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Mucus Glycoprotein Content of Chronic Bronchitis Sputum[1] (39864)

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Introduction. Excessive secretion of mucus in the airways is a characteristic feature of chronic bronchitis (CB), as indicated by the chronic production of sputum by patients with this disease. The elastic consistency of the sputum is largely dependent on the presence of mucus glycoproteins (1), whose characteristics have been reviewed in detail (2, 3). Purulent sputum also contains deoxyribonucleic acid from disintegrated leukocytes (1), which may become part of the mucus gel structure. The manner in which these constituents of the sputum are crosslinked to form a viscoelastic gel remains to be clarified; however, reduction with disulfide agents disperses the gel structure of both mucoid and purulent sputum and frees and sputum components (4, 5).

Previous studies have investigated the characteristics of mucus glycoproteins isolated from secretions of the respiratory tract (6-8). The present report describes quantitative studies on the concentration of mucus glycoproteins in the sputum of patients with chronic bronchitis and in sputum induced in normal subjects.

Methods. Selection of patients and collection of sputum. For these studies, patients were selected on the basis of history, clinical examinations, and pulmonary function tests consistent with chronic bronchitis, as defined by the American Thoracic Society (9). Sputum production was induced in normal subjects by inhalation of 15% saline aerosol from a jet nebulizer for 15 to 20 min. Each subject was trained to produce sputum from the lower respiratory tract with a minimum of admixed saliva. Sputum samples were frozen within 1 hr of expectoration, stored at −20°, and thawed immediately before use; samples from individual patients were pooled.

Preparation and solubilization of sputum. Three volumes of distilled water were added to each sputum sample, and this mixture was allowed to stand with intermittent gentle stirring for 1 hr, centrifuged at 1000*g* for 30 min, and the aqueous supernatant was separated from the sedimented mucus gel. When the water wash was subjected to the same treatment used with sputum samples, the hexose content of the eluate fractions expected to contain the mucus glycoproteins was less than 5% of that of the mucus glycoproteins found subsequently in the washed sputum, indicating that washing the sputum with water did not remove significant amounts of bronchial mucus glycoprotein.

Mercaptoethanol (0.2% final concentration) was added to the washed mucus gel; the mixture was allowed to stand at room temperature with intermittent thorough mixing for 4 hr, and then overnight at 4°. The resulting suspension was centrifuged at 12,000*g* for 1 hr at 4°, and the mercaptoethanol soluble fraction was decanted, dialyzed, and lyophilized. When solubilized by digestion with pronase and papain, and subjected to gel filtration on Sepharose 4B, the sedimented residue fraction yielded none of the mucus glycopeptides that are produced when mucus glycoproteins from sputum are subjected to this treatment (10, 11), indicating that no significant quantity of bronchial mucus glycoproteins remained undispersed in the residue following the treatment with mercaptoethanol.

Separation of mucus glycoprotein frac-

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tions by gel filtration. The lyophilized mercaptoethanol-soluble fraction (50–100 mg) was resuspended in 0.01 *M* Tris-HCl, pH 7.0 (5 ml), containing NaCl (0.1 *N*), urea (6 *M*), and mercaptoethanol (0.2%), centrifuged at 12,000*g* for 30 min, and passed through an 0.2-μm Millipore filter to remove any traces of suspended material still present. The resulting solution was loaded on a Sepharose 4B gel filtration column (2.5 × 70 cm), equilibrated, and subsequently eluted with 0.01 *M* Tris-HCl, pH 7.0, containing NaCl (0.1 *N*) at 10 ml/hr. The eluate was collected automatically in 5-ml cuts, and the hexose content was measured colorimetrically in order to locate the fractions containing mucus glycoproteins, since the latter contain about 25% hexose. This procedure yielded two major fractions containing mucus glycoproteins: Fraction A, of very large molecular size, essentially excluded from the Sepharose, merging with Fraction B, somewhat retarded in the column. For each of these fractions, the appropriate eluate cuts were pooled, dialyzed against distilled water, lyophilized, weighed, and their carbohydrate and amino acid composition were determined. In the gel filtration procedure, mercaptoethanol and urea were not included in the eluting medium routinely, since our initial experiments had shown that their presence did not reduce the amount of mucus glycoprotein that appeared in the fraction of very large molecular size. With the procedure used, alkylation with iodoacetamide after the sputum had been reduced with mercaptoethanol did not alter the yield of mucus glycoproteins in our two fractions. Consequently, the preparations were treated with mercaptoethanol at each step, but alkylation was not carried out routinely, in order to avoid permanent alteration of the mucus glycoproteins.

Purulent sputum may have a substantial content of leukocytes. However, we found that leukocytes isolated from blood yielded no significant quantities of hexose in the eluate region corresponding to our Fractions A and B when carried through the same procedure used with the sputum samples.

Fractionation of solubilized sputum by equilibrium density gradient centrifugation. Frozen samples of mucoid sputum from a patient with CB were thawed, pooled, washed, and solubilized as described in the previous section. To 62.0 ml of the solubilized sputum, guanidine hydrochloride (38.2 g) and cesium chloride (40.0 g) were added to make a solution of density 1.4 containing 4 *M* guanidine hydrochloride. The resulting solution was centrifuged in eight tubes in a Beckman No. 65 rotor at 56,000 rpm for 64 hr and the resulting gradients were separated into 10 fractions by means of a peristaltic pump and fraction collector. The hexose content of each fraction was determined and solution densities were measured gravimetrically using a 200-μl micropipet. The fractions containing the mucus glycoproteins, as indicated by density and high hexose content, were pooled, applied to Sepharose 4B columns with the dispersing agents mercaptoethanol, guanidine hydrochloride, and CsCl still present, and eluted as described in the previous section. In these experiments the first half of Fraction B and the second half were pooled separately (B_1 and B_2) in order to facilitate detection of trends in composition that might parallel differences in molecular size. Fractions A, B_1, and B_2 were dialyzed and lyophilized and their amino acid and carbohydrate compositions were determined.

Analysis. Glycoprotein fractions were analyzed for hexose (12), hexosamine (13), fucose (14), sialic acid (15), and sulfate (16); amino acid analysis was carried out on a Beckman Model 120C analyzer after hydrolysis *in vacuo* in 6 *N* HCl at 110° for 20 hr. For estimation of the content of half-cystine residues, samples were treated with performic acid before hydrolysis (17). For estimation of lipid content (18), the dried mucus glycoprotein preparations were extracted with chloroform–methanol (2:1).

Results. Typical results obtained on Sepharose 4B gel filtration of the sputum mucus components solubilized with mercaptoethanol are shown in Fig. 1. The sputum from the patients with CB yielded a fraction containing mucus glycoproteins which was excluded from the column (Fraction A, Fig. 1), merging with a larger polydisperse retarded fraction also containing mucus glyco-

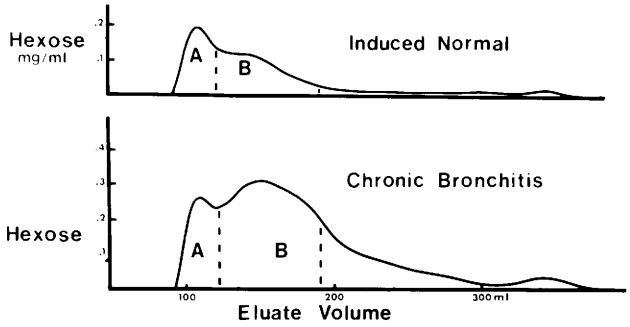

FIG. 1. Sepharose 4B gel filtration of sputum solubilized with mercaptoethanol. The mucus glycoprotein fraction excluded from the gel, Fraction A, was separated from the fraction retarded in the column, Fraction B.

proteins (Fraction B). This pattern is qualitatively similar to results reported in other laboratories (8, 19). In contrast, we found that the induced sputum from the normal subjects yielded relatively little of the retarded fraction (Fraction B), and the fraction excluded from the column (Fraction A) was predominant (Fig. 1).

The content of hexose in Fraction A (reflecting the content of mucus glycoproteins) in the sputum from the 21 patients with CB was the same as that in the induced sputum from the 9 normal subjects studied: 0.267 ± 0.029 and 0.264 ± 0.46 mg/g of washed sputum, respectively. In contrast, the content of hexose in Fraction B in the sputum from the patients was markedly higher (CB 1.27 ± 0.13 vs induced normals 0.166 ± 0.16 mg/g of washed sputum, $P < 0.001$). The hexose content of Fraction B (1.27 mg) is much higher than that of Fraction A (0.267 mg, $P < 0.01$) in the patients' sputum, whereas the reverse situation applies to the induced sputum from the normal subjects, where the hexose content of Fraction B is less than that of Fraction A (0.166 and 0.264 mg, respectively, $P < 0.05$).

The hexose content of Fraction B in the CB sputum increased in proportion to the content of solids (solid circles, Fig. 2; $r = 0.64$, $P < 0.005$). The increase in mucus glycoproteins in Fraction B accounts for approximately 10% of the increase in solids, based on hexose content of approximately 25% in the mucus glycoproteins. The balance of the increase in solids probably is due primarily to the increases in leukocytes and to a lesser extent to increases in cell debris which accompany increases in the purulence of the sputum (20).

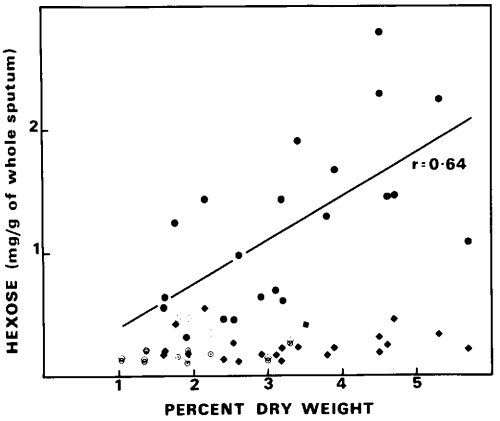

FIG. 2. Hexose content of mucus glycoprotein Fractions A and B (expressed as milligrams of hexose in each fraction per gram of washed sputum) vs percentage dry weight (dry weight of sputum as percentage of wet weight). Solid diamonds and circles represent Fractions A and B, respectively, from sputum of patients with chronic bronchitis. Open diamonds and circles represent corresponding values for sputum induced in normal subjects.

The composition of Fractions A and B obtained by direct gel filtration of solubilized CB sputum is shown in Table I, columns 1 and 2. The high carbohydrate content, particularly of hexose and hexosamine, and the high content of threonine, serine, proline, alanine, and glycine indicate that Fractions A and B are both rich in mucus glycoproteins, since they resemble typical values that have been reported for mucus glycoproteins (2, 5, 6, 8, 19).

Following the equilibrium density gradient centrifugation in the presence of 4 *M* guanidine hydrochloride, the mucus glycoproteins were concentrated in the density range 1.41 to 1.51, as shown in Fig. 3.

TABLE I. COMPOSITION OF MUCUS GLYCOPROTEIN FRACTIONS FROM SOLUBILIZED SPUTUM OF PATIENTS WITH CHRONIC BRONCHITIS.

	A[a]	B[a]	A[b]	B_1[b]	B_2[b]
		Grams/100 grams			
Hexose	24.8 ± 1.1[c]	26.3 ± 1.7[c]	24.2	22.9	21.0
Fucose	6.2 ± 0.6	7.9 ± 0.8	14.9	15.8	14.5
Sialic acid	7.5 ± 1.2	10.0 ± 2.3	9.8	9.6	10.5
Hexosamine	26.5 ± 0.3	26.1 ± 0.9	33.9	34.8	35.2
SO_4	–	–	1.5	1.4	1.4
Amino acids	24.2 ± 2.1	20.1 ± 1.9	10.3	11.1	10.6
		Moles/100 moles			
Lysine	5.1 ± 0.3	2.8 ± 0.2	2.2	2.7	2.6
Histidine	3.1 ± 0.2	1.7 ± 0.1	3.1	2.3	3.0
Arginine	5.5 ± 1.1	2.5 ± 0.4	2.3	2.4	2.7
Aspartic	6.1 ± 0.1	4.5 ± 0.5	3.5	2.9	3.2
Threonine	17.4 ± 1.2	23.4 ± 0.6	26.6	25.2	23.2
Serine	13.5 ± 0.4	15.8 ± 0.7	17.8	15.6	18.0
Glutamic	7.7 ± 1.2	6.3 ± 0.6	6.1	3.9	4.6
Proline	10.9 ± 0.9	12.5 ± 0.6	13.9	13.2	12.9
Glycine	8.3 ± 0.4	7.9 ± 0.5	8.3	8.2	10.1
Alanine	8.3 ± 0.5	8.6 ± 0.4	9.8	9.6	8.7
Valine	4.1 ± 0.9	5.0 ± 0.4	4.6	5.4	3.3
Isoleucine	3.2 ± 0.1	2.9 ± 0.3	2.4	3.7	2.0
Leucine	3.6 ± 0.1	4.1 ± 0.7	3.9	3.9	2.9
Tyrosine	1.4 ± 0.2	1.2 ± 0.3	1.0	1.0	0.9
Phenylalanine	1.5 ± 0.1	1.5 ± 0.1	1.2	1.4	1.2
Cystine (1/2)	–	–	1.3	1.1	1.4

[a] Mucus glycoprotein fractions from Sepharose 4B gel filtration of solubilized sputum.

[b] Mucus glycoprotein fractions from Sepharose 4B gel filtration of material from solubilized sputum after purification in CsCl–guanidine hydrochloride gradient. A fractions excluded from gel. B, B_1, and B_2 fractions retarded in column.

[c] Mean ± SEM of data for five sputum samples.

Extraction with Folch's reagent (18) indicated that these fractions were devoid of lipid; DNA and typical proteins also were absent, as indicated by absence of any optical absorption maximum near 260 or 280 nm. The fractions in the density range 1.25 to 1.34 showed a maximum in optical absorbance near 280 nm, indicating the presence of typical proteins having a considerable content of aromatic amino acids. After dialysis and lyophilization, the pooled fractions in the density range 1.25 to 1.29 were found to contain 25% lipid, as indicated by extraction with Folch's reagent (18).

The three mucus glycoprotein fractions obtained on gel filtration of the mucus glycoprotein material from the density gradient (Fractions A, B_1, B_2) were remarkably similar to one another in composition, as shown in Table I, columns 3, 4, and 5. All three had the typical high content of carbohydrate and low content of amino acids characteristic of mucus glycoproteins; and all had the typical high content of serine, threonine, proline, glycine, and alanine.

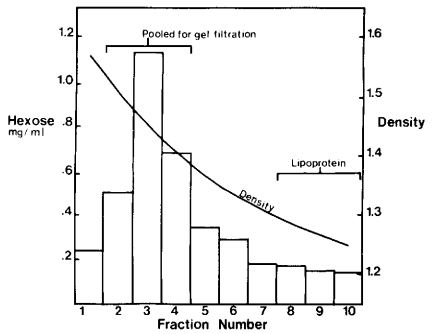

FIG. 3. Fractionation of solubilized sputum from a patient with chronic bronchitis in CsCl density gradient containing 4 *M* guanidine hydrochloride.

Discussion. The high content of the smaller-molecular-size mucus glycoproteins, which increases in proportion to the content of solids, appears to be a characteristic feature of CB sputum; it has been observed in all CB sputum samples we have studied. Even in the CB sputum samples with relatively low content of solids, the content of

the smaller-molecular-size mucus glycoproteins per gram of washed sputum was higher than that found in the induced sputum from the normal subjects. Since induced sputum might differ from sputum produced spontaneously, patients with chronic bronchitis were instructed to clear the sputum from their airways as completely as possible by repeated coughing, and further sputum production then was induced by inhalation of the hypertonic saline aerosol. The induced sputum from these patients with chronic bronchitis gave the same pattern in gel filtration as that given by the sputum they had produced spontaneously.

The very large mucus glycoproteins were not dispersed in the present experiments, despite the presence of the dispersing agents guanidine hydrochloride and CsCl and the removal of the lipoproteins and other proteins in the density gradient. It is still possible that they are present in aggregates that resist dispersal by the agents employed thus far, perhaps accompanied by a relatively small amount of crosslinking protein. If the mucus glycoproteins in the fraction of very large molecular size are present in aggregates, these aggregates may have some biological significance, since the major portion of the mucus glycoproteins from the induced sputum of the normal subjects appeared in this form. In the present experiments, we found no trend toward a higher content of half-cystine residues in the large mucus glycoproteins (Table I, columns 3, 4, and 5), and, consequently, no basis for increased disulfide crosslinking.

On the other hand, the smaller-molecular-size mucus glycoproteins might be derived from the larger forms as a result of various types of degradation, especially in purulent sputum. For example, proteolysis by agents like pronase and papain would be expected to result in lower protein content and higher content of carbohydrate, threonine, and serine (10, 11), but we found no trend in this direction in the smaller-molecular-size mucus glycoproteins (Table I, column 3, 4, and 5). It is possible that proteolytic enzymes from leukocytes or bacteria in purulent sputum might cleave the very-large-molecular-size mucus glycoproteins, or might cleave small amounts of crosslinking protein still present, with little loss of amino acids or peptide fragments. This would reduce the apparent molecular size without altering the composition. While proteolytic cleavage may be the mechanism producing the smaller-molecular-size mucus glycoproteins, it seems remarkable that the concentration of the very-large-molecular-size mucus glycoproteins remaining intact in the CB sputum, whether induced or produced spontaneously, always falls in the same range as that in the induced sputum from the normal subjects.

The smaller-size mucus glycoproteins also might arise through removal of carbohydrate groups by glycosidases present in purulent sputum, or because of incomplete glycosylation accompanying the characteristic hypersecretion in patients with CB. However, we see no trend toward lower carbohydrate content and consequent higher protein content in the smaller-molecular-size mucus glycoprotein fractions emerging later from the column; nor is there any trend toward a lower content of the terminal residues, fucose and sialic acid.

Fractionation of the CB mucus glycoproteins on the basis of molecular size did not separate them into classes differing in their ionic characteristics, as indicated by their content of fucose, sialic acid, or sulfate (Table I, columns 3, 4, and 5) or by their behavior in ion-exchange chromatography (8).

In summary, the induced sputum from the normal subjects yielded a major mucus glycoprotein fraction of very large molecular size merging with a minor mucus glycoprotein fraction of smaller molecular size when solubilized with mercaptoethanol and subjected to gel filtration on Sepharose 4B. The sputum from the patients with CB, whether induced or produced spontaneously, gave a different pattern. It showed the same concentration of the very-large-molecular-size mucus glycoprotein fraction, but differed in having a much higher content of the smaller-molecular-size mucus glycoproteins, which increased in proportion to the percentage dry weight of the sputum. Thus, the CB sputum was characterized by a higher total content of mucus glycoproteins than the sputum from the normal subjects, and the

increase was due to a large increase in the content of the smaller-molecular-size mucus glycoproteins.

Additional experiments will be needed to determine whether the mucus glycoproteins in the fraction of very large molecular size are merely present in artifactual aggregates (even though this was the predominant form in the sputum from the normal subjects) or whether the mucus glycoproteins of smaller molecular size arise through degradation of, or failure to assemble them into, the structures of very large molecular size.

Summary. When solubilized with mercaptoethanol and subjected to gel filtration on Sepharose 4B, sputum from patients with chronic bronchitis consistently yielded an elution pattern in which a portion of the mucus glycoproteins emerged in a fraction of very large molecular size excluded from the gel, but most emerged later in a portion of smaller molecular size retarded in the column. The concentration of the mucus glycoprotein fraction of lower molecular size increased in proportion to the content of total solids in the chronic bronchitis sputum. The elution pattern obtained with induced sputum from normal subjects differed in having relatively little of the smaller-molecular-size mucus glycoprotein fraction. Thus, the chronic bronchitis sputum was characterized by a higher total content of mucus glycoproteins than the sputum from the normal subjects, and the increase was due to a large increase in the content of the mucus glycoproteins of smaller molecular size.

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