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Isolation, Purification, and Properties of Respiratory Mucus Glycoproteins†

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ABSTRACT: The major glycoprotein from human tracheobronchial secretions and from primary explant cultures of human tracheal epithelium has been purified to apparent homogeneity. Mucin was solubilized in buffer and fractionated on Sepharose CL-4B, followed by CsBr density gradient centrifugation of the void volume fraction. High- and low-density fractions were obtained in ratios ranging from 2:1 to 5:1. The high-density (1.46) fraction appeared homogeneous by exclusion chromatography and recentrifugation in CsBr and had an amino acid composition characteristic of a mucin-type structure (threonine, serine, proline, glycine, and alanine comprise two-thirds of the amino acid residues). The carbohydrate, which is nearly 80% by weight, was O-glycosidically linked via GalNAc, sulfated (5.4% by weight), and contained fucose, galactose, glucosamine, galactosamine, and sialic acid. The low-density fraction had an amino acid composition distinct from that of the high-density fraction (threonine, serine, proline, glycine, and alanine comprise 51% of the amino acid residues) and a lower sulfate content. The size distribution of the saccharides in the low-density fraction was similar to that of the high-density fraction; the same sugars were present although the ratios were different. The low-density fraction contained 3 times more noncovalently associated lipid than did the high-density fraction. Several distinct classes of lipids were identified. Neutral lipids (mono-, di-, and triglycerides, cholesterol, and cholesteryl esters) comprised 56% by weight of the total lipid. Glycolipids and phospholipids were also identified. Palmitate (16:0), stearate (18:0), and oleate (18:1) were the major fatty acids in all classes of lipids.

Human tracheobronchial secretions consist of a heterogeneous population of macromolecules, salts, and water. Respiratory mucus, as well as mucus from other sources (e.g., gastric, salivary, and cervical mucus), serves as a lubricative and protective barrier and, in addition, provides a clearance mechanism for particulate matter from the respiratory tract.

Human tracheobronchial mucus has been characterized in terms of total protein, carbohydrate, lipid, and inorganic salts (Basch et al., 1941; Masson et al., 1965; Chernick & Barbero, 1959). Rheological properties have also been studied (Mitchell-Heggs, 1977; Charman & Reid, 1972, 1973).

The major glycoprotein present in tracheobronchial secretions is a large, carbohydrate-rich glycoprotein whose macromolecular structure regulates the viscoelastic properties of mucus. Isolation and characterization of the individual components from mucus are required to understand structure–function relationships.

Mucus contains several components in addition to the major mucin glycoprotein, most of which are lower molecular weight proteins and serum-type glycoproteins (Havez et al., 1968; Roussel et al., 1975; Boat et al., 1976; Feldhoff et al., 1976, 1979; Rose et al., 1979). Resolution of these components often involves the use of exclusion chromatography (Boat et al.,

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1976; Feldhoff et al., 1979; Rose et al., 1979) or ion-exchange chromatography (Lamblin et al., 1977; Roussel et al., 1975) to separate the high molecular weight, polyanionic mucin glycoprotein from lower molecular weight serum components. Another separation technique which has proved quite valuable is density gradient centrifugation in CsBr/CsCl solutions. This technique has been used preparatively for bronchial mucin glycoproteins (Creeth et al., 1977) as well as for blood group substances (Creeth & Denborough, 1970), pig gastric and intestinal mucus (Starkey et al., 1974; Mantle & Allen, 1981), and human gastric mucus (Pearson et al., 1980). Studies on these types of secretions have shown the presence of tightly, but noncovalently bound, protein and have provided detailed information on the macromolecular organization of mucus components.

Detailed studies on "normal" human tracheobronchial secretions, however, are often incomplete because of difficulties in collecting adequate amounts of secretions for chemical characterization. Previous studies from our laboratory have accordingly reported on mucin glycoproteins isolated from the tracheobronchial secretions of an asthmatic patient (Feldhoff et al., 1979). This paper describes the isolation, purification, and characterization of mucin glycoprotein from bronchial secretions of patients who have undergone surgery for nonpulmonary illness and from primary explant organ cultures of human tracheal epithelium.

Materials and Methods

Collection of Bronchial Aspirate Material. Mucus was collected every 8 h by sterile aspiration at room temperature from seven different patients who had undergone surgery for nonpulmonary illness. Blood group status of these patients was determined by hemagglutination inhibition using anti-A and anti-B serum and a 2% suspension of red blood cells (Morgan & Watkins, 1951). Three patients exhibited blood group A activity, one patient blood group B activity, and three patients no detectable blood group A or B activity. Samples were stored in the freezer if not used immediately. Samples used immediately were diluted 1:1 with phosphate-buffered saline (PBS)[1] containing 0.02% NaN_3 and stirred at 4 °C for 24 h and then centrifuged at 10000 rpm (12000*g*) for 20 min to remove a small amount of cellular debris. No visible gellike material was present in the pellet. This pellet contained less than 10% by weight of the starting material and was not examined further. The resulting supernatant which contained 90–95% of the carbohydrate content of the original material was dialyzed against distilled H_2O at 4 °C for 48 h and then lyophilized.

Primary Explant Culture of Tracheal Epithelium. Segments of trachea were obtained from one subject at autopsy within 4 h after death and were cultured on the basis of the procedure of Boat et al. (1977). Epithelial cells were removed from the segments by gentle scraping with a scalpel and tweezers and then transferred to Gibco medium 199 + glutamine (685 μM) in culture plates (2–3 mL of medium/plate). [^{3}H]Glucosamine (100 μCi; New England Nuclear, specific activity = 20.2 Ci/mmol) and [^{14}C]leucine (10 μCi; Amersham, specific activity = 35 mCi/mmol) were added to each plate. The plates were incubated at 37 °C in a 5% CO_2/95% air atmosphere, and medium (containing isotope) was changed every 24 h. The epithelial cells were cultured for 3–4 days. Pooled, spent medium was dialyzed against H_2O at 4 °C for 3 days and then lyophilized.

Analytical Methods. Chromatographic column effluents and CsBr density gradient fractions were screened for neutral sugar by the phenol/H_2SO_4 method (Dubois et al., 1956) with galactose as a standard and for protein by measuring the absorbance at 280 nm. Specific sugar components were identified by gas–liquid chromatography of their alditol acetates after hydrolysis in either 2 N HCl or 2 N trifluoroacetic acid (Sawardekar et al., 1965; Lehnhardt & Winzler, 1968) or by methanolysis of sugars in 1.4 N methanolic HCl at 85 °C for 24 h, followed by conversion to the trimethylsilyl derivatives prior to chromatography (Clamp et al., 1971). Glucosamine and galactosamine were also determined on the amino acid analyzer after hydrolysis for 8 h at 100 °C in 4 N HCl.

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Samples for amino acid analysis were hydrolyzed in sealed ampules for 24 h at 110 °C with 6 N HCl. Analyses were performed on a Beckman 120C or 121 amino acid analyzer or on a Dionex amino acid/peptide analyzer MBFSS kit.

Sulfate was determined by the method of Spencer (1960) after hydrolysis for 12 h at 110 °C in 6 N HCl. Uronic acid was determined by a modified carbazole method (Davidson, 1966).

Ouchterlony Immunodiffusion of Sepharose CL-4B Included Fractions. Ouchterlony immunodiffusion was performed according to the procedure of Williams (Williams & Chase, 1971). Two samples of unfractionated bronchial mucus (samples C.L. and K.W.) and one sample of fractionated lower molecular weight components (C.L.) in 0.15 M phosphate buffer, pH 7.2, were placed in the immunodiffusion wells, covered, and incubated in a humid atmosphere at room temperature for 24 h. Gels were stained with Buffalo Black for 2–6 h and destained with 2% acetic acid.

The antisera used were from commercial sources. Rabbit anti-whole human serum, rabbit anti-α_1-acid glycoprotein (lot 78M), and goat anti-human serum albumin (lot G389) were from Miles Biochemicals. Goat anti-IgG (lot 231-78-13), goat anti-IgA (lot 231-78-21), and goat anti-whole human serum (lot 231-74-7) were from Bionetics.

Electrophoretic Studies. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed in 5% polyacrylamide gels according to the method of Weber & Osborn (1969). Mucin (100–200 μg) was incubated in $NaDodSO_4$/βME at 110 °C for 3 min and then applied to the gels. After electrophoresis, gels were stained for protein with Coomassie Brilliant Blue and for carbohydrate by the periodate–Schiff method (Fairbanks et al., 1971).

CsBr Density Gradient Centrifugation. Sepharose CL-4B void volume material from fractionation of crude bronchial aspirates or spent culture medium was subjected to CsBr density gradient centrifugation on the basis of the procedure of Creeth et al. (1977). Void volume material was suspended in phosphate buffer (16.7 mM $Na_2HPO_4 \cdot 7H_2O$, 16.7 mM $NaH_2PO_4 \cdot H_2O$, 33 mM NaCl, and 0.02% NaN_3) containing 42% (w/w) CsBr at a loading density of 1.43 g/cm^3 and stirred for 24 h at 4 °C. After the mixture was stirred, it was precentrifuged at 5000 rpm (3000*g*) for 5 min at 4 °C, and then the supernatant was transferred to 1 in. × 3.5 in. cellulose nitrate centrifuge tubes. After this centrifugation, 97% of the total neutral sugar remained in the supernatant. Tubes were filled to the top and capped. The samples were centrifuged for 67 or 85 h at 14 °C and 42000 rpm in a Beckman 60Ti

[1] Abbreviations: PBS, phosphate-buffered saline; MeOH, methanol; DPC, dipalmitoylphosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate; βME, β-mercaptoethanol; HOAc, glacial acetic acid; TLC, thin-layer chromatography.

rotor. After centrifugation, tubes were punctured and 1-mL fractions collected and assayed (after diluting 1:10 with H_2O) for protein and for neutral sugar. Fractions from centrifugation of labeled materials were counted in ACS II (Amersham). Densities were determined by weighing a 100-μL aliquot of each fraction.

In some cases, centrifugation was performed in 0.625 in. × 2.5 in. tubes in a Beckman type 50 rotor. All other conditions were identical with those described above except that 0.7–0.8-mL fractions were collected.

Alkaline Borohydride Treatment. High- and low-density fractions were treated with 1 M $NaBH_4$ in 0.1 N NaOH according to the procedure of Carlson (1966). A 0.1% solution of mucin in 1 M $NaBH_4$ in 1 N NaOH was incubated for 72 h at 37 °C in the dark under N_2. Following incubation, mixtures were neutralized with 4 N HOAc and evaporated to dryness by rotary evaporation. The residue was washed repeatedly (5 times) with 1000:1 MeOH:concentrated HCl, evaporating to dryness by rotary evaporation after each washing. The residue was then analyzed by chromatography on a Bio-Gel P-10 column (column dimensions 1 × 30 cm) in 0.1 N pyridinium acetate, pH 5.1.

Lipid Extraction. Mucin samples (50 mg) were extracted sequentially for 12 h at 4 °C in 30-mL Corex test tubes with 20 mL of 2:1 $CHCl_3$:MeOH, 10 mL of 2:1 $CHCl_3$:MeOH, and finally 5 mL of 1:2 $CHCl_3$:MeOH, based on a procedure discussed by Esselman et al. (1972). After each extraction, samples were centrifuged at 10000 rpm (12000*g*) for 20 min, and the supernatants were removed with a Pasteur pipet. Addition of a small volume of methanol (usually 5 mL) aided in pelleting the insoluble glycoprotein. The combined supernatants were filtered through coarse-grade filter paper and evaporated to dryness by rotary evaporation at 25 °C. Lipid residues were dried for 24 h in vacuo over P_2O_5 and then weighed. The delipidated mucins were suspended in H_2O and then lyophilized.

Silicic Acid Column Chromatography. Lipid extracts were chromatographed on a Bio-Sil A (200–400 mesh) silicic acid column (Esselman et al., 1972). Heat-activated silicic acid (100 °C, 8 h) was equilibrated in $CHCl_3$, and then a 4-mL (1 × 5 cm) column was poured. The column was washed sequentially with 10 column volumes of $CHCl_3$, 10 column volumes of 9:1 acetone:MeOH, 10 column volumes of MeOH, and finally 10 column volumes of $CHCl_3$. Lipid residue, up to 15 mg suspended in 1 mL of $CHCl_3$, was applied to the column, and then the column was eluted sequentially with 5 bed volumes each of $CHCl_3$, 9:1 acetone:MeOH, and MeOH. Effluents from each different wash were collected separately and evaporated to dryness by rotary evaporation. The residues were resuspended in a small volume of elution solvent, transferred to weighed tubes, evaporated to dryness with N_2, dried in vacuo over P_2O_5, and then weighed.

The $CHCl_3$ fraction, containing neutral lipid, was further chromatographed on a 4-mL (1 × 5 cm) Bio-Sil A column (200–400 mesh) equilibrated in 6% benzene in hexane (Lo-Chang & Sweeley, 1963). Neutral lipid in 1 mL of 6% benzene in hexane was applied to the column, and the column was eluted sequentially with 5 column volumes each of 6% benzene in hexane, 20% benzene in hexane, 65% benzene in hexane, and $CHCl_3$. Effluents were collected and evaporated to dryness by rotary evaporation. Residues were resuspended in a small volume of elution solvent, transferred to weighed tubes, evaporated to dryness with N_2, dried in vacuo over P_2O_5, and then weighed. Lipids in these fractions were identified by TLC. Lipids in the acetone/MeOH fraction (glycolipid enriched) and the MeOH fraction (phospholipid enriched) were also identified by TLC, without further purification.

TLC of Lipid Fractions. Whatman silica gel K5 plates (250-μm thickness) used for lipid separation were heated at 100 °C for 12 h and used immediately upon being cooled to room temperature. Solvent systems used (all solvents were redistilled before use) were 90:10:1 hexane:diethyl ether:glacial acetic acid for separation of neutral lipids and 65:25:4 $CHCl_3$:MeOH:H_2O or 65:35:8 $CHCl_3$MeOH:2.5% aqueous ammonia for separation of phospholipids and glycolipids. All fractions were dissolved in 2:1 $CHCl_3$:MeOH before being applied to the plates.

All lipids were visualized by 50% sulfuric acid charring or with I_2 vapors followed by spraying with a 1% starch solution. Glycolipids were specifically visualized with 2% α-naphthol in ethanol followed by H_2SO_4 charring. Phospholipids were visualized with 2.5% ammonium molybdate in 2 N H_2SO_4 followed by 0.25% ascorbic acid. Phospholipids were also visualized with Phospray (Supelco). Choline-containing lipids were visualized with Chargaff's reagent (Stahl, 1965).

Fatty Acid Analysis. Fatty acids were determined as their methyl esters after treatment with 14% w/v BF_3/MeOH for 3 h at 110 °C. Methyl esters were separated and identified by gas–liquid chromatography on a column of 10% DEGS-PS 80/100 Supelcoport. The chromatography was conducted isothermally at 200 °C at a flow rate of 30 mL/min with N_2 as a carrier gas. The column was attached to a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector.

Equilibrium Sedimentation Analysis. High- and low-density delipidated glycoproteins were examined by analytical equilibrium ultracentrifugation by using the meniscus depletion method (Yphantis, 1964). Prior to centrifugation, the sample in PBS was dialyzed against PBS for 24 h.

Results

Results of all the analyses correspond to the mean value of the seven different samples. After centrifugation of crude bronchial aspirate material at 12000*g*, 90–95% of the total neutral sugar remained in the supernatant. Chromatography of the supernatant on Sepharose CL-4B (Figure 1) separated the material into an excluded fraction (peak I) containing a mucin-type glycoprotein and a heterogeneous included fraction (peak II) consisting of serum proteins and glycoproteins. The excluded fraction accounted for 65–75% of the total neutral sugar. Blood group activity in the crude aspirate material, measured by hemagglutination inhibition, was recovered in the excluded fraction. Amino acid analysis of this fraction (Table I) indicated a composition characteristic of a mucin-type structure: threonine, serine, proline, glycine, and alanine comprised 54% of the total amino acid residues. Aromatic amino acids (phenylalanine and tyrosine) and cysteine were low. Fucose and galactose comprised 30–40% by weight of the mucin glycoprotein. The hexosamines were present in equimolar ratios and accounted for 25–30% by weight of the mucin glycoprotein. Fraction II had an amino acid composition characteristic of serum-type components. The presence of mannose and extensive cross-reactivity with serum components after double immunodiffusion identified components in this fraction as circulating serum proteins and glycoproteins (Figure 2).

A similar type of elution profile was observed when labeled, spent medium from organ culture was chromatographed (Figure 3). A high molecular weight, excluded fraction (80% of the total [^{3}H]glucosamine) and a lower molecular weight included fraction (30% of the total [^{14}C]leucine) were observed.

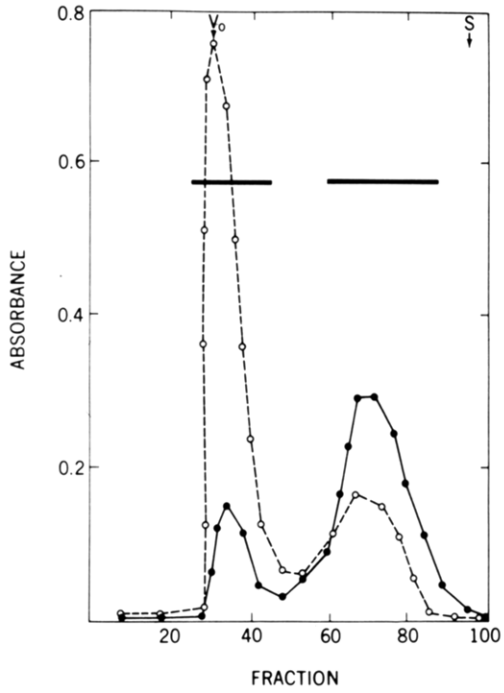

FIGURE 1: Sepharose CL-4B (5 × 95 cm) chromatography of crude bronchial aspirate material in 50 mM Tris, pH 8, containing 0.02% NaN_3. A 10 mg/mL solution of mucin in buffer was stirred at room temperature for 3 h and then centrifuged at 12000*g* for 10 min. The supernatant was applied to the column equilibrated in the buffer. 100 × 20 mL fractions were collected and screened for protein (●) and neutral sugar (○).

Table I: Amino Acid Composition of Bronchial Secretions after Chromatography on Sepharose CL-4B

amino acid	residues/1000 residues peak I	peak II
lysine	29	64
histidine	21	22
arginine	40	40
cysteic acid	4	1
aspartic acid	70	115
threonine	170	59
serine	110	59
glutamic acid	78	138
proline	103	79
glycine	72	63
alanine	86	75
half-cystine	15	35
valine	69	66
methionine	10	11
isoleucine	29	22
leucine	63	85
tyrosine	11	27
phenylalanine	21	41
glucosamine[a]	13	6
galactosamine[a]	12	1.5

[a] Expressed as percent by weight of the total glycoprotein.

$NaDodSO_4$–polyacrylamide gel electrophoresis of the excluded fractions in 5% polyacrylamide gels indicated the presence of lower molecular weight contaminants in both the bronchial aspirate and the organ culture materials. Rechromatography of the excluded fraction of Sepharose CL-4B also indicated the presence of contaminating protein, although greater than 95% of the total neutral sugar in the bronchial aspirate material remained in the excluded fraction. CsBr density gradient centrifugation was used to separate this contaminating protein from the mucin glycoprotein. A high-density (1.46) and a low-density (1.34) fraction were obtained from both bronchial aspirate material and tracheal organ culture (Figure 4A,B).

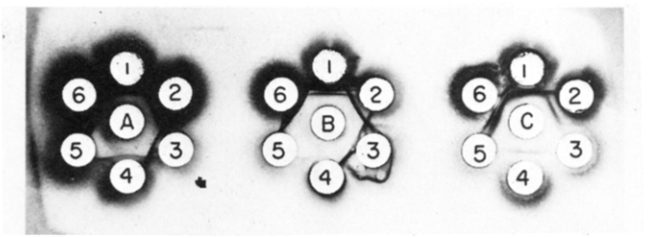

FIGURE 2: Ouchterlony immunodiffusion reactions of human tracheobronchial components (center walls) against antisera (outer wells). Center wells: A, unfractionated bronchial secretion (K.W.); B, low molecular weight fraction from Sepharose CL-4B chromatography; C, unfractionated bronchial secretion (C.L.). Outer wells: 1, rabbit anti-whole human serum; 2, anti-α_1-acid glycoprotein; 3, anti-IgG; 4, anti-IgA; 5, blank; 6A, goat anti-whole human serum; 6B, anti-human serum albumin.

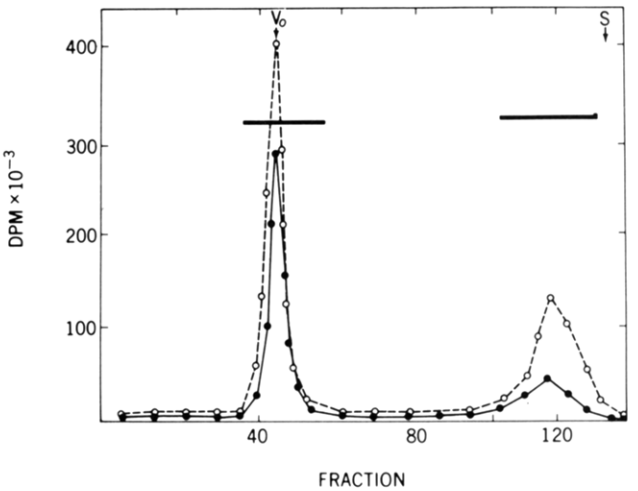

FIGURE 3: Sepharose CL-4B (2 × 65 cm) chromatography of labeled spent culture medium in 50 mM Tris, pH B, containing 0.02% NaN_3. 140 × 1.5 mL fractions were collected and aliquots of each fraction counted in ACS II with a liquid scintillation spectrometer. (○) [^{3}H]Glucosamine; (●) [^{14}C]leucine.

Table II: Amino Acid Composition of High- and Low-Density Fractions from Bronchial Aspirates

amino acid	residues/1000 residues high density	low density
lysine	26	45
histidine	26	27
arginine	35	43
aspartic acid	46	72
threonine	210	112
serine	127	110
glutamic acid	63	82
proline	120	87
alanine	96	96
glycine	91	104
valine	44	55
methionine	3	10
isoleucine	33	34
leucine	52	63
tyrosine	10	28
phenylalanine	10	33
threonine + serine + proline + glycine + alanine	644	509
glucosamine[a]	14	13
galactosamine[a]	11	4

[a] Expressed as percent by weight of the total glycoprotein.

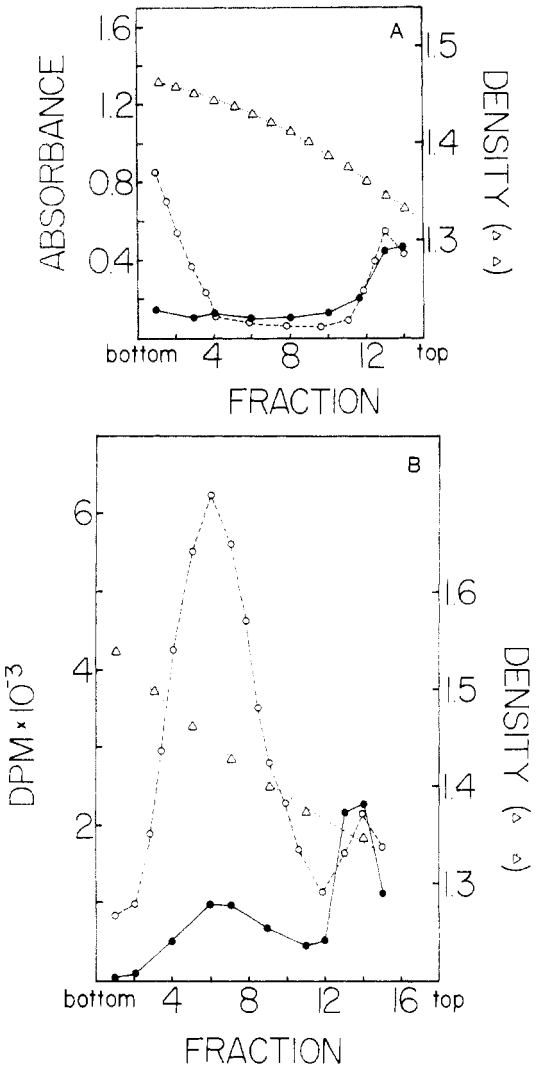

FIGURE 4: CsBr density gradient centrifugation of void volume material. (A) Bronchial aspirate material excluded on Sepharose CL-4B was loaded at a concentration of 1–2 mg/mL and centrifuged for 85 h at 42000 rpm. Starting density was 1.43 mg/mL. Fractions (0.7–0.8 mL) were collected and analyzed for protein (●) and neutral sugar (○). (B) Tracheal organ culture material excluded on Sepharose CL-4B was centrifuged as above but for 67 h. Fractions (0.7–0.8 mL) were collected and aliquots screened for [^{3}H]glucosamine (○) and [^{14}C]leucine (●).

Table III: Carbohydrate Analysis[a] of High- and Low-Density Fractions from Bronchial Aspirates

sugar	% by weight of total glycoprotein: high density	% by weight of total glycoprotein: low density
fucose	19	21
galactose	20	23
N-acetylglucosamine	14	13
N-acetylgalactosamine	11	4
sialic acid (as NeuNAc)	4	3
glucose	1	2
sulfate	5.4	3.6

[a] These analyses are on nondelipidated samples. Glucose is decreased in delipidated samples (less than 1% by weight of total glycoprotein in both the high- and low-density fractions).

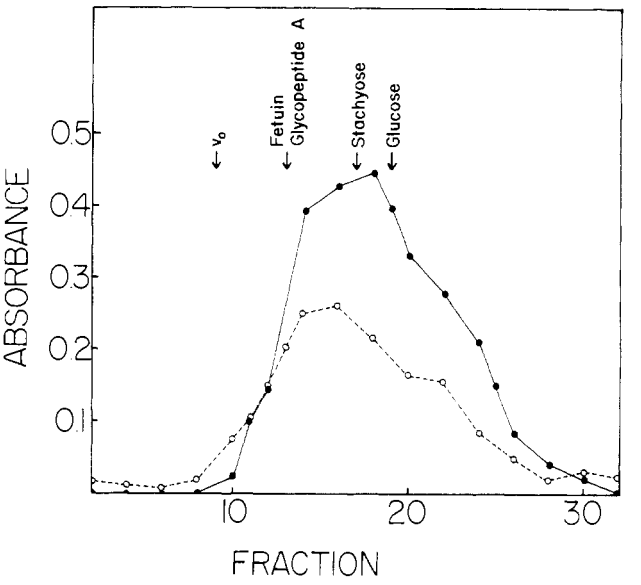

FIGURE 5: Bio-Gel P-10 (1 × 30 cm) chromatography in 0.1 N pyridinium acetate, pH 5.1, of high- (●) and low-density (○) fractions from bronchial aspirates after treatment with alkaline sodium borohydride. Aliquots of each fraction were screened for neutral sugar.

Both high- and low-density fractions from bronchial aspirate material contained 30–40% neutral sugar and 10–20% protein by weight. The high-density fraction had an amino acid composition characteristic of a mucin-type structure (Table II): threonine, serine, proline, glycine, and alanine totaled 64% of the amino acid residues; aromatic amino acids were low (phenylalanine and tyrosine totaled 2% of the amino acid residues). The low-density fraction had an amino acid composition distinct from that of the high-density fraction: threonine, serine, proline, glycine, and alanine totaled 51% of the amino acid residues; aromatic amino acids (phenylalanine and tyrosine) were greater (6% of the total amino acid residues) than in the high-density fraction. The high- and low-density fractions from bronchial aspirate material had similar carbohydrate compositions, but the ratios of monosaccharides in the two fractions were different (Table III). The neutral sugar:hexosamine ratio was 2:1 in the high-density fraction and 3:1 in the low-density fraction. Sulfate was present in both the high-density (5.4% by weight) and low-density (3.6% by weight) fractions. Glucose was also detected in both the high- and low-density fractions. Delipidation removed most of the glucose, suggesting the presence of glycolipid. The remaining glucose may be a result of contamination from the dialysis tubing used during purification. Only trace amounts of mannose (<0.5% of the total carbohydrate) were detected in the high-density fraction. A slight amount of mannose was detected in the low-density fraction (1% of the total carbohydrate). Neither xylose nor uronic acid was detected in either the high- or the low-density fractions. The carbohydrate in both the high- and low-density fractions is linked to protein via Ser/Thr on the basis of susceptibility to alkaline sodium borohydride (Figure 5). The oligosaccharides of the high- and low-density fractions were similar in size distribution and ranged from 2–14 sugars.

Neither the high- nor the low-density fractions from tracheal organ culture were susceptible to chondroitinase ABC digestion as judged by their elution profile on Sepharose CL-4B. These data indicate the absence of proteoglycan in these fractions.

Equilibrium sedimentation analysis of the high- and low-density *delipidated* fractions (Figure 6) yielded molecular weights of 2.3×10^6 for the high-density fraction and 1.5×10^6 for the low-density fraction. The curvature of the plots showed a significant degree of polydispersity and/or aggregation with molecular weights ranging to 4.6×10^6 for the high-density component and to 5.6×10^6 for the low-density component.

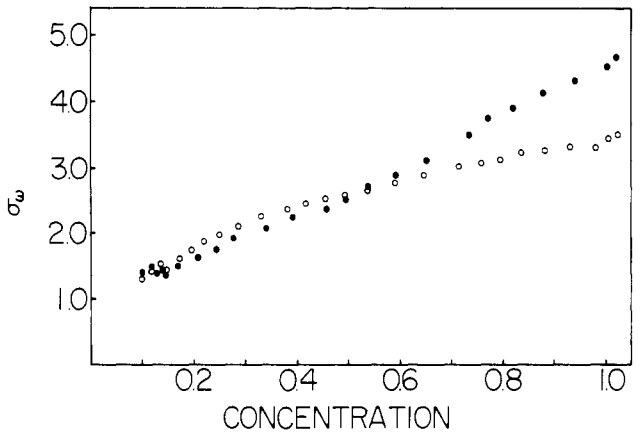

FIGURE 6: Equilibrium sedimentation analysis of the high- (○) and low-density (●) *delipidated* fractions from bronchial aspirate material excluded on Sepharose CL-4B.

Table IV: Lipid Composition of Mucin

class of lipid	weight % of total lipid	% of total fatty acids as palmitate + stearate + oleate
neutral lipids	56	
monoglycerides	5	61 for glycerides
diglycerides	4	
triglycerides	20	
cholesterol	15	
cholesteryl esters	12	64
glycolipid-enriched fraction	26	63
phospholipid-enriched fraction	18	70
phosphatidylcholine		
other phospholipids		

The presence of noncovalently associated lipid was demonstrated by extraction of the high- and low-density fractions from bronchial aspirates with $CHCl_3$/MeOH. Three times as much lipid was associated with the low-density fraction than with the high-density fraction. This result suggested that variations in lipid binding gave rise to the different buoyant densities exhibited by the glycoproteins. As a test of this, (1) bronchial aspirate material excluded from Sepharose CL-4B was delipidated prior to density gradient centrifugation, and (2) the low-density fraction from bronchial aspirate material was delipidated and then rerun in density gradient centrifugation. The results of these two experiments are indicated in Figure 7. Both delipidation of bronchial aspirate material excluded from Sepharose CL-4B before centrifugation and delipidation of the low-density fraction, followed by recentrifugation, yielded only a high-density fraction. Analyses of material thus treated were substantially unchanged.

Lipid extraction and analysis were performed for seven different bronchial aspirate void volume fractions (Table IV), and the results are expressed as the mean value of the samples. The total weight percent of lipid associated with these fractions ranged from 5 to 15%. In all cases, neutral lipids (mono-, di-, and triglycercides, cholesterol, and cholesteryl esters) comprised the major class (56% of the total lipid). A glycolipid-enriched fraction (26% of the total lipid) and a phospholipid-enriched fraction (18% of the total lipid) were also identified. α-Naphthol-positive spots on TLC plates were designated glycolipids. Specific glycolipids were not identified, but those present had R_f values similar to a commercially available (Sigma) preparation of crude cerebrosides. No gangliosides were detected, based on comparison of the mobilities of known gangliosides with the isolated lipids. In addition, no periodate–resorcinol-positive lipids were detected.

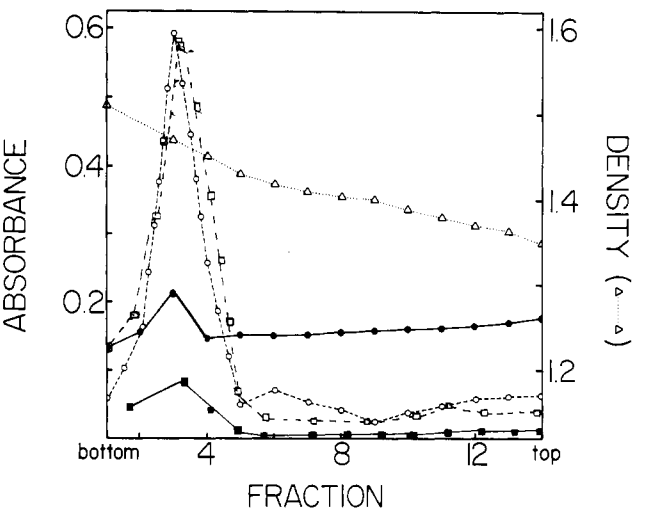

FIGURE 7: CsBr density gradient centrifugation of the low-density fraction after delipidation [(□) neutral sugar; (■) protein] and bronchial aspirate void volume material after delipidation [(○) neutral sugar; (●) protein]. Centrifugation conditions were identical with those described for Figure 4A.

Phosphatidylcholine was identified by comparison of the R_f values of standard dipalmitoylphosphatidylcholine with those lipids present. Several lipids with R_f values less than those for DPC were visualized but not identified.

The glycolipid fraction was estimated to be contaminated with 15–20% phospholipid based on spectrodensitometric scanning of the TLC plates. The phospholipid fraction was contaminated with 5–8% glycolipids. Palmitate ($C_{16:0}$), stearate ($C_{18:0}$), and oleate ($C_{18:1}$) were identified as the major fatty acids in all classes of lipids.

Discussion

Mucin from bronchial aspirate material was chemically characterized after solubilization in aqueous buffer without the use of detergents or prior reduction. A similar result was reported from this laboratory in a study on mucus from an asthmatic patient (Feldhoff et al., 1979). This mild procedure allowed the isolation of mucin glycoprotein in a near-native state from seven different patients. Structurally similar macromolecules were isolated and characterized from these different secretions. Sepharose CL-4B chromatography separated mucin glycoprotein from lower molecular weight serum components, but entrapped contaminants were still present as judged by the presence of lower molecular weight components on $NaDodSO_4$–polyacrylamide gel electrophoresis.

CsBr density gradient centrifugation removed these contaminating components, and in addition, in four of the samples characterized, high-density and low-density mucin glycoproteins were identified. The high-density mucin glycoprotein appeared homogeneous by rechromatography on Sepharose CL-4B, by recentrifugation in CsBr, and by $NaDodSO_4$–polyacrylamide gel electrophoresis. In general, the isolated glycoprotein fraction met the following criteria: a single, albeit broad, peak on exclusion (A150M) or ion-exchange (DEAE-Sephadex A25) chromatography; no detectable amino-terminal end group; failure to enter a 0.5% agarose/2% acrylamide gel; no other protein component detectable by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (500 μg loading); absence of uronic acid (proteoglycan) and mannose (N-glycosidically linked glycoprotein); and complete susceptiblity of saccharides to alkaline borohydride catalyzed elimination. This component had an amino acid composition characteristic of a mucin-type structure similar to that reported

for bronchial glycoproteins obtained from a patient with cystic fibrosis (Rose et al., 1979) but a somewhat higher protein content than that reported from bronchial glycoproteins obtained from a patient with asthma (Feldhoff et al., 1979).

Carbohydrate analysis indicated the presence of fucose, galactose, glucosamine, galactosamine, and *N*-acetylneuraminic acid. The relative ratios of sugars were different than those reported in cystic fibrosis bronchial secretions (Rose et al., 1979; Roussel et al., 1975) and asthmatic bronchial secretions (Feldhoff et al., 1979). Sulfate was identified, probably esterified to galactose (Roussel et al., 1975). Mannose, xylose, and uronic acid, which are constituents of serum glycoproteins and proteoglycan, respectively, were not present. Equilibrium sedimentation analysis indicated the presence of aggregation and/or polydispersity of the glycoprotein whose molecular weight ranged from 2.3×10^6 to 4.6×10^6.

The low-density fraction present in some samples contained a mucin glycoprotein with a slightly altered composition from that of the high-density component, although the protein/carbohydrate ratios of the two components were the same (1:4). In addition, Sepharose CL-4B rechromatography indicated the presence of lower molecular weight components which were not further characterized. This low-density glycoprotein appeared homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Previous studies using density gradient centrifugation have reported the presence of low buoyant density protein/glycoprotein components in blood group substances (Creeth & Denborough, 1970), bronchial glycoproteins from asthmatic bronchitic patients (Creeth et al., 1977), and glycoproteins from pig small intestinal mucus (Mantle & Allen, 1981; Mantle et al., 1981). These studies have not reported the presence of mucin-like glycoproteins of low buoyant density. The apparent low density appears to be a result of variations in lipid binding which can give rise to aggregates with different buoyant density. There is a slight alteration in carbohydrate and/or protein composition, but the overall mucin structure is present. These data suggest that in addition to the clustered, hydrophilic sugar domain a hydrophobic region is present which can bind lipid and may thus provide a mechanism for clearance of such components from the respiratory tract.

It is not known why some of the bronchial aspirate samples did not contain a low-density glycoprotein fraction. Lack of this component is probably not a result of a lack of lipid binding specificity since all seven aspirate samples qualitatively contained the same lipids. The slight quantitative differences in the samples (5–8% variations in any given class of lipid) may be sufficient to prevent resolution of a low-density fraction by the techniques employed. Quantitative differences of total associated lipid, however, do not seem to affect whether a low-density component appears upon centrifugation.

Lipids have been identified in pulmonary lavage of patients with alveolar proteinosis (Sahu et al., 1976), in secretions (Chernick & Barbero, 1959) and sputum (Matthews et al., 1963; Galabert et al., 1981) from cystic fibrosis patients, and from lung lavage of patients with asthma and cystic fibrosis (Sahu & Lynn, 1977). In these studies, phosphatidylcholine was identified as the major lipid. Neutral lipids such as mono-, di-, and triglycerides, cholesterol, and cholesteryl esters were identified, and some glycolipids were identified (Sahu & Lynn, 1977).

Lipids present in mucus obtained from an asthmatic patient were found to be different than those described in the above studies. Lewis (1971) found neutral lipids (mono-, di-, and triglycerides, free fatty acids, cholesterol, and cholesteryl esters) to be the major class (64.8% of total lipid weight); phospholipids were also identified.

The lipids associated with the mucin glycoprotein from normal bronchial secretions were similar to those described by Lewis. Neutral lipids comprised the major class of lipid (56% of the total lipid weight); phospholipids and glycolipids were present. It should be noted that these lipids were isolated from fractionated mucus (i.e., Sepharose CL-4B void volume material) whereas lipids described in the above studies were isolated from whole mucus. Lhermitte et al. (1977) reported the presence of lipid associated with mucin glycoproteins obtained from a patient with chronic bronchitis. They found that two fractions obtained from affinity chromatography had associated lipid identified as neutral lipids (steroids, free fatty acids, and mono-, di-, and triglycerides) and polar lipids (cardiolipids, cephalins, lecithins, and sphingomyelins).

It is clear from the lipid analysis that the contribution of lipid to the overall architecture of mucus secretions cannot be overlooked. The analysis does not indicate any specificity of a given lipid (or class of lipid) for interacting with mucin glycoprotein nor does it address the structural requirement of the glycoprotein for lipid binding. In vitro lipid binding experiments using intact mucin glycoproteins and mucin glycoprotein fragments may yield answers to these questions.

Experiments with primary explant culture of human tracheal epithelium suggest that these cells produce macromolecular components similar to those isolated from bronchial aspirate materials. The high- and low-density components incorporate glucosamine into glycoconjugates. In addition, the high-density component is susceptible to alkaline sodium borohydride treatment. Fractionation of the alkaline borohydride treated high-density component on Bio-Gel P-10 gave an elution profile similar to that obtained by fractionation of the oligosaccharides obtained from alkaline borohydride treated bronchial aspirate high-density mucin glycoprotein.

These preliminary data suggest that tracheal epithelial cells in culture produced macromolecules with physical properties similar to mucin glycoproteins from bronchial secretions.

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Growth of Synthetic Myosin Filaments from Myosin Minifilaments†

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ABSTRACT: Addition of KCl to a solution of synthetic myosin minifilaments in 10 mM citrate–Tris buffer (pH 8.0) induces the growth of filaments. These filaments, at pH 8.0, resemble in their morphological and hydrodynamic properties the synthetic filaments described by Josephs and Harrington [Josephs, R., & Harrington, W. F. (1966) *Biochemistry 5*, 3474–3487]. The rate of filament growth depends critically on the KCl concentration in the solution. Low rates of filament formation are noted in the presence of both low (below 80 mM KCl) and high (above 0.15 M KCl) salt concentrations, whereas at the intermediate KCl concentrations the filaments are formed at a fast rate. The formation of filaments from minifilaments is a reversible process, and under moderate salt concentrations, these two polymeric systems appear to exist in a dynamic equilibrium. Small amounts of minifilaments can induce rapid polymerization of dissociated myosin; i.e., they can act as a seeding material. These and other observations are discussed in terms of a direct route for filament formation from myosin minifilaments.

The electron microscopy studies of Huxley (1963) established the structural similarities of native myosin filaments and the synthetic filaments obtained by decreasing the ionic strength of a myosin solution. These findings implied that the same principles might govern the assembly of both filaments. The bipolar character of myosin filaments, and the presence of a smooth central region in these structures, indicated a two-step process of their formation: initial antiparallel (tail to tail) aggregation of myosin molecules to form short bipolar structures, and the subsequent addition of myosin in a parallel fashion (head to tail) at each end of the bipolar unit (Huxley, 1963; Kaminer & Bell, 1966; Josephs & Harrington, 1966; Huxley & Brown, 1967; Pepe, 1967; Harrington, 1972; Katsura & Noda, 1971, 1973; Hinssen et al., 1978). Although this model of assembly is widely accepted, no detailed study has been undertaken to confirm it. Until now, very little is known about the formation of myosin filaments and the factors which determine their length.

Most of the previous work on myosin filaments was concerned with their structural properties and the effect of experimental parameters on the final assembly products. The composition and myosin content of the thick filaments have

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