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ISOLATION AND CHARACTERIZATION OF TRACHEOBRONCHIAL MUCIN FROM A LARYNGECTOMEE*

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ABSTRACT

A tracheobronchial mucin was isolated from the tracheobronchial secretion of a laryngectomee. It was purified by gel filtration on Sepharose CL-6B in Tris–urea buffer and rechromatography of excluded materials through the same gel matrix. It was homogeneous in 0.7% agarose–2% polyacrylamide electrophoresis under nonreducing conditions. Comparable analysis with 2-mercaptoethanol revealed at least 3 subunits. Based upon recoverable weight, the mucin was composed of 75% carbohydrate, 21% protein, and 3% sulfate. Oligosaccharides obtained by alkaline β-elimination indicated *O*-glycosyl linkage to the peptide component. Marked heterogeneity of the carbohydrate side-chains was reflected in the preparation of 20 distinct oligosaccharides ranging in size from 4 to 17 residues.

INTRODUCTION

Tracheobronchial secretion (TBS) is the fluid that bathes the respiratory tree. It is derived from four primary sources[1], namely, mucous secreting cells of the surface epithelium and submucosal glands, submucosal serous secreting cells, secretions from such specialized cells as mast cells and Type II pneumocytes, and plasma exudate from the primary vascular bed. Of particular functional significance are the high-molecular-weight mucins which are believed to be responsible for the unique physiochemical and rheological properties of TBS[2]. The isolation and characterization of mucins derived from TBS have been hampered largely by the inability to collect sufficient amounts of this secretion. Although it has been estimated that as much as 350 mL of TBS are produced daily[3], most of it is swallowed and therefore inaccessible. Consequently, many investigators have used sputum as a source of TBS mucins[4–12]. However, the presence of leukocytic hydrolases and proteases[13] leads to the possibility of exogenous degradation of TBS constituents. Additionally,

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the pharmacological agents used to induce sputum expectoration may alter TBS. Finally, although the contribution of salivary components to sputum is reportedly small[1,14], it may be difficult to distinguish between salivary and TBS mucins. Therefore, it would appear that sputum may be an inappropriate starting material for the definitive characterization of TBS glycoproteins. More recently, bronchial-lavage techniques have been employed[14–18]. However, these procedures are invasive and yield only limited amounts of material. Various explant systems have been proposed; they are derived from species as diverse as dog[19], rat[20], cat[21], and hamster[22,23]. A recent survey, however, indicates the wide range of TBS-mucin compositions observed across species[24]. Several groups have used a canine model which employs either a closed, subcutaneous-tracheal pouch[25–29], or a fiberglass screen plugged with cotton[30]. Although this system appears promising, the phylogenetic distance from man may be a drawback. The human analog of this canine model are individuals who have been tracheotomized[6] or laryngectomized[31]. Collection of TBS from such individuals is noninvasive and provides sufficient amounts of material to initiate chemical characterization. We report herein the isolation and characterization of a mucin derived from the TBS of a single laryngectomee.

EXPERIMENTAL

Materials. — The materials were obtained as follows: Sephadex G-25 and G-200, and Sepharose CL-6B from Pharmacia Fine Chemicals (Div. of Pharmacia Inc., Piscataway, NJ 08854), reagents for gel electrophoresis, Bio-Gel P-2, Dowex 50-X4 (200–400 mesh), and Dowex 1-X8 (200–400 mesh) from Bio-Rad Laboratories (Richmond, CA 94804), 3MM paper from Whatman Products, Inc.; *p*-nitrophenyl α- and β-L-fucopyranoside, and *p*-nitrophenyl α- and β-D-galactopyranoside from Sigma Chemical Co. (St. Louis, MO 63178); *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and -α-D-galactopyranoside from Pierce Chemical Co. (Rockford, IL 61105); Econofluor and [^{14}C]formaldehyde (1.9 GBq/mol) from New England Nuclear, Boston, MA 02118; prior to use, anionic contaminants were removed from the formaldehyde by passage through columns of Dowex 1-X8 (AcO^-); NaB^3H_4 (0.37 TBq/mol) from Research Products International Corp. (Mt Prospect, IL 60056); Betafluor from National Diagnostics, Inc. (Somerville, NJ 08876); and BF_3-methanol (14%) from Pierce Chemical Co.

Analytical procedures. — Protein and neutral sugars were determined by the method of Lowry *et al.*[32] and anthrone reaction[33], respectively. Neutral and amino sugars were determined after hydrolysis with 2M HCl for 6 h at 100° and passage of the hydrolyzate through coupled columns[34] of Dowex 50 and 1. The neutral sugars were determined as alditol acetates by g.l.c.[35], and hexosamine, amino acid, sialic acid, and sulfate as described previously[34].

Collection and handling of tracheobronchial secretion. — Human TBS was collected from a 69-year old male Caucasian (blood type O) who had undergone a

TABLE I

SPECIFIC ACTIVITY OF TBS GLYCOSIDASES[a]

Enzyme	*In buffered TBS*		*In saline soluble TBS*	
	4°	*37°*	*4°*	*37°*
α-L-Fucosidase	39	320	17	303
β-L-Fucosidase	17	18	[b]	[b]
α-D-Galactosidase	14	14	[b]	[b]
β-D-Galactosidase	9	61	[b]	35
N-Acetyl-β-D-galactosaminidase	91	939	56	719
N-Acetyl-β-D-glucosaminidase	30	2930	14	65

[a]Expressed as pmol of *p*-nitrophenol released (at pH 6.8)/min/mg of protein determined according to Lowry *et al.*[32]. [b]Not detected.

laryngectomy 4 years previously. Materials (8–12 mL) were "expectorated" onto a Teflon pad and were immediately transferred into ice-cold 0.1M Tris · HCl (2 mL, pH 7.5) containing 2% disodium ethylenediaminetetraacetate–2mM phenylmethanesulfonyl fluoride for solubilization (buffered TBS). In preliminary studies, microscope examination was performed to assess the presence of white cells and microorganisms. Although small numbers of white cells were seen, few if any microorganisms were detected. Buffered TBS was gently stirred for 18 h at 4° in 0.154M NaCl (30 mL). The insoluble material, including cellular debris, was removed by centrifugation (12 000 *g* for 20 min at 4°) and not studied further. The resulting, saline-soluble TBS was dialyzed against 0.2% EDTA and then distilled water, and the retentate lyophilized for subsequent studies to give ~20–30 mg of solubilized material per collection, as compared to 3.2–4.8 mg of insoluble material per collection.

To determine whether degradation of oligosaccharide chains of TBS had occurred during handling, the presence of glycosidase activities in buffered TBS was assessed. These activities were tested by incubating buffered TBS or saline, soluble TBS with several *p*-nitrophenyl glycosides at pH 6.8 (the pH of solubilized TBS) for 72 h at 4 and 37°. The specific activity of glycosidases associated with TBS are summarized in Table I. Significantly, there was little glycosidase activity at the temperature (4°) used to solubilize the buffered TBS. Centrifugation of the TBS (saline soluble TBS) removed a significant amount of the *N*-acetyl-α-D-glucosaminidase activity, suggesting that it is associated with cellular debris.

Purification of tracheobronchial mucin (TBM). — (*a*) *Gel filtration.* Lyophilized TBS was redissolved by gentle stirring at 4° at a concentration of 20 mg/mL in 0.1M Tris · HCl buffer, pH 7.5, in 6M urea (Tris–urea). The undissolved material was removed by centrifugation at 12 000 *g* for 20 min at 4°. After being warmed to room temperature, each sample was applied to a column (2.5 × 116 cm) of Sepharose CL-6B equilibrated in Tris–urea buffer. Fractions (11.0 mL) were collected at room temperature and monitored for protein and hexose content.

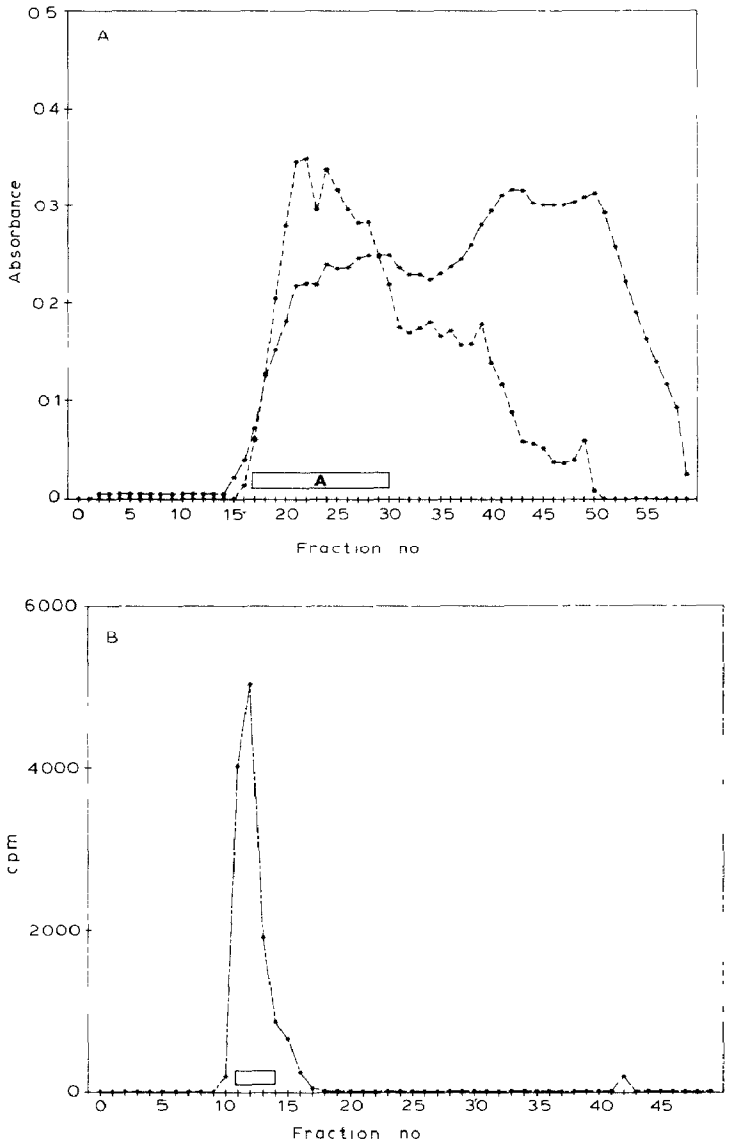


Fig. 1. (A) Chromatography by gel filtration of TBS (200 mg) on Sepharose CL-6B (11.0-mL fractions); absorbance at 620 nm (----) and 280 nm (——). (B) Rechromatography by gel filtration of ^{14}C-TBM [4.7 mg; Peak A, fractions 17–30 of (A)] on Sepharose CL-6B (1.2-mL fractions)

Materials were pooled as indicated in Fig. 1A, dialyzed against distilled water, and lyophilized.

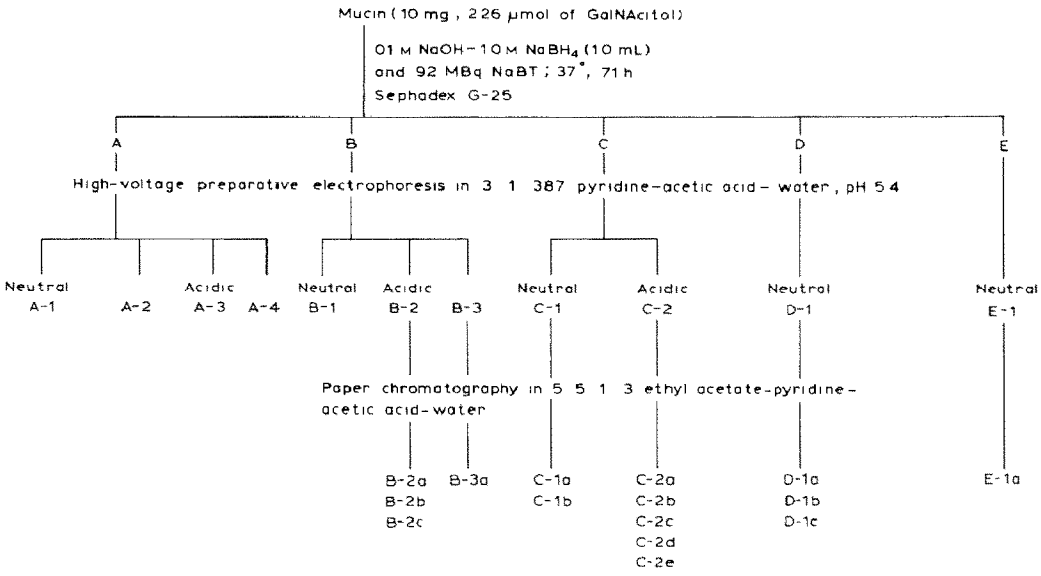
(*b*) *Radiolabeling of TBM by reductive methylation.* To facilitate subsequent isolation procedures and to provide a sensitive means of assessing purity, each void-volume material (Peak A, Fig. 1A) was radiolabeled by ^{14}C-reductive methylation[35,36]. In a typical experiment, the material was dissolved at a concentration of

10 mg/mL in 0.1M HEPES–6M urea buffer, pH 7.5, by gentle stirring for 16–18 h at 4°. Recrystallized $NaCNBH_3$ was added to a final concentration of 20nM. [^{14}C]formaldehyde (~9 MBq) was added to the reaction mixture which was incubated for 16 h at 37°. The radiolabeled material was separated from unreacted products by gel filtration on a column (1.0 × 43 cm) of Bio-Gel P-2 (200–400 mesh) equilibrated with Tris–urea buffer.

(*c*) *Gel filtration of* ^{14}*C-TBM.* Final purification of TBM was achieved by rechromatography of the desalted, ^{14}C-labeled material on a column (1.5 × 115 cm) of Sepharose CL-6B equilibrated with Tris–urea buffer. Fractions (1.2 mL) were collected at room temperature and monitored by liquid-scintillation spectrophotometry (Fig. 1B). The void-volume material, designated A-1, represented purified TBM.

Mild alkaline methanolysis. — Purified TBM (10 mg) and nonadecanoic acid (5 nmol, internal standard) were suspended in 0.3M KOH (3 mL) and gently shaken by sonication for 15 min at room temperature. The mixture was then incubated for 30 min at 37° and acidified with 6M HCl with phenolthalein as indicator[37]. Free fatty acids were extracted with petroleum ether (3 × 5 mL, 30–60°) and the combined extracts washed with distilled water (3 × 2 mL). The petroleum ether layer was passed through a Pasteur pipette containing anhydr. Na_2SO_4 and dried by a stream of nitrogen. BF_3–methanol (14%, 1 mL) was added to the dried mixture, and the reaction tube closed and heated for 2 min at 100°. After cooling, distilled water (4 mL) was added and the material extracted with petroleum ether (3 × 2 mL). The extract was washed and dried as just described, the residue dissolved in chloroform (20 μL) and an aliquot (2 μL) analyzed with a Varian Model 3700 gas chromatograph equipped with columns (2 mm × 2 m) of SP 2100 DOH, at a temperature gradient from 145 to 225° (4°/min) and a nitrogen-flow rate of 20 mL/min. The peaks were quantitatively determined relative to the internal standard by use of a Cyborg Model 42A Chemistry Module and Chromatext software.

Isolation and purification of ^{3}*H-oligosaccharides.* — Alkali-labile oligosaccharides from TBM (10.0 mg) were released and tritiated by incubation with 0.1M NaOH–M $NaBH_4$ (1.2 mL) containing 0.92 GBq of NaB^3H_4 (370 TBq/mol) for 72 h at 37°. The released oligosaccharides were desalted by gel filtration on a column (1.5 × 170 cm) of Sephadex G-25 equilibrated with 0.1M pyridine acetate, pH 5.1. The desalted oligosaccharides were fractionated into neutral and acidic fractions by high-voltage paper electrophoresis on Whatman 3 MM paper in 3:1:387 pyridine–acetic acid–water buffer, pH 5.4, for 4 h at 33 V/cm. For localization of ^{3}H-oligosaccharides, one-cm strips were cut from the dried chromatogram and counted directly, in Betafluor. After washing the paper strips with toluene, the ^{3}H-oligosaccharides were recovered by elution with water[38]. Additional purification of the ^{3}H-oligosaccharides was achieved by either descending paper-chromatography on Whatman No. 1 paper in 5:5:1:3 ethyl acetate–pyridine–acetic acid–water or by high-voltage paper electrophoresis in a 10mM borate buffer, pH 9.5, at 33 V/cm. Localization and recovery of the ^{3}H-oligosaccharides were achieved as



Scheme 1. Preparation of TBM oligosaccharides.

just described. Stachyose or D-glucose were cochromatographed on guide strips as standards and localized by silver nitrate staining[39]. Scheme 1 summarizes the preparation of the TBM oligosaccharides.

Electrophoretic procedures. — $NaDodSO_4$–polyacrylamide gel electrophoresis with and without 2-mercaptoethanol (5% v/v) was performed in 4 and 10% gels according to the method of Weber and Osborn[40]. Glycoproteins were stained for protein with Coomassie blue and for carbohydrate with the periodic acid–Schiff reagent[41]. Relative molecular masses were estimated from plots of the log of molecular weight *vs.* mobility of standard proteins. TBM was also subjected to 2% polyacrylamide–0.7% agarose $NaDodSO_4$–gel electrophoresis as described by Liao *et al.*[29]. Immunoelectrophoresis was performed[42] in slides of 0.8% agarose in barbital acetate buffer, pH 8.2.

RESULTS

Purification of TBM. — Approximately 73% (by weight) of lyophilized, saline-soluble TBS could be dissolved (15 mg/mL) in 0.1M pyridine acetate buffer, pH 6.0. In contrast, 94% of lyophilized TBS went into solution in 0.1M Tris · HCl (pH 7.5)–6M urea buffer. Attempts to increase the solubility of TBS in aqueous solution by delipidation using either a chloroform–methanol extraction[43] or a phenol–water–urea partitioning[44] proved unsuccessful.

In Fig. 1A, a typical-elution profile of TBS fractionated on a column of Sepharose CL-6B, equilibrated with the Tris–urea buffer is illustrated. The mucin-containing fraction (Peak A) contained ~57% of the total hexose and 29% of the

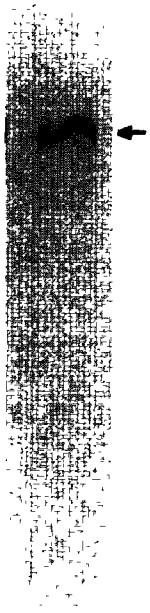


Fig. 2. Gel electrophoresis in 0.7% agarose 2% polyacrylamide of unreduced TBM (100 μg). The gel was stained by the periodic acid–Schiff reagent.

total protein content applied to the column. Following dialysis and lyophilization, the materials were radiolabeled by reductive methylation and, after removal of salt, rechromatographed on Sepharose CL-6B (Fig. 1B). The resulting void-volume material represents purified TBM. In a typical experiment, 23 mg of purified TBM was obtained from a total of 200 mg of lyophilized TBS, which represents a yield of 11.5%.

$NaDodSO_4$–polyacrylamide gel electrophoresis of TBM in the absence of reducing agent revealed that this material did not penetrate a 4% acrylamide gel. TBM stayed at the origin and stained intensely with the periodic acid–Schiff reagent, but stained only faintly with Coomassie brilliant blue. However, in the presence of 2-mercaptoethanol, at least 3 subunits penetrated the gel. Analysis by $NaDodSO_4$–polyacrylamide electrophoresis on a 10% gel, with and without 2-mercaptoethanol, did not reveal any smaller-molecular-weight components (data not shown).

When subjected to $NaDodSO_4$–polyacrylamide electrophoresis on 0.7% agarose–2% acrylamide without reducing agent, a single, discrete band that penetrated the gel was made visible by the periodic acid–Schiff reagent (Fig. 2). The mobility of this material was between those of the major and minor components of monkey-salivary mucin[34] and, as such, a tentative relative molecular mass between 0.9 and 1.0×10^6 may be assigned to TBM. Evidence for size homogeneity was also

TABLE II

CHEMICAL COMPOSITION OF TBM

Component	%
Protein	20.2
2-Acetamido-2-deoxygalactose	5.0
2-Acetamido-2-deoxyglucose	16.9
Fucose	9.0
Galactose	18.4
Sialic acid	10.6
Sulfate	3.0
Fatty acids[a]	
$C_{16:0}$	3.2
$C_{18:1}$	0.7
$C_{18:0}$	1.4

[a]As nmol/mg of TBM

TABLE III

AMINO ACID AND CARBOHYDRATE COMPONENTS OF TBM

Component	*Residues/1000 amino acid residues*
Aspartic acid	59
Threonine	141
Serine	105
Glutamic acid	70
Proline	107
Glycine	77
Alanine	75
Half-cystine	32
Valine	42
Methionine	+
Isoleucine	50
Leucine	86
Tyrosine	16
Phenylalanine	26
Lysine[a]	35
Histidine	29
Arginine	50
2-Acetamido-2-deoxygalactose	259
2-Acetamido-2-deoxyglucose	882
Fucose	467
Galactose	986
Sialic acid	55
Sulfate	55

[a]Determined as mono- and di-methyllysine.

seen by rechromatography of the ^{14}C-TBM on a column of Sepharose CL-6B (Fig. 1B). Immunochemical homogeneity of TBM was examined by immunoelectrophoresis using a guinea pig anti-TBS serum. TBM reacted to give a single, anodal-precipitin arc characteristic of a mucin-like molecule[34].

Chemical composition of TBM. — The chemical composition of TBM was typical of mucin-type glycoproteins (Tables II and III). The protein content, as determined by amino acid analysis, comprised 20% of the recoverable weight. The amino acids threonine, serine, proline, and glycine contributed ~40% of the total amino acids, and 32 residues of half-cystine per 1000 amino acid residues were observed. The carbohydrate content corresponded to 75% of the recoverable weight of TBM, 2-acetamido-2-deoxygalactose, 2-acetamido-2-deoxyglucose, fucose, galactose, and *N*-acetylneuraminic acid being present in the ratios 5:17:9:19:1. TBM contains 3% by weight of inorganic sulfate. The content of fatty acids comprised only 0.14% (w/w) of TBM, the preponderant fatty acids being $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$ derivatives.

Isolation and purification of TBM oligosaccharides. — To cleave off the oligosaccharide chains, TBM was treated with mild alkali followed by reduction. After being desalted with Sephadex G-25, ^{3}H-oligosaccharides were separated into neutral and acidic fractions by high-voltage paper electrophoresis. Each fraction was then subjected to a combination of descending paper chromatography and borate-buffered, high-voltage electrophoresis as a means of purifying the individual oligosaccharides. The marked heterogeneity of the TBM side-chains is reflected in the preparation of 20 distinct oligosaccharides (Fig. 3). Table IV summarizes the yield, electrophoretic mobility, and composition of the 10 preponderant compounds. They ranged in size from 4 to 17 residues. Hydrolysis and subsequent borate-buffered, high-voltage paper electrophoresis at pH 9.5 revealed that the radioactivity was exclusively incorporated into the 2-acetamido-2-deoxy-D-galacti-

TABLE IV

CARBOHYDRATE COMPOSITION OF TBM OLIGOSACCHARIDES[a]

Oligosaccharide	*NeuAc*	*Fuc*	*Gal*	*GlcNAc*	*GalNAcitol*	*Yield (nmol)*	$R_{Stachyose}$
A-1		5.9	3.9	4.7	1.0	145	
A-2	1.0	3.2	3.9	4.3	1.0	262	
A-3	1.8	4.1	4.6	4.7	1.0	222	
B-1		3.1	3.1	2.7	1.0	90	0.11
B-2c	1.0	0.4	1.8	1.9	1.0	38	0.35
C-1b		2.3	2.3	1.9	1.0	78	0.48
C-2a	0.9	1.4	1.9	1.5	1.0	101	0.36
C-2e	1.3		1.0	1.0	1.0	33	1.21
D-1a		0.4	1.1	1.1	1.0	81	1.36
D-1b		1.2	1.1	1.0	1.0	81	1.86

[a]Molar ratio relative to GalNAcitol.

tol residue indicating that the released chains were *O*-glycosyl-linked to serine or threonine residues. Preliminary data indicated that oligosaccharides were either neutral or acidic. Two oligosaccharide fractions (A-3 and A-4) could contain both sialyl and sulfate groups since mild acid treatment of these materials[34] to remove sialic acid resulted in products that were still acidic in high-voltage paper electrophoresis.

DISCUSSION

We report herein the purification of a mucin obtained from the tracheobronchial secretions of a laryngectomee. To minimize alterations of the native TBM structure, the secretion was solubilized under relatively mild conditions. Although the enzyme specificity of some glycosidases precluded their assay by low-molecular-weight (*p*-nitrophenyl glycosides) substrates, minimal glycosidase activity was demonstrable under the conditions employed.

In a preliminary report, we showed that the use of 6M urea during gel filtration was necessary to minimize interaction of the mucin with the chromatographic matrix[45]. Moreover, several low-molecular-weight peptides were shown to form complexes with the mucin glycoproteins during gel filtration in aqueous buffers. For example, gel filtration of TBS in 0.1M pyridine acetate buffer, pH 6.0, produced an excluded mucin fraction that, when examined by immunoelectrophoresis with guinea pig anti-TBS sera, revealed the presence of anionic (mucin) and cationic components. The cationic component was identified as lysozyme by immunoelectrophoresis with rabbit antisera to human-leukemic lysozyme. In contrast, excluded materials obtained by gel filtration in the presence of 6M urea revealed only the mucin, indicating that the mucin–lysozyme complex is dissociated under these conditions. These findings are in agreement with the observations of Rose *et al.*[10] who pointed out the importance of noncovalent forces in the maintenance of TBM suprastructure.

The homogeneity of the TBM was assessed by several complementary criteria. Electrophoresis in 4 and 10% polyacrylamide gels in the presence of sodium dodecyl sulfate failed to reveal contaminating species. When subjected to 0.7% agarose–2% polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate, with or without reducing agent, a discrete band of material (stained only by the periodic acid–Schiff reagent) was observed. Previously, we have pointed out the efficacy of immunological reagents for assessing the purity of mucin glycoproteins[34]. In studies to be reported elsewhere, we have shown that TBM reacts with guinea pig anti-TBS to form a single, anodal-precipitin arc. Finally, the absence of any demonstrable mannose (Table II) indicates the absence of appreciable contamination by *N*-proteins, such as secretory IgA. Collectively, these observations argue for the relative homogeneity of the present TBM preparation.

Protein and carbohydrate content accounted for only 83% of the dry weight of TBM. The presence of lipids associated with respiratory mucins has been re-

ported[7,18,46]. In the present study, a very small proportion (0.14%) of fatty acids was detected. This is in contrast to the observation of Woodward *et al.*[18] who found that TBM prepared from bronchial lavage samples contained 5–15% of total lipid. The reason for these differences is not clear, but could possibly be due to the different methods of preparation.

Several reports have demonstrated the heterogenous nature of TBM oligosaccharides[4,47]. In the present study, oligosaccharides ranging in size from 4 to 17 residues were isolated. The fact that the oligosaccharides obtained in greatest yield were the largest argues against the heterogencity observed being due to exogenous breakdown during handling and purification steps.

Interestingly, the chemical structures of oligosaccharide chains reported for pooled sputum samples of patients suffering from cystic fibrosis[47] are similar to those found for oligosaccharide chains of the lower-molecular salivary mucin[48] and not with those found in the present study. It remains to be determined whether these differences are due to cystic fibrosis *per se* or to the presence of saliva in sputum samples.

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