid binding lectin from *Tritrichomonas mobiliensis* (TML) and an anti-TML monoclonal antibody were obtained from Calbiochem-Novabiochem Corp. (San Diego, Calif.).

Preparation and radioiodination of mucin. Mucin samples used in this study were partially purified by gel exclusion chromatography to obtain a single protein band on sodium dodecyl sulfate (SDS)-polyacrylamide gels using the following protocol. Bovine submaxillary mucin (10 mg) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) was applied to a Sephadex G-200 column (PD-10, 9.1-ml bed volume; Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) equilibrated in 0.1 M PBS to remove low-molecular-weight contaminants. The proteins in the void volume of the column (2.5 ml), which consist of the high-molecular-weight mucins, were collected. The protein content of the fractions was determined with the Bio-Rad (Hercules, Calif.) protein assay reagent, using the procedure outlined by the manufacturer and bovine serum albumin (BSA) as the standard. Fractions of the void volume containing the highest protein concentration were radioiodinated with $Na^{125}I$ (17 Ci/mg; NEN Life Science Products, Boston, Mass.) by the chloramine-T method, using Iodobeads (Pierce Chemical Co., Rockford, Ill.) as described (32). Labeled proteins were separated from free iodine by passage over a Sephadex G-25 column (PD-10; Pharmacia) equilibrated in 0.1 M PBS, pH 6.5. The protein concentration was determined as described above. Purity of the labeled mucin was confirmed by autoradiography of SDS-polyacrylamide gels. After labeling, the specific activity of the mucin sample was 1.5×10^5 cpm/μg.

Solid-phase mucin binding assay. To assess the ability of group A streptococci to bind to bovine submaxillary mucin, two solid-phase assays were performed.

(i) Immobilized mucin assay. A modified solid-phase mucin binding assay was performed with bovine mucin as described previously (54). Preliminary experiments (using iodinated mucin) revealed that 400 ng of the bovine mucin per well was the optimum concentration needed to coat the wells of Maxisorp plates with BreakApart modules (Nunc, Naperville, Ill.). To ensure that all of the sites were bound, 1 μg of the partially purified bovine mucin (prepared as described above) was added to wells of microtiter plates (in duplicate), and the plates were incubated for 24 h at 37°C. Overnight bacterial cultures of D471 were pelleted by centrifugation (1,100 × *g* for 10 min at 4°C), washed twice in PBS, and adjusted to an OD of 1.0 at 660 nm. Triplicate wells in both plates were inoculated with 5×10^6 bacteria in a volume of 100 μl. Wells that contained only BSA served as controls. The microtiter plates were incubated for 1 h, one plate at 37°C and the second plate at 30°C. Following this incubation, the supernatant containing the unbound bacteria was collected. The wells were washed 8 to 10 times with sterile PBS, and each wash was collected, pooled with the unbound streptococci, and plated on blood agar. Bound bacteria were desorbed with 250 μl of 0.5% Triton X-100 in sterile PBS for 30 min at room temperature and plated on blood agar. The plates were incubated at 37°C for 12 to 14 h, and the colonies were counted. Preliminary experiments revealed that streptococcal adherence to BSA was significantly less than that to mucin and not an effect of differences in desorption by Triton X-100.

(ii) Immobilized bacterial assay. An overnight culture of strain D471 was adjusted to an OD of 1.0 at 660 nm, washed twice in PBS, and heat killed at 55°C for 4 h. This procedure was necessary to perform since streptococci secrete numerous enzymes which could potentially interfere with these initial binding assays. The bacterial suspensions were slowly cooled to room temperature to preserve the conformation of surface-exposed molecules. Previous studies from our laboratory have shown that after subjecting streptococci to this treatment, the reactivity of antibodies to surface-exposed proteins and epitopes is retained. Poly-L-lysine was prepared in PBS (100 μg/ml), and 100-μl aliquots were added to the wells of Maxisorp microtiter plates with BreakApart modules and incubated for 1 h at room temperature. The poly-L-lysine was aspirated, the wells were washed three times with PBS, and the heat-killed bacteria were added (5×10^6 CFU in a volume of 50 μl) followed by the addition of 50 μl of a dilute (2%) glutaraldehyde solution prepared in PBS. Following a 20-min incubation period at room temperature, the plates were centrifuged (1,500 × *g*) for 20 min and the wells were washed twice with sterile PBS. A 100-μl aliquot of 0.1 M lysine solution was added to each well of the plate, and the plate was incubated at room temperature for 1 h to block excess glutaraldehyde sites. The wells were again washed with PBS, and residual protein binding sites were blocked with 2% BSA in 10 mM Tris-HCl overnight at 4°C. Various concentrations of ^{125}I-bovine mucin were added to the wells, and the plates were incubated for 4 h at room temperature. All concentrations of mucin were tested in triplicate wells. Wells that did not contain immobilized bacteria served as control. Following this incubation, the wells were washed three times with PBS and the radioactive counts in both the wash buffer (representing free mucin) and the wells (representing bound mucin) were determined in a gamma counter.

Identification of streptococcal cell wall proteins that bind mucin. Crude extracts of the cell walls of strains D471 and JRS75 were prepared using the amidase enzyme lysin as described previously (20). Cell wall extracts of all other class I and class II strains were prepared using the enzyme mutanolysin (25). All extracts were prepared in 30% raffinose to stabilize the protoplasts after the cell walls were removed. Proteins in the streptococcal cell wall extracts were subjected to electrophoresis and Western blotting techniques as described before (41). The streptococcal cell wall extracts and the recombinant M6 protein were separated (in triplicate) on SDS–8% polyacrylamide gels and were either visualized by Coomassie stain or transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore Corp., Bedford, Mass.). Blots were probed with radiolabeled mucin as described before (34). Duplicate membranes to be probed with antibodies were first blocked with BSA (3% in PBS) and then incubated with the anti-M-protein-specific antibody, 10B6 (26). Bound antibody was visualized with alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin G, followed by the substrate 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Localization of the mucin binding region of the M protein. The N- and C-terminal portions of the recombinant M6 protein from *E. coli* were isolated after pepsin digestion, separated (in duplicate) by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to PVDF membranes. One set of Western blots was probed with MAb 10A11 (reactive with the N-terminal portion of the M protein) or MAb 10B6 (reactive with the C-terminal portion of the M protein) (26) and developed as described above. The duplicate blot was probed with ^{125}I-bovine mucin in order to localize the mucin binding region of the M molecule.

Identification of the component of mucin to which streptococci bind. To determine if any of the individual sugar components of mucin were important in the adherence of streptococci, the five monosaccharide constituents of the oligosaccharide side chains of mucin, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), *N*-acetylneuraminic acid (sialic acid, NANA), D-galactose and L-fucose (47, 56), were used in a solid-phase assay.

Wells of microtiter plates were coated with bovine mucin as described above.

Fifty millimolar solutions of GlcNAc, GalNAc, galactose, and fucose were prepared in PBS, whereas sialic acid (50 mM) was prepared in 10 mM Tris-HCl buffered to pH 6.9 (55) Suspensions (500 μl) of strain D471 prepared in either PBS or Tris-HCl were mixed with an equal volume of the individual sugar solutions to yield 5×10^7 CFU/ml in 25 mM sugar solutions. Bacteria added to PBS or Tris-HCl without sugar served as the control. The mixtures were incubated for 30 min at 37°C to allow bacteria to bind to the monosaccharide. The bacteria were then pelleted by centrifugation, washed three times, and resuspended to the original volume in the same buffer. Samples (100 μl) of each bacterium-buffer or bacterium-sugar suspension were added to mucin-coated wells to yield a final concentration of approximately 5×10^6 CFU/well. The mucin adherence assay was continued as described above. An additional set of mucin-coated wells was inoculated with sialic acid-treated D471 and incubated at 30°C to assess the effect of this temperature on adherence.

Pharyngeal cell adherence and invasion assay. Streptococcal adherence to and invasion of the human pharyngeal cell line, Detroit 562, was assayed by modification of a procedure described previously (50). Overnight cultures of streptococci were pelleted by centrifugation and washed twice in sterile PBS. The cultures were resuspended in MEM and diluted to a final concentration of 5×10^7 CFU/ml, and 1-ml aliquots were inoculated into each well containing washed Detroit cell monolayers. At least three wells were used for each bacterial strain or culture condition. Following a 2.5-h incubation period with the bacteria at 37°C, the monolayers were washed three times in PBS.

For the bacterial invasion assay, 1 ml of MEM supplemented with penicillin (10 μg/ml) and gentamicin (200 μg/ml) was added to each well (to kill extracellular bacteria) and incubated for 1 h at 37°C. For wells used to determine total numbers of adherent and invasive bacteria, MEM without antibiotics was added to each well and incubated for an additional hour. Following the incubation, the pharyngeal cells were detached from the wells by the addition of 100 μl of 0.025% trypsin–0.02% EDTA and lysed with 400 μl of 0.025% Triton X-100. The lysates were diluted appropriately and plated on blood agar. The total number of adherent streptococci was calculated as the difference between the total number of adherent and invasive bacteria and the number of invasive bacteria alone.

Effect of monosaccharides on streptococcal adherence to pharyngeal cells. The effect of various monosaccharides on streptococcal adherence to pharyngeal cell monolayers was assessed in inhibition assays. Solutions of GlcNAc, GalNAc, and fucose were prepared in PBS, whereas sialic acid was prepared in 10 mM Tris-HCl buffered to pH 6.9 (55). Suspensions of strains D471 and JRS75 prepared in either PBS or Tris-HCl were mixed with the individual sugar solutions to yield 5×10^7 CFU/ml in 25 mM sugar solutions. In separate experiments, strain D471 (5×10^7 CFU) was preincubated with various concentrations of sialic acid (25, 10, 2, 1, and 0.5 mM) prior to inoculating pharyngeal cell monolayers. Bacteria added to PBS or Tris-HCl without sugar served as the control. Following preincubation with the individual sugars, the bacteria were washed, resuspended in MEM, and added to monolayers of pharyngeal cells (5×10^7 CFU/well). The adherence assay was continued as described above.

Sialylated compounds. The sialylated compounds, fetuin and transferrin, as well as NeuAcα2-3Galβ1-4Glc (3′sialyllactose [3′SL]) and NeuAcα2-6Galβ1-4Glc (6′SL) were tested in inhibition assays to determine the effect of each compound on streptococcal binding to pharyngeal cells. Streptococci (5×10^7 CFU) were separately incubated (in a final volume of 1 ml) with sialic acid (25 or 2 mM), 6′SL (1.7 mM), 3′SL (1.7 mM), fetuin (5 mg, 1.8 mM total sialic acid), or transferrin (5 mg) (all prepared in 10 mM Tris-HCl, pH 7.4) for 30 min at 37°C. Following this treatment, the bacteria were washed twice, resuspended in MEM, and added to the pharyngeal cell monolayers. Bacteria treated with buffer alone served as control. An aliquot of each of the treated bacterial samples was plated onto blood agar to ensure that the treatment had no effect on the viability of organism.

Neuraminidase treatment of pharyngeal cells. Confluent monolayers of Detroit 562 pharyngeal cells (in 24-well tissue culture plates) were washed three times with PBS and then treated with 1 U of *Clostridium perfringens* neuraminidase (Sigma) in 50 mM Na acetate (pH 5.5) buffer containing 5 mM $CaCl_2$. Cells incubated in buffer alone served as a control. Cells were incubated for 30 min at 37°C, washed three times with PBS, and used in the adherence assay as described above. The neuraminidase from *C. perfringens* cleaves, at decreasing rates, sialic acid linked α2-3, α2-6, and α2-8 (45).

Preparation of Detroit 562 pharyngeal cell membranes. Detroit 562 cells were grown to confluence and washed extensively with PBS. The adherent cells were scraped with a disposable rubber cell scraper into PBS, pelleted by centrifugation for 10 min at 1,100 × *g*, and resuspended in lysis buffer (10 mM NaH_2PO_4, pH 8, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Cells were disrupted by sonication (5-s pulses for 2 min), and cellular debris was removed by centrifugation (2,000 × *g* for 10 min). The membrane fraction was pelleted by ultracentrifugation at 100,000 × *g* (60 min at 4°C) and then resuspended in 0.2% Triton X-100 and stored at −20°C until further use.

Identification of sialylated proteins on pharyngeal cells which bind M protein. The sialic acid binding lectin, TML, was employed to identify the pharyngeal cell membrane proteins that contain sialic acid. Pharyngeal cell membrane proteins were separated by SDS-PAGE, blotted in duplicate to PVDF membranes, separately probed with the M protein (50 to 100 μg) and TML (1 to 2 μg) for 2 h at 37°C, and then washed three times with PBS. Bound M protein and lectin were detected by incubating the blots with the M-protein-specific MAb 10B6 and with the anti-TML MAb, respectively, for 2 h at 37°C. After washing, bound antibody was visualized using alkaline phosphatase-conjugated secondary antibodies and the 4-nitroblue tetrazolium chloride–BCIP substrate system described above.

Statistical analysis. Differences between groups in each experiment were determined by Student's *t* test. The results are expressed as means ± standard deviation.

RESULTS

Binding of whole streptococci to mucin. The ability of *S. pyogenes* D471 to adhere to bovine mucin was examined in a solid-phase assay using mucin-coated microtiter wells. As shown in Fig. 1, the binding of strain D471 to bovine mucin was significantly higher than that to BSA at both 37 and 30°C. To determine the specific binding activity of streptococci to mucin, a quantitative solid-phase assay was employed, in which strain D471 (M6 serotype) was first immobilized to the microtiter wells and then various concentrations of radiolabeled mucin were added. As can be seen, this strain was able to bind mucin in a dose-dependent manner (Fig. 1, inset).

Identification of a binding protein in streptococcal cell wall extracts. To determine which proteins on the surface of group A streptococci are capable of binding to mucin, cell wall-associated proteins from streptococcal strain D471, released after lysin digestion, were probed with radiolabeled bovine mucin. This analysis revealed two proteins (57 and 40 kDa) that bound mucin (Fig. 2A). The size and migration pattern of the 57-kDa protein suggested that it might be streptococcal M protein; therefore, an M-protein-specific antibody and the recombinant M protein were included in a second analysis. A blot of cell wall-extracted proteins probed with the M-protein-specific MAb 10B6 confirmed the identity of the 57-kDa protein as the M protein (Fig. 2B). In addition, the cell wall proteins extracted from the isogenic M-negative mutant, JRS75, subjected to the same analysis, did not contain the 57-kDa protein present in the extracts of strain D471, further confirming the identity of the 57-kDa protein as the M protein. Characterization and identification of the 40-kDa protein are currently in progress.

The cell wall-extracted proteins from eight different streptococcal serotypes, representing both class I and class II strains, were subjected to the same SDS-PAGE and Western blot analyses. The M protein from each strain was able to bind iodinated mucin (not shown), indicating that the mucin binding property of the M protein is ubiquitous in nature and is not restricted to serotype or class designation.

Localization of the mucin binding region in the M protein. To determine if the mucin binding region of the M protein could be localized to the N- or C-terminal half of the molecule, the recombinant M6 molecule was digested with the enzyme pepsin. At suboptimal pH, pepsin cleaves the molecule twice, once around its center and once within the C-terminal domain (19). The resulting three fragments, which represent essentially the intact N-terminal half and two C-terminal segments of the

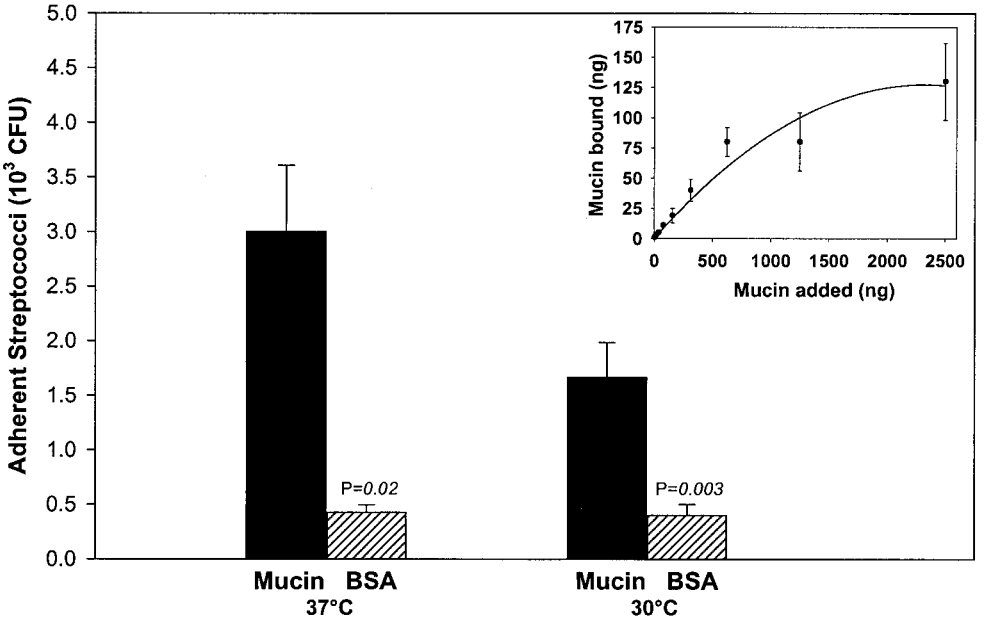

FIG. 1. Binding of group A streptococci to bovine submaxillary mucin. The figure shows adherence of *S. pyogenes* D471 to mucin- and BSA-coated wells of a microtiter plate at 37 and 30°C. Wells were coated with 1 μg of mucin, blocked with BSA, and inoculated with 5×10^6 bacteria. The plates were incubated for 1 h and washed, and the bacteria were desorbed with 0.5% Triton X-100 and plated. The mean ± standard deviation (error bars) was derived from triplicate wells in at least three independent experiments. *P* values are indicated above corresponding bars. The inset shows the adherence of strain D471 to ^{125}I-mucin. Increasing concentrations of labeled mucin were added to microtiter plate wells containing immobilized heat-killed bacteria (5×10^6 bacteria/well) and incubated for 4 h. The wells were washed, and the counts in each well (representing bound mucin) were determined in a gamma counter. Nonspecific binding was assessed as binding to BSA-coated wells and subtracted from the total binding. Means ± standard deviations (error bars) obtained from triplicate wells in at least two independent experiments are shown.

protein (17), were separated by SDS-PAGE and analyzed by Western blotting to determine which fragment bound radiolabeled mucin. The binding was clearly localized only to the N-terminal 30-kDa fragment (Fig. 3), which was further verified by the N-terminal specific MAb 10A11 (26) (not shown).

Effect of sugars on streptococcal adherence. To determine if a monosaccharide component of mucin was the M protein receptor, the sugars found in mucin, GlcNAc, GalNAc, galactose, fucose and NANA (sialic acid) were used in inhibition experiments. Pretreating strain D471 with sialic acid reduced adherence to bovine mucin at 37°C by 80% ($P = 0.04$) compared to the untreated bacterial control (Fig. 4A), indicating that sialic acid is a constituent of the receptor on mucin for the M protein.

GalNAc, galactose, and fucose did not significantly decrease adherence at the concentration tested ($P = 0.36$, 0.75, and 0.67, respectively). However, when the bacteria were pretreated with GlcNAc, the number of adherent bacteria doubled compared to untreated bacteria ($P = 0.3$) (Fig. 4A), suggesting that GlcNAc may act as a bridge between the organism and mucin, thereby enhancing adherence. Further investigation with different concentrations of these monosaccharides is necessary to confirm this hypothesis.

In a separate set of experiments, sialic acid (25 mM) was found to decrease streptococcal adherence to bovine mucin at both 30 and 37°C compared to the untreated control (Fig. 4B). At 30°C, sialic acid decreased adherence to bovine mucin by approximately 70%.

These results indicate that sialic acid inhibited adherence by binding to the bacterial adhesin(s) rather than to the receptors on mucin, since the unbound sugar was subsequently removed when the bacteria were washed prior to their addition to the mucin-coated wells. To confirm this, mucin-coated wells were individually pretreated with the monosaccharides before the addition of strain D471. None of the sugars significantly affected bacterial adherence to mucin-coated wells (not shown), again suggesting that sialic acid inhibited adherence by interacting with the bacterial adhesin(s) rather than with the mucin.

Role of sialic acid in adherence to cultured pharyngeal cells. The ability of the M6 protein to bind sialic acid prompted us to investigate if sialic acid plays a role in the adherence of group A streptococci in vitro to Detroit 562 pharyngeal cells, since many epithelial cell membrane proteins are sialylated and since the M protein has previously been shown to be involved in adherence to these cells (21). The adherence of streptococcal strains D471 and JRS75 to this cultured pharyngeal cell line was established by other investigators (21, 33) and our initial results with untreated bacteria were consistent with these previously published reports.

Streptococcal strains D471 and JRS75 were each separately preincubated with sialic acid and other monosaccharides (at 25 mM) prior to their addition onto pharyngeal cell monolayers. Pretreating strain D471 with this concentration of sialic acid significantly affected bacterial associations with the pharyngeal cells, decreasing adherence of streptococci by approximately 70% (Fig. 5). The number of internalized streptococci was not directly affected and remained consistent at 10 to 15% of the number of adherent bacteria in both treated and untreated

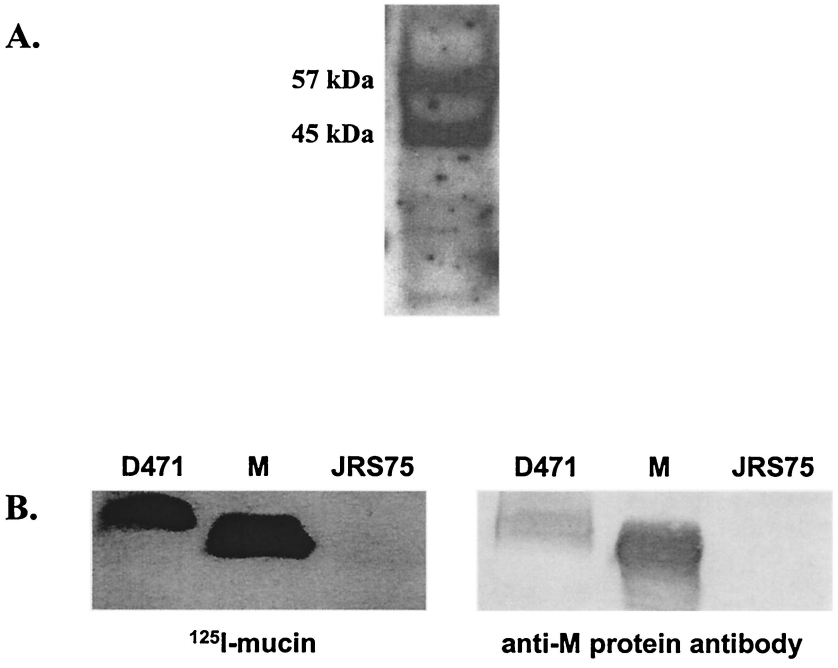

FIG. 2. Identification of group A streptococcal cell wall proteins that bind bovine submaxillary mucin. Cell wall-associated proteins were extracted with lysin enzyme from streptococcal strains D471 and JRS75 (the isogenic M-negative mutant). Extracted proteins (40 μg of total protein/strain) and the recombinant M protein from *E. coli* (2 μg) were separated on an SDS–8% polyacrylamide gel and transferred to PVDF membranes. (A) Autoradiogram of blot of D471 cell wall proteins probed with ^{125}I-bovine mucin. (B) At left is shown an autoradiogram of blots of cell wall proteins from D471 and JRS75, as well as purified M protein, probed with ^{125}I-bovine mucin. The right panel shows a Western blot of extracted proteins probed with the M-protein-specific MAb 10B6.

samples. If sialic acid had affected the internalization process, one would expect to see a lower percentage of the adherent bacteria become internalized in the treated cells. The other monosaccharides did not decrease adherence or internalization of this strain at the concentration tested.

To determine the minimum concentration of sialic acid that was able to significantly decrease adherence of strain D471 to Detroit 562 pharyngeal cells, the bacteria were separately preincubated with various concentrations of the monosaccharide (ranging from 25 to 0.5 mM) prior to inoculating the monolayers. We found that 1 mM sialic acid was the lowest concentration tested that could significantly decrease adherence of 5×10^7 CFU streptococci to pharyngeal cells (Fig. 6). Although streptococcal adherence decreased by approximately 40% after treatment with 0.5 mM sialic acid, the difference in binding in this set of experiments is not statistically significant ($P = 0.13$) as determined by Student's t test.

Binding of the M6 protein to sialic acid on pharyngeal cells. As the treatment with sialic acid significantly decreased adherence of strain D471 to pharyngeal cells, we were interested in determining if the M protein was involved in this interaction. To this end, strain JRS75, the M-negative mutant of D471 (which differs only in its ability to produce the M protein), was used in the binding assays (Fig. 7A). As can be seen, the adherence of JRS75 to pharyngeal cells is not affected by sialic acid. The effect of sialic acid on D471, therefore, is most likely the result of the monosaccharide interacting with the M protein.

Sialylated linkages. 3′SL and the glycoproteins, fetuin and transferrin, were used as potential inhibitors in vitro to determine the effect of each compound on the adherence of streptococcal strain D471 to pharyngeal cells. Since these compounds contain different sialylated linkages, our hope was to determine if a specific linkage is important in the binding of the M6 protein to the Detroit cells. 3′SL contains sialic acid linked α2-3 to galactose while fetuin contains both α2-3 and α2-6 linkages (28), although according to the manufacturer (Sigma), the ratio of the two linkages has not been determined. Transferrin contains oligosaccharides that terminate only in α2-6-linked sialic acid (45).

Figure 7B shows that 3′SL (1.7 mM) had no significant effect on adherence of strain D471 to the cultured pharyngeal cells. Fetuin (1.8 mM total sialic acid) was able to decrease adherence by approximately 30%. Even though the ratio of sialylated linkages in fetuin has not been determined, it is likely that this decrease in adherence was not the result of the α2-3-linked sialic acid since 3′SL had little effect on adherence. However, pretreatment of strain D471 with transferrin reduced adherence to cultured pharyngeal cells by approximately 90%. Since transferrin contains only α2-6-linked sialic acid, it seemed likely that this configuration was important in M-protein interactions.

To further verify that sialic acid and not another component of transferrin was responsible for the decrease in adherence of strain D471, 6′SL (which contains sialic acid linked α2-6 to galactose) was used in inhibition assays. 6′SL (1.7 mM) was

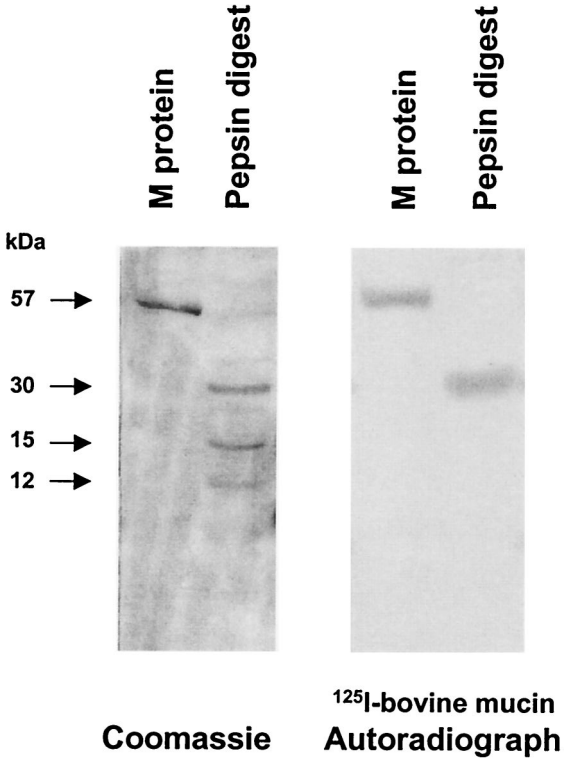

FIG. 3. Identification of the mucin binding region of the M protein. The recombinant M protein from *E. coli* (5 μg) was digested with pepsin at pH 5.9 for 30 min, separated by SDS-PAGE, stained with Coomassie blue (left panel), or electroblotted onto a PVDF membrane. The right panel shows an autoradiogram of a Western blot that was probed with ^{125}I-bovine mucin. Following hybridization, the membrane was washed and autoradiographed for 12 h at −80°C. Untreated M protein (2 μg) from *E. coli* served as control. Arrows indicate the positions of the intact M protein (57 kDa) and the three peptide bands that represent the N-terminal portion (30 kDa) and the two C-terminal regions (15 and 12 kDa) of the pepsin-digested M protein.

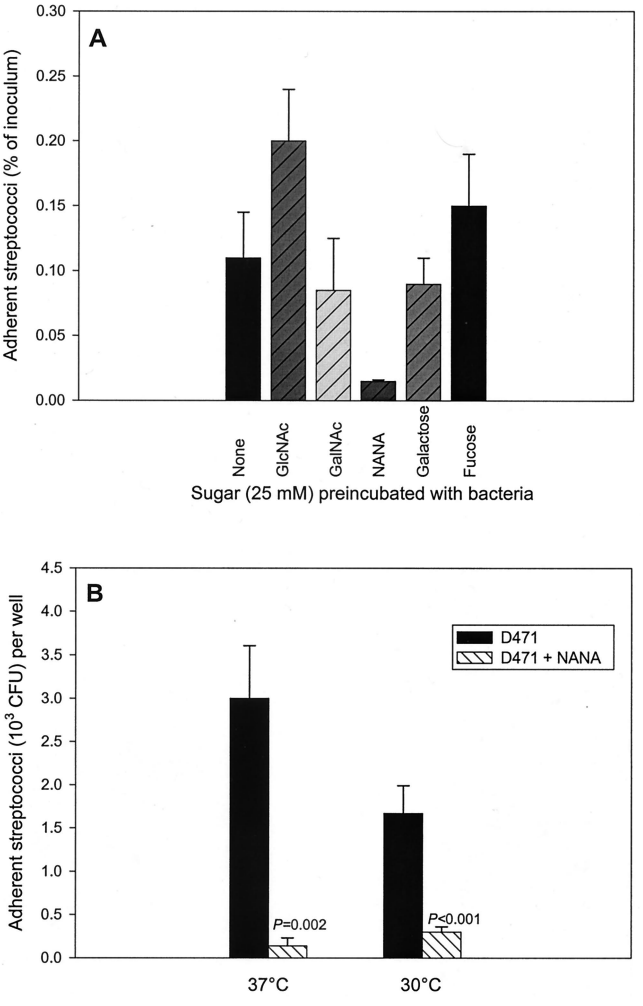

FIG. 4. Effect of monosaccharides on the binding of group A streptococci to bovine mucin. (A) Streptococcal strain D471 (5×10^7 CFU) was preincubated with 25 mM aliquots of GlcNAc, GalNAc, fucose, galactose, or NANA (sialic acid) for 30 min at 37°C. Bacteria were used to inoculate mucin-coated wells (1 μg of mucin/well inoculated with 5×10^6 CFU/well). (B) Mucin-coated wells were inoculated with strain D471, which was treated with 25 mM NANA (as described above) and incubated at 37 or 30°C to assess the effect of temperature on mucin binding. In all experiments, untreated bacteria served as control. The number of adherent bacteria was determined as described in Materials and Methods. In both panels, means ± standard deviations (error bars) were derived from triplicate wells in at least two independent experiments. *P* values are indicated above the corresponding bars in panel B.

able to reduce binding of this strain to pharyngeal cells by approximately 85% (Fig. 7C). Because 6′SL and transferrin contain only α2-6-linked sialic acid, this sialylated linkage is directly implicated in the interaction of the M6 serotype with sialic acid-containing receptors on the Detroit 562 pharyngeal cell. Bacterial plate counts determined that none of the compounds tested affected the viability of the organisms.

Effect of neuraminidase on streptococcal adherence to pharyngeal cells. To determine if the removal of sialic acid from the pharyngeal cells affected bacterial attachment, Detroit cell monolayers were treated with *C. perfringens* neuraminidase prior to being inoculated with streptococcal strain D471. This treatment decreased streptococcal adherence by approximately 80% (Fig. 7D), further implicating sialic acid in the adherence process.

Identification of the sialylated pharyngeal cell proteins that bind the M protein. The preceding experiments provide strong evidence that sialic acid on the surface of the pharyngeal cells plays an important role in the adherence of strain D471 through the M-protein molecule. Our next goal was to identify the sialylated membrane proteins on pharyngeal cells and to determine which of these glycoproteins bind the M protein. To this end, a lectin from *T. mobiliensis*, which exclusively binds sialic acid, was used to probe blots of pharyngeal cell membrane proteins in parallel with duplicate blots probed with the purified M protein. This analysis revealed three pharyngeal cell proteins (65, 43, and 35 kDa) which bound the M protein as well as the sialic acid-specific lectin (Fig. 8). All three proteins are under investigation as potential receptor molecules.

DISCUSSION

The sequence of events leading to group A streptococcal infection of the upper respiratory mucosal tissue has not been

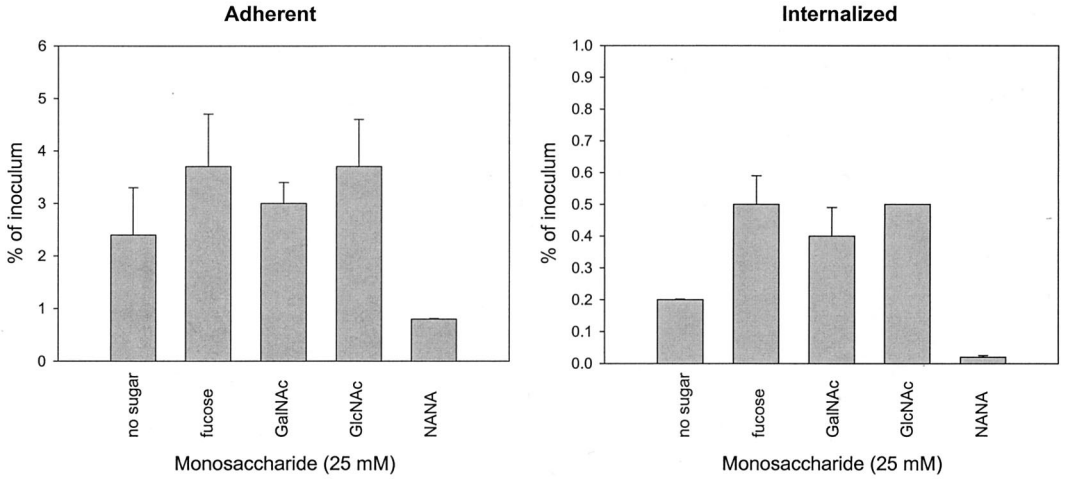

FIG. 5. Effect of monosaccharides on adherence and internalization of streptococci to Detroit 562 pharyngeal cells. Streptococcal strain D471 (5×10^7 CFU) was preincubated with 25 mM aliquots (prepared in 10 mM Tris-HCl, pH 7.4) of fucose, GalNAc, GlcNAc, or NANA for 30 min at 37°C. Bacteria were washed and then used to infect confluent wells of Detroit 562 pharyngeal cells (5×10^7 CFU/well). Untreated bacteria served as a control. The adherence and internalization assays were performed for 2.5 h at 37°C, as described in Materials and Methods. Means ± standard deviations (error bars) were derived from triplicate wells in at least two independent experiments. See Results for *P* values.

fully elucidated, although it is clear that microbes which infect through mucosal surfaces (which account for >90% of all infections) share two initial goals. First, they must overcome the mucous layer that coats the mucosal epithelium, and second, they must be able to attach to, and infect, the underlying target tissue. The results described here are the first to identify sialic acid as a receptor for group A streptococci, and this interaction lends insight into how streptococci reach the initial goals necessary to establish infection.

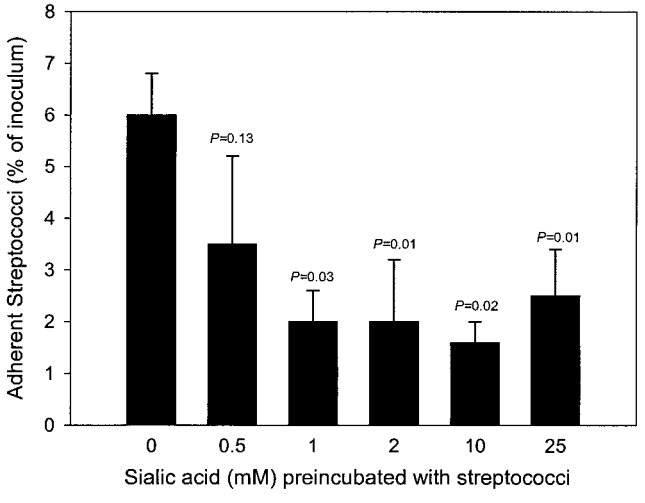

FIG. 6. Effect of different concentrations of sialic acid on adherence to pharyngeal cells. Streptococcal strain D471 (5×10^7 CFU) was preincubated with different concentrations of sialic acid (25 to 0.5 mM) for 30 min at 37°C and then used to infect confluent wells of Detroit 562 pharyngeal cells (5×10^7 CFU/well). Untreated bacteria served as control. The adherence and internalization assays were performed for 2.5 h at 37°C, as described in Materials and Methods. Means ± standard deviations (error bars) were derived from triplicate wells in at least two independent experiments. *P* values are indicated above the corresponding bars.

Mucus and, in particular, mucin, the major glycoprotein component of mucus, are presumably the first barriers that group A streptococci encounter upon entering the human upper respiratory tract. Mucins not only coat the pharyngeal mucosa but are also similar in structure to cell surface glycoproteins (2). Hence, by defining the streptococcal interaction with mucin and the structural components involved, we hoped to identify potential pharyngeal receptors used in the adherence process.

Our results show that the N-terminal region of the M6 protein is the molecule responsible for binding streptococci to the sialic acid residues of bovine mucin. This binding was not altogether surprising, as pathogens such as *P. aeruginosa* (55), *Helicobacter pylori* (52), and *H. influenzae* (39) also bind to the sialic acid residues on mucin; however, the adhesins involved in these interactions have not been well characterized. Thus, sequence comparisons to identify common features between the M molecule and other bacterial mucin binding proteins were not possible.

Although the mechanism by which these particular bacterial proteins interact with sialic acid has not been elucidated, advances have been made in understanding the molecular basis for the binding of bacterial adhesins to other sialic acid-containing molecules. A sialic acid binding motif has been identified in adhesins from *E. coli* and *H. pylori* (15, 23). Close examination of the amino acid sequence of the M6 protein N-terminal region revealed no significant homology to this motif, suggesting that the interaction between the M protein and sialic acid is mediated by a different mechanism. One such mechanism has been proposed for the SspB polypeptide (formerly SSP-5) from *Streptococcus gordonii*, which binds to sialic acid on the salivary agglutinin glycoprotein but does not contain the sialic acid binding motif (13). Interestingly, the N-terminal portions of the SspB protein and the M protein are similar, not on the amino acid level, but rather due to similarities in the periodic distribution of hydrophobic amino acids

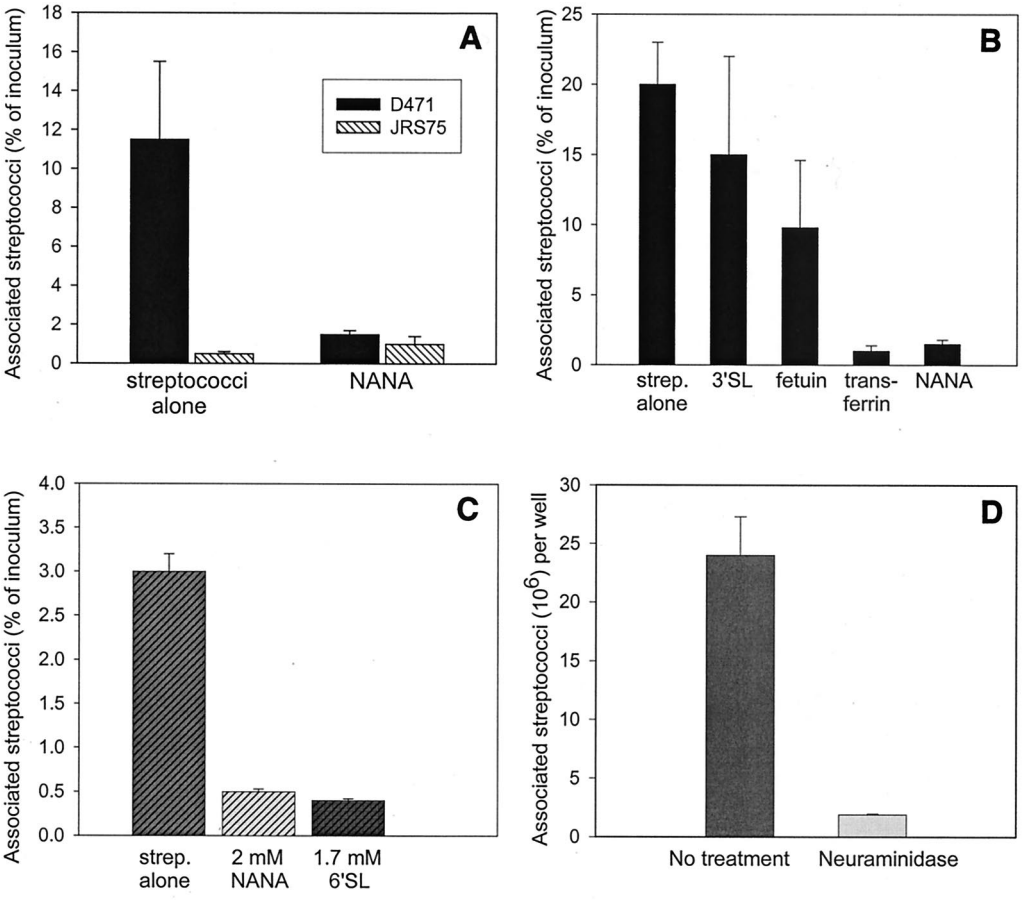

FIG. 7. Effect of sialic acid containing glycoconjugates and neuraminidase on adherence of streptococci to Detroit 562 pharyngeal cells. (A) *S. pyogenes* D471 and the isogenic M-negative strain JRS75 (5×10^7 CFU/ml) were preincubated with sialic acid (25 mM) and used in a pharyngeal cell adherence assay as described in Materials and Methods. (B) To determine the sialylated linkages that play a role in adherence of D471 to pharyngeal cells, the bacteria (5×10^7 CFU) were individually incubated for 30 min at 37°C with the following compounds: 3′SL (1.7 mM), fetuin (5 mg, 1.8 mM total sialic acid), transferrin (5 mg), and sialic acid (NANA) (25 mM); cells were then washed and used in the adherence assays. Sialylated linkages of each compound are reported in Results. Streptococci incubated with Tris-HCl (10 mM, pH 7.4) served as a control (strep. alone). (C) Strain D471 (5×10^7 CFU) was preincubated with sialic acid (NANA) (2 mM) and 6′SL (1.7 mM) as described above prior to performing the adherence assay. (D) Detroit cell monolayers were treated at confluence with *C. perfringens* neuraminidase (as described in Materials and Methods) to determine the effect on streptococcal adherence. After treatment, the monolayers were inoculated with strain D471 (5×10^7 CFU/well) and the adherence assay was performed as described. In all panels, means ± standard deviations (error bars) were derived from duplicate wells in at least two independent experiments. *P* values are indicated above the corresponding bars.

found in these coiled-coil proteins (24) and the presence of amino acids with alpha-helical potential (13). Thus, the conservation of secondary structure in the N-terminal regions of these two sialic acid binding proteins suggests that the coiled-coil structure may in some way be involved in binding the monosaccharide (13).

The terms sialic acid and NANA are often used interchangeably, although it is important to make a distinction between the two. Sialic acid refers to a family of nine-carbon monosaccharides, of which NANA is both the most common member and the metabolic precursor of a group of more than 40 nine-carbon sugars (53). The diversity of sialic acids lies in the various substitutions at different carbon positions, in addition to various linkages from carbon-2 to different underlying sugar chains. It is generally accepted that the recognition of a particular sialic acid is based on its specific structure and that any substitution or change in linkage will also alter the recognition of a particular sialic acid (53).

The streptococcal M protein has been shown previously to be important in the adherence process to pharyngeal epithelial cells and many epithelial membrane proteins contain sialic acid. Since the results presented here indicate that the M protein from strain D471 binds sialic acid, we were prompted to investigate if sialic acid (and a particular sialylated linkage) are important in the adherence of this M6 serotype to pharyngeal cells. Because there is no animal model available, we chose an in vitro assay using cultured pharyngeal cells to study this aspect of streptococcal colonization.

The human pharyngeal epithelial cell line Detroit 562 was chosen for these studies for a number of reasons. This cell line has been used in numerous previously published studies on streptococcal adherence (21, 33) and is derived from pharyn-

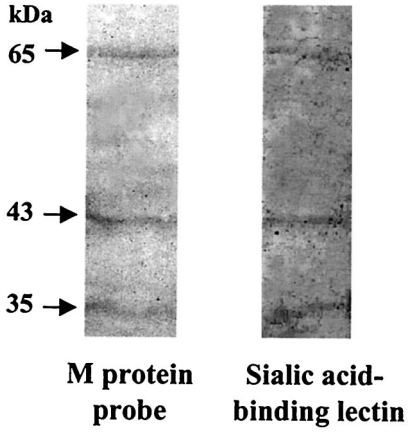

FIG. 8. Identification of sialylated pharyngeal cell membrane proteins which bind the streptococcal M protein. Membrane-associated proteins from pharyngeal cell line Detroit 562 were extracted and solubilized as described in Materials and Methods. Extracted proteins (50 μg of total protein/lane) were separated (in duplicate) on SDS–8% polyacrylamide gels and transferred to PVDF membranes. The left panel shows a blot probed with 25 μg of the purified M protein, and the right panel shows a blot probed with 2 μg of the sialic acid-specific lectin, TML. Bound M protein and TML were detected as described in Materials and Methods. Arrows indicate the three pharyngeal cell membrane proteins that bound to both the M protein and TML.

geal tissue of humans, which is the only known reservoir of group A streptococci. Since many transformed or immortalized cell lines do not necessarily display the same repertoire of carbohydrates on their surfaces as the native cells from which they are derived, our choice of a cell line was important in the identification of epitopes involved in streptococcal adherence. The Detroit 562 pharyngeal cell line has been reported to more faithfully display the carbohydrate epitopes which are representative of the native cells than other cell lines (2). We understand the inherent limitations of using in vitro systems to study in vivo events and have limited the interpretation of the data presented here to Detroit 562 pharyngeal cells until further investigation is performed on other epithelial cell lines.

The Detroit 562 pharyngeal cells were used to determine the role of sialic acid in streptococcal adherence in vitro, and we found that sialic acid was able to inhibit the adherence of an M6 streptococcal strain. Sialic acid did not exhibit an effect on an isogenic M-negative mutant. Thus, the decrease in adherence of the M-protein-producing strain after the addition of sialic acid is likely due to sialic acid binding to the M protein, making those epitopes unable to bind to the sialylated ligand on the pharyngeal cell (competitive inhibition). To ensure that exogenous sialic acid was not interfering with adherence by binding directly to the pharyngeal cell, unbound sialic acid was removed from the streptococci prior to inoculating the pharyngeal cell monolayers.

Many epithelial membrane proteins contain sialic acid, and we previously reported the identification of the sialylated membrane-bound mucin, MUC-1, on the surface of pharyngeal cells (P. A. Ryan, V. Pancholi, and V. A. Fischetti, Abstr. 100th Gen. Meet. Am. Soc. Microbiol, abstr. D-13, 2000). In the work presented here, we identified three pharyngeal cell membrane proteins that bind both the M6 protein and the sialic acid specific lectin from *T. mobiliensis*. These sialylated molecules are under investigation as potential receptor molecules for the M protein.

Previous reports have shown that various bacterial and viral adhesins have a preference for particular sialylated linkages. The S fimbriae from *E. coli* (35) and adhesins from *H. influenzae* (2) and *H. pylori* (28, 45) bind to α2-3-linked sialic acid, whereas the influenza virus hemagglutinin binds specifically to α2-6-linked sialic acid (2). In addition, an adhesin from *Streptococcus pneumoniae* has been shown to bind to either α2-3- or α2-6-linked sialic acid (2). We ascertained that, by identifying the sialylated linkages that are involved in streptococcal adherence, we might be able to more easily identify the potential receptors for the M protein.

In the results presented here, treatment of streptococcal strain D471 with 6′SL (which contains sialic acid linked α2-6 to galactose) decreased the adherence of the bacteria to Detroit cells, implicating this particular sialylated linkage in adherence to this cell line. Furthermore, transferrin, which contains oligosaccharides that terminate in α2-6-linked sialic acid, also decreased adherence to pharyngeal cells. Compounds containing sialic acid linked in other configurations such as 3′SL (which contains NeuAcα2-3Gal) failed to significantly decrease streptococcal adherence to the same extent in these inhibition experiments. Note that we report here that the M6 protein binds to sialic acid on bovine submaxillary mucin, and structural studies on this type of mucin have determined that sialic acid is also linked in an α2-6 configuration (to GalNAc) (12).

In a second approach to study the involvement of sialic acid in streptococcal adherence, we show that neuraminidase treatment of the Detroit 562 cells decreased the adherence of streptococcal strain D471. These data further emphasize the role of sialic acid in the adherence process. Although the enzyme from *C. perfringens* preferentially cleaves α2-3-linked sialic acid, α2-6- and α2-8-linked sialic acids are also enzyme substrates. It seems unlikely that the decrease in streptococcal adherence after neuraminidase treatment can be attributed to the cleavage of an α2-3-linked sialic acid, since 3′SL (which contains the preferred substrate, NeuAcα2-3Gal) failed to decrease streptococcal adherence in inhibition experiments. Many commercially available preparations of neuraminidase are frequently contaminated with trace amounts of proteases or glycosidases that are difficult to remove by standard purification procedures; thus, results based solely on treatment with these enzymes are not entirely conclusive. However, the results from the competitive inhibition studies substantiate the data from the neuraminidase experiments, implicating sialic acid (in particular, α2-6-linked sialic acid) in streptococcal adherence. Previous studies have confirmed that glycoconjugates found on the surface of the respiratory epithelium contain α2-6-linked sialic acid (2). Thus, the observed role of sialic acid in streptococcal adherence in vitro may likely represent one of the numerous events that occur in vivo in the upper respiratory tract of the human host.

An examination of the initial events that occur when group A streptococci enter the upper respiratory tract of the human host is of primary importance in our understanding of streptococcal colonization of pharyngeal tissue. A strong binding to mucin in the upper respiratory tract of humans would at first

seem to be counterproductive to streptococci in terms of its ability to initiate infection. However, the binding to sialylated mucin, such as in the case of MUC-1 (a bound form of mucin), might allow the streptococci to be positioned closer to the epithelial cell surface, facilitating adherence. Alternatively, if the soluble mucin of certain individuals was not sialylated or did not contain the sialylated linkage that we show here is necessary for streptococcal binding, the bacteria would not bind to this form of mucin and thus avoid efficient clearance from the airways. Work is currently in progress to investigate both hypotheses so that we may better understand the role that both mucin and sialic acid play in streptococcal colonization.

It is worth noting that a number of the cell surface glycoproteins that have been implicated in streptococcal adherence, such as the integrins (31), fibronectin (6, 30, 40), and plasminogen (34), are sialylated. It would be interesting to know if the sialic acid moieties of these proteins are responsible in some cases for the observed binding of certain streptococcal adhesins. Although some of the interactions between these glycoproteins (such as fibronectin) and certain streptococcal proteins have been defined, many have yet to be elucidated. One such example is PFBP, a recently characterized fibronectin binding protein (40). As adherence is a multifactorial process that involves many adhesin-receptor pairs, we are not suggesting that all streptococcal adhesins that bind sialylated glycoproteins do so through sialic acid moieties. We are simply noting the presence of a common monosaccharide on many potential streptococcal receptors. By investigating these initial adherence events and by identifying the receptors involved, we will be better able to understand the extraordinarily complex process of colonization such that we may design therapeutics for the prevention of streptococcal infection.

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