

Human Tracheobronchial Mucin: Purification and Binding to *Pseudomonas aeruginosa*

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Colonization of the respiratory tract with *Pseudomonas aeruginosa* is a serious problem in cystic fibrosis and seriously ill hospitalized patients. Human tracheobronchial mucin (HTBM), the major glycoprotein of human tracheobronchial secretions, is known to interact with this pathogen, which may then be cleared by mucociliary action. However, the mechanism of interaction is not known. To understand this process, pure HTBM was isolated from tracheobronchial secretions of a laryngectomee. Following initial fractionation on Sepharose CL-2B, the HTBM-containing fraction was subjected to reductive methylation and then gel filtration. Pure HTBM was employed in an overlay binding assay to identify the bacterial adhesin(s) and mucin receptors that participate in mucin-*P. aeruginosa* interactions. An ~16-kDa nonpilus protein component(s) of *P. aeruginosa* was found to be the adhesin(s) for HTBM. The mucin receptor for the 16-kDa component(s) was found in the peptide moiety. This study confirms that *P. aeruginosa* utilizes the nonpilus adhesin(s) to bind to HTBM. Identification of the specificity of the HTBM-*P. aeruginosa* interactions can lead to a better understanding of the predominance of *P. aeruginosa* colonization in individuals with cystic fibrosis.

Pseudomonas aeruginosa is a serious respiratory pathogen in patients with cystic fibrosis (CF) and seriously ill hospitalized patients. Its adherence to the respiratory tract mucosal surface initiates colonization, which may lead to infection. Adherence of *P. aeruginosa* to the epithelium is mediated by pili and cell surface sialic acid (6, 18, 19). Human tracheobronchial secretions (HTBS) that coat the respiratory tract epithelium protect the airways by binding or trapping the pathogens, which are then cleared by mucociliary action. Human tracheobronchial mucin (HTBM), the major glycoprotein of HTBS, contains numerous oligosaccharide side chains. Some of the structures may be similar to those present on epithelial cells. *N*-Acetylneuraminic acid (30) and oligosaccharides with type 1 (Gal β 1-3GlcNAc) and type 2 (Gal β 1-4GlcNAc) units (14) have been suggested as mucin receptors for *P. aeruginosa*. *P. aeruginosa* appears to bind to mucin by pilus (15) and nonpilus (17) adhesins. However, these studies do not identify a mucin receptor for a particular adhesin. This is partly due to the difficulty in isolating pure HTBM. The large size of HTBM has been exploited for its isolation by gel permeation chromatography (8, 12, 20, 22, 27, 28). Since mucins interact very strongly with other proteins, HTBM isolated by gel filtration has always been associated with lower-molecular-weight (glyco) proteins (3, 12, 20, 22, 27). The amino acid compositions of these (glyco)protein components are similar to those of link proteins identified in other mucins (21). Link proteins of intestinal mucin contain *N*-glycosidically linked oligosaccharides and can bind pilated *Escherichia coli* (24). Therefore, in order to determine the role of HTBM in bacterial adhesion, one has to employ link protein-free mucin in binding assays.

In this report, a procedure for the isolation of HTBM is described. HTBS were initially fractionated on Sepharose CL-2B to obtain a high-molecular-weight fraction containing HTBM with the link proteins. The HTBM-link protein complex was dissociated by reductive methylation. HTBM was then isolated by gel filtration on Sepharose CL-2B and utilized for the characterization of HTBM-*P. aeruginosa*

interactions. Mucin-bacterium binding was found to be a protein-protein interaction between the HTBM peptide and an ~16-kDa nonpilus protein component(s) of *P. aeruginosa*.

MATERIALS AND METHODS

Materials. The following materials were obtained from the indicated sources: Trypticase soy broth and MacConkey no. 2 prepared-medium agar plates (BBL Microbiology Systems, Cockeysville, Md.); yeast extract, 1.5% nutrient agar, and nutrient broth (Difco Laboratories, Detroit, Mich.); Chloramine T, bovine serum albumin (BSA; fraction V), sodium cyanoborohydride, *N*-2-hydroxyethylpiperzine-*N'*-2-ethanesulfonic acid (HEPES), Tris (TRIZMA), Coomassie brilliant blue R-250, urea, *N*-acetylneuraminic acid (synthetic), α -L-(-)-fucose, D-(+)-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-(+)-mannose, phenylalanine, methionine, α -amino- β -guanidinopropionic acid (AGP), D-(-)-arabinose, and sodium metaperiodate (Sigma Chemical Co., St. Louis, Mo.); acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate, Dowex 50-X4 (H⁺; 200 to 400 mesh), and Dowex 1-X8 (HCOO⁻; 200 to 400 mesh) (Bio-Rad Laboratories, Richmond, Calif.); OV-225 on 80/100 Supelcoport (Supelco, Inc., Bellefonte, Pa.); Spectrapor 3 dialysis membrane (molecular weight cutoff, 35,000; Spectrum Medical Industries, Los Angeles, Calif.); Sephadex and Sepharose gels (Pharmacia Fine Chemicals, Piscataway, N.J.); hydrogen fluoride (HF) (Matheson, Twinsburg, Ohio); Na¹²⁵I (14.3 mCi/ μ g; Amersham Corp., Arlington Heights, Ill.); Immobilon P membrane (Millipore Corp., Bedford, Mass.); SE 200 Mighty Small electrophoresis and semiphor TE 70 transfer units (Hofer Scientific Instruments, San Francisco, Calif.); [¹⁴C]formaldehyde (57.5 mCi/mmol; ICN Radiochemicals, Irvine, Calif.); formaldehyde and cesium bromide (Baker analyzed; J. T. Baker Chemical Co., Phillipsburg, N.J.); X-ray film (X-OMAT; Eastman Kodak Co., Rochester, N.Y., and Fuji Photo Film Co., Tokyo, Japan, supplied by Fisher Scientific, Fair Lawn,

N.J.); X-ray film developer and fixer (Eastman Kodak). Purified pili of *P. aeruginosa* K and O were provided by W. Paranchych, University of Alberta, Edmonton, Canada.

Bacterial strains, culture conditions and preparation of bacterial extract. *P. aeruginosa* 1244 (piliated nonmucoid isolate provided by R. Ramphal, University of Florida, Gainesville, Fla.), ATCC 19142 (mucoid HTBS isolate), ATCC 17933 (encapsulated sputum isolate), ATCC 17648 (HTBS isolate), K and O (both provided by W. Paranchych) were employed in this study. Strains 1244, 19142, 17933, and 17648 were maintained as frozen cultures at -70°C in Trypticase soy broth supplemented with 0.5% yeast extract and 10% glycerol. For routine use, they were subcultured on MacConkey no. 2 prepared plates under limited-oxygen tension. Isolated colonies were grown in Trypticase soy broth supplemented with 5% yeast extract. Strains K and O were maintained as frozen cultures at -70°C in nutrient broth and 10% glycerol and subcultured on nutrient agar plates under limited-oxygen tension. Isolated colonies were grown in nutrient broth. After static incubation for 8 to 16 h under limited-oxygen tension, bacteria were inoculated (1% [vol/vol]) into larger batch cultures. They were harvested by centrifugation at $1,900 \times g$ and washed three times in 0.01 M sodium phosphate with 0.154 M sodium chloride, pH 7.2 (PBS). Bacterial suspensions (1 to 3 g in 50 ml of PBS) were sonicated for 5 min at 50% of the duty cycle at a microtip output of 5 (Vibra cell; Branson Ultrasonics Corp., Danbury, Conn.). After centrifugation at $12,000 \times g$, the clear supernatant was dialyzed against cold water and lyophilized. The lyophilized sample was redissolved in 25 ml of water and centrifuged, and the clear supernatant was lyophilized. Sixteen to 38 mg of solids per gram (wet weight) of bacteria was obtained.

Isolation of HTBM. HTBS from a 71-year-old laryngectomized Caucasian male (blood type O) were collected as described previously (28). HTBS (8 to 12 ml) were transferred into a beaker containing 15 ml of 0.1 M Tris-HCl with 6 M urea, pH 7.5 (Tris-urea; the urea contained traces of insoluble material and was therefore filtered through a Büchner funnel with a fritted disk of medium porosity), and solubilized by gentle stirring for 30 to 60 min at 10°C . Insoluble material was removed by centrifugation at $12,000 \times g$ for 20 min at 4°C . Clear supernatant was dialyzed against cold deionized water and lyophilized. Lyophilized HTBS were redissolved in Tris-urea and fractionated on a column of Sepharose CL-2B. The higher-molecular-weight fraction, which contained most of the carbohydrate, was subjected to reductive methylation (9). The sample was solubilized in 0.1 M HEPES with 6 M urea, pH 7.5, and treated with [^{14}C]formaldehyde (1 mCi) for 1 h at 37°C in the presence of 0.1 M sodium cyanoborohydride. Unlabeled formaldehyde was then added to a total 50-fold molar excess over lysine and incubated at 37°C for an additional 23 h. The reaction mixture was carefully dialyzed against water with Spectrapor 3 membrane tubing, lyophilized, and fractionated on Sepharose CL-2B.

Radiolabeling by iodination. Iodination was performed by the Chloramine T method (7). To a mixture of the sample to be iodinated ($\sim 20 \mu\text{g}$ of protein in $25 \mu\text{l}$ of 0.5 M sodium phosphate, pH 7.2 [phosphate buffer]) and [^{125}I]sodium iodide (1 mCi; $10 \mu\text{l}$), Chloramine T ($20 \mu\text{l}$ of a 1-mg/ml solution in 0.1 M phosphate buffer) was added and mixed immediately. After 90 s, the reaction was terminated by the addition of sodium metabisulfite ($25 \mu\text{l}$ of a 2-mg/ml solution in 0.1 M phosphate buffer). Potassium iodide ($40 \mu\text{l}$ of a 10-mg/ml solution in 0.1 M phosphate buffer) was added to

dilute the residual iodine and subjected to gel filtration on Sephadex G-25 (by using a 10-ml disposable pipette and water as the eluent). Prior to chromatography, about 40 mg of BSA in 2 ml of water was passed through the column to saturate any protein-binding sites on the gel, which was then washed with water. Column fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, and fractions containing the iodinated sample were pooled and stored at 4°C until use.

Electrophoresis. SDS-PAGE (11) was performed at a constant 100 V. Proteins were detected by a 0.1% solution of Coomassie brilliant blue R250 in methanol-water-glacial acetic acid (4:5:1) and were then destained in ethanol-water-glacial acetic acid (1:8:1). For autoradiography, dried gels were exposed to X-ray film. Western (immunoblot) transfers (4, 29) to an Immobilon P membrane were made at room temperature for 40 min at 100 mA by employing 0.1 M Tris-0.192 M glycine in 20% methanol as the transfer buffer. Transferred proteins on the Immobilon P membrane were identified by staining with 0.1% Coomassie blue in methanol-glacial acetic acid-water (4:1:5) for 30 min at room temperature and were then destained in the same solvent.

Overlay binding assay. After the Western transfer, the Immobilon P membrane was blocked with BSA (2% [wt/vol] in 0.01 M Tris-HCl-0.154 M sodium chloride [TBS]) for 1 h at room temperature. The membrane was then washed three times for 10 min each with TBS and incubated with [^{125}I]HTBM ($\sim 4 \times 10^6$ to 5×10^6 cpm in 10 ml of TBS) overnight at room temperature. Unbound radioactivity was removed by washing the membrane with TBS three times for 10 min each. The membrane was gently blotted on a filter paper, air dried, and subjected to autoradiography. For inhibition of binding of [^{125}I]HTBM to the bacterial component by monosaccharides, the final incubation with [^{125}I]HTBM was preceded by incubation with the appropriate sugar at a concentration of 0.01 M for 1 h at room temperature. Since the *N*-acetylneuraminic acid solution had a pH of ~ 2.5 , it was adjusted to pH 7.2 with 1 M Tris prior to use. All the monosaccharide inhibition experiments were performed by employing sonicate from *P. aeruginosa* 19142.

Manipulation of HTBM. For periodate oxidation, [^{125}I]HTBM ($75 \mu\text{l}$; 7×10^6 cpm) was treated with an equal volume of sodium metaperiodate (0.05 M in 0.05 M sodium acetate, pH 4.5) for 2 h at room temperature. Excess periodate was then destroyed by the addition of $20 \mu\text{l}$ of ethylene glycol, and the mixture was employed in the overlay assay. In the control experiment, sodium metaperiodate was first destroyed by incubation with ethylene glycol and then mixed with [^{125}I]HTBM and employed in the assay. For deglycosylation, a mixture of HTBM (96 mg), phenylalanine (250 mg), and methionine (50 mg) was dried in a vacuum at 60°C for 3 days over phosphorus pentoxide and hydrolyzed with HF (10 ml) for 3.5 h in a closed system (5). HF was removed by distillation in a vacuum. The residue was dissolved in water, dialyzed against cold distilled water, and lyophilized. Deglycosylated HTBM (14 mg) was dissolved in water at a concentration of 1 mg/ml and stored in aliquots at -20°C until further use. Chemical analysis indicated a loss of 98.5% of *N*-acetylgalactosamine (a glycopeptide linkage sugar) because of hydrolysis with HF. Electrophoretically deglycosylated HTBM migrated as a zone with an estimated molecular mass of ~ 24 kDa at the midpoint.

Manipulation of bacterial sonicate. For hydrolysis with trypsin, *P. aeruginosa* sonicate (150 μg in $7.5 \mu\text{l}$ of water) was treated with trypsin ($7.5 \mu\text{g}$ in $7.5 \mu\text{l}$ of 0.05 M ammonium bicarbonate, pH 7.6) for 18 h at 37°C . The

reaction mixture was mixed with 4× SDS-PAGE sample solubilizing solution (5 μ l) and employed in the binding assay. In the control experiment, bacterial sonicate was treated as described above, but employing heat-inactivated trypsin (i.e., treated with a boiling water bath for 5 min).

Analytical and preparative methods. For density gradient ultracentrifugation of HTBM, 45 g of cesium bromide was solubilized in an aqueous solution of HTBM (55 mg in 62 ml). The solution was filtered through a plug of glass wool and subjected to centrifugation at 79,500 \times *g* in a Beckman L8-70M ultracentrifuge by using an SW28 rotor for 88 h at 18°C. Starting from the bottom of the tube, fractions (1.2 ml) were collected and the optical density at 280 nm was determined. A single peak was obtained. Appropriate fractions were pooled, dialyzed against water, and lyophilized. To determine the amino acid composition, samples were hydrolyzed with constant boiling HCl for 24 h at 110°C and analyzed by a Beckman 6300 Amino Acid Analyzer employing AGP as an internal standard. For the determination of neutral sugars and hexosamines, samples were hydrolyzed in 2 M HCl at 100°C for 6 h. The hydrolyzate was passed through coupled columns of Dowex 50-X4 (H⁺) and Dowex 1-X8 (HCOO⁻). Neutral sugars in the effluent water wash were quantitated as alditol acetates by gas-liquid chromatography on an OV-225 column, by using a Varian 3700 gas chromatograph with arabinose as an internal standard. Hexosamines were eluted from Dowex 50 columns with 2 M HCl and quantitated on the amino acid analyzer employing AGP as an internal standard. Sialic acid was determined by the thiobarbituric acid method (31).

RESULTS

Isolation of HTBM. The gel permeation chromatographic profile of HTBS on Sepharose CL-2B is shown in Fig. 1A. Most of the carbohydrate-containing (anthrone reaction positive) material was of high molecular weight and eluted at the void volume. The chemical composition indicated this material to be a mucin glycoprotein, as evidenced by the high content of hydroxy amino acids and *N*-acetylgalactosamine (Table 1). It was designated as pool A. The homogeneity of pool A was examined by SDS-PAGE of [¹²⁵I]pool A. In the absence of disulfide bond reducing agent, pool A was present mainly in the stacking gel while only a small portion entered the separating gel. The reduction of disulfide linkages with 2-mercaptoethanol lowered the molecular weight of pool A and allowed migration to the interface of the stacking and separating gels, with a simultaneous release of lower-molecular-weight (glyco)protein components (Fig. 2, lanes 1 and 2). This is in agreement with the observation of Rose et al. (22) that solubilization of HTBS in dissociating solvents and subsequent chromatography on Sepharose 4B and 2B columns do not dissociate the low-molecular-mass proteins (13 to 66 kDa) from the mucin.

As an additional step of purification, pool A was subjected to density gradient ultracentrifugation in cesium bromide. It migrated as a single peak and had an identical chemical composition after centrifugation, suggesting that no dissociation of the low-molecular-weight components occurred (results not shown).

To dissociate the lower-molecular-weight (glyco)proteins from HTBM, pool A was subjected to reductive methylation. Reductively methylated pool A was fractionated on a column of Sepharose CL-2B (Fig. 1B). Three pools (A1 to A3) were obtained. The amino acid composition indicated that only pools A1 and A2 were mucins (Table 1). The results

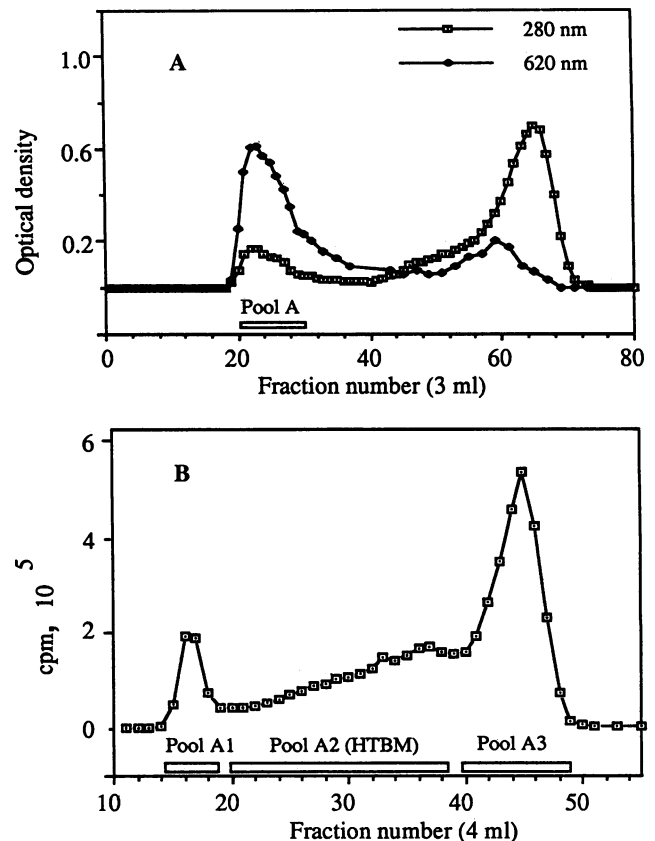


FIG. 1. Isolation of HTBM. (A) Fractionation of HTBS. HTBS (40 mg) of a laryngectomee were solubilized in 4 ml of 0.1 M Tris-HCl, pH 7.5, with 6 M urea buffer and subjected to gel filtration on a column (1.5 by 120 cm) of Sepharose CL-2B equilibrated with the above buffer. Fractions (3 ml) were monitored by measuring the A_{280} and by the anthrone reaction (A_{620}) on 0.2 ml from each fraction. Fractions were pooled as indicated in the figure, dialyzed against water, and lyophilized. Pool A yielded 10 mg. (B) Fractionation of reductively methylated pool A. Reductively methylated pool A (88 mg) was solubilized in 4 ml of the above buffer and fractionated on a column (1.6 by 95 cm) of Sepharose CL-2B. Fractions (4 ml) were monitored by liquid scintillation spectrometry of 10 μ l from each fraction, pooled as indicated in the figure, dialyzed against water, and lyophilized. Yields: pool A1, 9 mg; pool A2 (HTBM), 60 mg; pool A3, 7 mg.

suggest that reductive methylation dissociated pool A into mucin and lower-molecular-weight protein components. However, amino acid analysis indicated that only 53% of the lysine residues of pool A1 were modified to dimethyl lysines. On the other hand, pool A2 did not contain any unmodified lysine residues. In addition, SDS-PAGE and fluorography indicated the presence of a lower-molecular-weight component(s) migrating past the dye front in pool A1 but not in A2. These results suggested that pool A1 represents partially depolymerized pool A (Fig. 2, lanes 3 to 5) while pool A2 represents pure HTBM. The purity of HTBM was confirmed by SDS-PAGE of [¹²⁵I]pool A2. The results also indicate the absence of disulfide-linked lower-molecular-weight subunits in HTBM (Fig. 2, lanes 6 and 7). However, it should be noted that lysine residues of HTBM isolated in this study have been modified to dimethyl lysines.

Pool A3 was a mixture of several components, as indicated by the presence of radioactivity throughout the length

TABLE 1. Chemical composition of pool A, as obtained by gel filtration of HTBS on Sepharose CL-2B, and the products of reductive methylation

Component	No. of residues/1,000 amino acids in pool:			
	A	A1	A2 ^a	A3
Aspartic acid	49	47	23	97
Threonine	226	191	330	106
Serine	85	93	104	59
Glutamic acid	72	65	46	118
Proline	99	79	115	92
Glycine	82	94	69	96
Alanine	90	93	100	77
Half-cystine	30	20	9	66
Valine	56	62	47	62
Methionine	7	9	7	10
Isoleucine	25	36	22	21
Leucine	62	84	52	70
Tyrosine	13	19	7	23
Phenylalanine	23	35	14	31
Histidine	23	18	21	18
Lysine	23	9	0	0
Dimethyllysine	0	10	10	15
Arginine	35	29	24	39
<i>N</i> -Acetylgalactosamine	270	ND ^b	ND	ND
<i>N</i> -Acetylglucosamine	942	ND	ND	ND
Fucose	747	ND	ND	ND
Mannose	ND	ND	ND	ND
Galactose	978	ND	ND	ND
Sialic acid	217	ND	ND	ND

^a Pool A2 is composed of pure HTBM, as described in the text.

^b ND, not determined.

of the acrylamide separating gel. Indeed, it was further fractionated on a column of Sephacryl S-300 into several components (results not shown). The amino acid composition of pool A3 revealed the presence of a higher content of acidic amino acids (21.5%) and cysteine (6.6%) than HTBM (acidic amino acids, 6.9%; cysteine, 0.9%). From the above results, it is speculated that HTBM in pool A is a polymer of highly glycosylated basic units stabilized by cysteine-rich acidic components. Reductive methylation did not reduce any of the disulfide linkages of HTBM (results not shown), suggesting that the stabilization of the polymeric structure involves ionic interactions between HTBM and components of pool A3.

Binding of HTBM to *P. aeruginosa* extract. Sonication of *P. aeruginosa* strains 1244, 19142, 17933, and 17648 released several protein components. A qualitative examination of the Coomassie blue-stained gels did not indicate any major differences among the four strains (results not presented). All of the protein bands could also be electrophoretically transferred to Immobilon P (results not shown). Overlay with [¹²⁵I]HTBM resulted in the binding of mucin to a component(s) of ~16 kDa in all four *P. aeruginosa* strains (Fig. 3, lanes 1 and 6). Treatment of *P. aeruginosa* sonicate with trypsin abolished binding, suggesting that the adhesin was a protein. It was thought that the 16-kDa adhesin(s) could be from pili. Therefore, purified pili and the bacterial sonicate of *P. aeruginosa* K and O were transferred to a Western blot and tested for binding to [¹²⁵I]HTBM. The results indicated binding to a 16-kDa component of the sonicate but not to purified pili, suggesting that the interaction between [¹²⁵I]HTBM and *P. aeruginosa* in the overlay assay system may not be pilus mediated (Fig. 3, lanes 2 to 5).

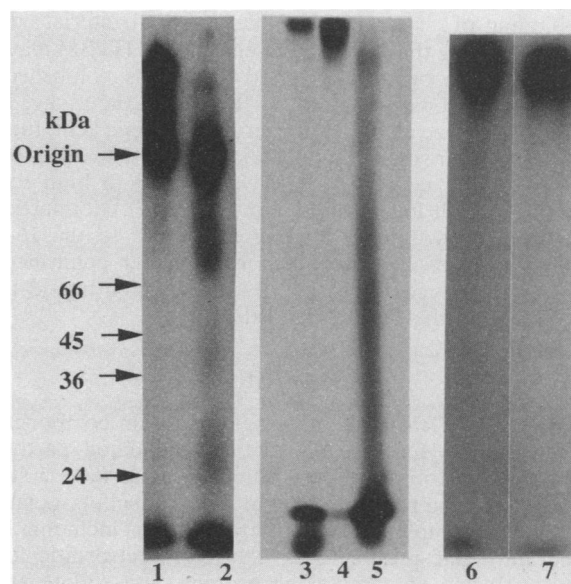


FIG. 2. Homogeneity of mucin-containing fractions at various stages of HTBM isolation. Samples were solubilized as indicated, and the homogeneity was determined by SDS-PAGE in 10% acrylamide gels. Lane 1, [¹²⁵I]pool A in nonreducing sample solubilizing buffer (0.064 M Tris-HCl, pH 6.8, with 2% SDS-10% glycerol); lane 2, [¹²⁵I]pool A in reducing sample solubilizing buffer (0.064 M Tris-HCl, pH 6.8, with 2% SDS-10% glycerol-5% 2-mercaptoethanol); lanes 3 to 5, [¹⁴C]pool A1, [¹⁴C]pool A2, and [¹⁴C]pool A3, respectively, in reducing sample solubilizing buffer; lane 6, [¹²⁵I]pool A2 in nonreducing sample solubilizing buffer; lane 7, [¹²⁵I]pool A2 in reducing sample solubilizing buffer. ¹⁴C- and ¹²⁵I-labeled components were visualized by fluorography and autoradiography, respectively.

N- and O-linked oligosaccharides of mucins are generally made up of fucose, galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, mannose, and *N*-acetylneuraminic acid. None of the above monosaccharides inhibited

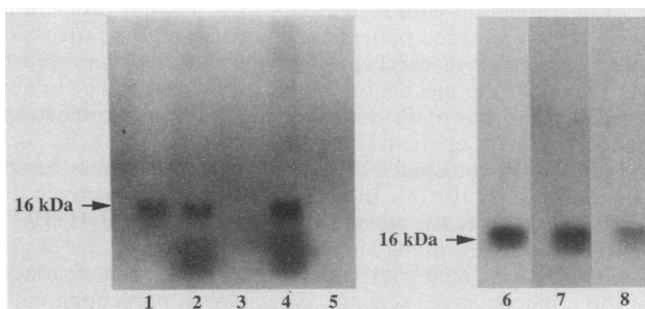


FIG. 3. Characterization of HTBM-*P. aeruginosa* interactions by an overlay binding assay. Lane 1, *P. aeruginosa* 1244 sonicate (100 µg); lane 2, *P. aeruginosa* K sonicate (100 µg); lane 3, purified pili from *P. aeruginosa* K (10 µg); lane 4, *P. aeruginosa* O sonicate (100 µg); lane 5, purified pili from *P. aeruginosa* O (10 µg). Samples were transferred (12% acrylamide gel) to an Immobilon P membrane and incubated with [¹²⁵I]HTBM. The bound bacterial component was visualized by autoradiography. Similarly, in lanes 6 to 8 (10% acrylamide gels), *P. aeruginosa* 1244 sonicate (100 µg) was examined for binding to [¹²⁵I]HTBM, periodate-treated [¹²⁵I]HTBM, and deglycosylated [¹²⁵I]HTBM, respectively. Similar results were obtained with *P. aeruginosa* ATCC 19142, ATCC 17933, and ATCC 17648.

the binding of [¹²⁵I]HTBM to the 16-kDa bacterial adhesin(s), suggesting that the oligosaccharides of HTBM may not be involved in bacterial binding. This was confirmed by employing periodate-treated [¹²⁵I]HTBM in the assay. Periodate will oxidize all of the *N*-acetylneuraminic acid, fucose, and all the other sugars that contain vicinal hydroxyl groups. Periodate-oxidized [¹²⁵I]HTBM was found to bind to the adhesin(s) of all four strains (Fig. 3, lane 7). Nonparticipation of carbohydrate in binding of HTBM to the 16-kDa component(s) of *P. aeruginosa* was further confirmed by employing deglycosylated [¹²⁵I]HTBM, which bound to all four strains in the assay (Fig. 3, lane 8).

DISCUSSION

Although HTBM is the major glycoprotein component of HTBS, its biological role has not been elucidated, partly due to an inability to isolate pure mucin. Mucins have a strong tendency to bind peptides and lipids and to self-associate (1, 23). HTBM complexes with several proteins including lysozyme and basic proteins (22, 25, 28). Electrostatic forces between lysine of complexing proteins and sulfate and/or sialic acid of mucins appear to be responsible for complexing (26). Therefore, blocking of the lysine residues by either citraconylation (26) or reductive methylation (13) has been employed in mucin purification protocols. In this study, the high-molecular-weight fraction obtained by chromatography of HTBS on Sepharose CL-2B was subjected to reductive methylation and then gel filtration again on Sepharose CL-2B. HTBM thus isolated was practically free from associated proteins. Since these experimental conditions for reductive methylation do not cleave the disulfide linkages, it can be concluded that the proteins bound to HTBM were noncovalently associated. Therefore, on the basis of the purification protocols employed for the isolation of mucins, it is tempting to speculate that the link proteins described for HTBM (3, 12, 20, 27) and human intestinal, pig gastric, rat intestinal, and human submandibular-sublingual salivary mucins (21) may not be disulfide linked to the respective mucins. However, this study does support the hypothesis that the link proteins may stabilize the polymeric structure of mucins.

Lung infection with *P. aeruginosa* is a major problem in CF and hospitalized patients. To understand the process of infection, most researchers have concentrated their efforts to elucidate the mechanism of bacterial attachment to epithelial cells. Pili of *P. aeruginosa* appear to participate in attachment to epithelial cells (6, 19, 32). Epithelial cell surface glycoconjugates appear to be the receptors for *P. aeruginosa* (2, 10, 18). In normal individuals, mucus is the first line of defense against invading pathogens. HTBM's oligosaccharides, mimicking those of glycoconjugates on the epithelial cells, may bind bacteria to facilitate their clearance by the mucociliary escalator system, thus preventing colonization and infection. In fact, sialylated (30) and neutral (14) oligosaccharides were found to be receptors for *P. aeruginosa*. Similarly, pili and nonpili components of *P. aeruginosa* (15, 17) were found to be adhesins for HTBM. However, the specificity of neither the bacterial adhesins nor the mucin receptors has been determined. A knowledge of the specificities of mucin-bacterium interactions will be of immense value in understanding the pathogenesis of *P. aeruginosa* in respiratory disease. This cannot be achieved by utilizing intact bacteria and impure mucin in the binding assays. Thus, this study employed bacterial components, highly purified mucin, and an overlay assay in which the test

material and the assay conditions can be individually manipulated. By utilizing [¹²⁵I]HTBM as a probe, a 16-kDa protein component(s) of *P. aeruginosa* was identified as an adhesin(s) for HTBM. By manipulating HTBM, the mucin-bacterium interaction in this assay was found to be a protein-protein interaction. The interpretation of the results of this study was based on the assumption that binding of HTBM to the 16-kDa component was not the result of the modification of lysine residues to dimethyllysines by reductive methylation. In order to rule out this possibility, HTBM has to be isolated without modifying its lysines. This can be accomplished by employing citraconylation in place of reductive methylation to dissociate the ionic interactions between HTBM and other proteins of HTBS (26).

Recent studies have suggested that oligosaccharides can be receptors for *P. aeruginosa* (2, 10, 14). In fact, Ramphal and Pyle (18) and Vishwanath and Ramphal (30) have shown that sialic acid of HTBM was a receptor for *P. aeruginosa*. In addition to sialic acid, HTBM contains type 1 and type 2 disaccharide structures that can interact with *P. aeruginosa* (23). In this study, the only mechanism of binding of *P. aeruginosa* to HTBM appears to be protein-protein interaction. Therefore, in addition to the 16-kDa component(s), other adhesins must exist. Failure to identify the *P. aeruginosa* adhesin for HTBM sialic acid may be due to the overlay assay conditions employed in this study. In thin-layer chromatography bacterial overlay assays, Baker et al. (2) and Ramphal et al. (14) have noted that BSA suppresses the binding of *P. aeruginosa* to all sialylated compounds. Since BSA was used in the present study, this could explain the failure of binding involving sialylated oligosaccharides but not neutral oligosaccharides. An explanation for failure of the latter to bind could be that neutral oligosaccharides of the glycolipids (2, 10, 14) may be more accessible for binding than those of HTBM. Conformational restraints determined by the distribution of the oligosaccharides on the HTBM peptide backbone might influence their access to the adhesin(s) in our assay system. Alternatively, the laryngectomy might have altered the HTBM carbohydrate moiety, affecting its interaction with *P. aeruginosa*. In a recent study, differences were indeed observed in the adhesion of *P. aeruginosa* to HTBM glycopeptides isolated from CF and chronic bronchitis patients (16).

It is clear from this and other studies that binding of *P. aeruginosa* to HTBM is specific and may involve more than one receptor and adhesin. Thus, the adhesion process is very complex. Such multiple interactions may firmly bind bacteria and facilitate bacterial clearance from the respiratory tract. Elucidation of the various adhesion mechanisms can lead to a better understanding of the predominance of *P. aeruginosa* colonization in CF individuals.

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