Mucins in airway secretions from healthy and chronic bronchitic subjects

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Little is known about whether the properties of respiratory mucins are altered as a result of airway irritation, but histochemical studies of respiratory tract secretory cells show a more 'acidic' staining pattern after exposure to tobacco smoke. Furthermore it has been suggested that proteoglycans are the major glycoconjugates in 'normal' respiratory secretions, whereas mucins predominate in sputum. To investigate these observations further, mucins from secretions collected from the tracheal surface of healthy non-smoking 'normal' subjects and sputum from patients with chronic bronchitis were compared. All samples contained one major mucin population after density-gradient centrifugation, and a small amount of 'denser' mucin was present in some chronic bronchitic and one of the 'normal' samples. Proteoglycans were not a major component of 'normal'

secretions. The major mucin population from chronic bronchitic samples had molecular masses between 10 and 30 MDa and behaved as random coils in solution. Whole mucins from 'normal' individuals and chronic bronchitic patients were excluded from Sepharose CL-2B, whereas reduced subunits were included. Proteolysis of subunits yielded two populations of high-molecular-mass glycopeptides differing in size, suggesting the presence of two different tandem repeat regions in the mucins. Finally, mucins from patients with chronic bronchitis are less, rather than more, acidic than those from 'normal' individuals. Mucins from bronchitic sputum and 'normal' secretions are thus similar in their macromolecular properties, but differ slightly in charge density.

INTRODUCTION

Respiratory mucins are linear, flexible thread-like molecules [1–3] with molecular masses between 10 and 25 MDa [4] composed of 'subunits' held together by disulphide bonds. Each subunit contains highly glycosylated regions (oligosaccharide clusters) interspersed with stretches of sparsely substituted peptide core [5]. Recently it was shown that subunits from respiratory mucins can be separated into two distinct populations by electrophoresis [6]. Studies with molecular biology techniques have identified several mucin genes (MUC-1 to MUC-7) and hybridization *in situ* has shown the expression of MUC-1 at the apical surfaces of the epithelial cells and in serous glands [7], MUC-2 in surface epithelial cells, and MUC-4, MUC-5B and MUC-5AC in both surface epithelial and gland cells [8]. Tandem repeat regions rich in serine and threonine (potential O-glycosylation sites) are a characteristic feature of mucin apoproteins and correspond to the highly glycosylated regions in the macromolecules. Respiratory mucins contain many different oligosaccharide structures (see, for example, [9,10]) and glycosylation, in particular with the initial GalNAc residue, seems to confer an extended conformation on the mucin apoprotein within the highly glycosylated regions [11].

In diseases such as chronic bronchitis and cystic fibrosis (CF), goblet cells increase in number and the mucous cells of the submucosal glands become hypertrophied [12,13] leading to mucus hypersecretion. However, of the few studies of secretions from healthy airways, some suggest that they contain mucins [14,15] whereas others report that such glycoproteins are absent and that proteoglycans are the major macromolecular constituents [16]. In sputum from patients with CF and chronic bronchitis, the presence of chondroitin sulphate was found to be associated with infection and was absent from non-purulent

secretions [17]. In the rat, the level of mRNA for MUC-2 has been shown to increase in response to both infection and airway irritation [18]. It is therefore possible that changes in the secretion of different mucins may be responsible, in part, for the changes in the physical properties of mucus that often accompany disease of the airways.

Histochemical investigations have shown a shift from 'neutral' to more 'acidic' secretory cell contents on exposure of the airways to tobacco smoke [19,20]. Such changes in 'acidity' may be caused by structural changes in the mucin oligosaccharides and, indeed, an increase in the content of sulphate has been shown in mucins from CF epithelial cells [21].

The purpose of this study is to determine whether mucins are a major component of secretions from the healthy respiratory tract in individual subjects and to investigate whether major changes in the structure and macromolecular properties of the mucins occur in chronic bronchitis. Furthermore, the charge densities of the highly glycosylated regions of mucins from healthy airways and chronic bronchitic patients were compared, to determine whether the 'acidity' is increased in bronchitis as suggested by previous histological studies.

EXPERIMENTAL

Materials

Guanidinium chloride was purchased from Riedel-de-Haen and stock solutions of approx. 8 M were treated with charcoal and filtered through a PM 10 filter (Amicon) before use. Ultrapure guanidinium chloride was bought from Bethesda Research Laboratories and was used in analytical ultracentrifugation. Superose 6 HR 10/30 as well as Mono Q HR 5/5 columns and Sepharose CL-2B and Sephacryl S-500 were obtained from

Abbreviation used: CF, cystic fibrosis.

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Figure 1 Isopycnic density-gradient ultracentrifugation in CsCl/4 M guanidinium chloride of mucins from a typical sample of (a) chronic bronchitic sputum and (b) 'normal' airway secretion, and isopycnic density-gradient ultracentrifugation in CsCl/0.2 M guanidinium chloride of mucins from (c) chronic bronchitic sputum and (d) 'normal' airway secretion

Mucins were subjected to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride (a,b) and pooled according to the horizontal brackets. Fractions containing the major mucin band were then re-centrifuged in CsCl/0.2 M guanidinium chloride (c,d). After centrifugation (for details, see text), fractions were collected from the bottom of the tubes and analysed for sialic acid (●), hexose (△), A_{280} (○) and density (■). Fractions with a buoyant density higher than the mucins in the CsCl/4 M guanidinium chloride gradient from all ten of the 'normal' samples were pooled (as shown in b) and analysed for the presence of proteoglycans (see text).

Pharmacia, and PMSF, *N*-ethylmaleimide, trypsin [EC 3.4.21.4, type XIII, tosylphenylalanylchloromethane ('TPCK')-treated], papain (EC 3.4.22.2), deoxyribonuclease I (EC 3.1.21.1) and ribonuclease A (EC 3.1.27.5) were purchased from Sigma. Di-isopropyl phosphorfluoridate was from Fluka. All other chemicals were of A.R. grade.

Analytical methods

Fractions from density gradients and column chromatography were analysed for hexose as described by Heinegård [22], for uronic acid as described by Bitter and Muir [23] and for sialic acid using an automated assay based on the method described by

Lohmander et al. [24]. For estimation of the amount of proteoglycan and mucin in the 'normal' samples, glucuronolactone and *N*-acetylneuraminic acid were used as standards. The sensitivity of these analyses was increased through the use of a 5 cm cuvette, optimizing the heating time and using a more sensitive photometer. These improvements allowed the detection of sialic acid down to approx. 0.2 $\mu\text{g}/\text{ml}$ and uronic acid to approx. 0.5 $\mu\text{g}/\text{ml}$.

Isolation of whole mucins, subunits and high-molecular-mass glycopeptides from chronic bronchitic sputum

Sputum from individual chronic bronchitic patients was collected, frozen immediately and stored at -20°C until use.

Samples were thawed with gentle mixing into an equal volume of cold 0.2 M NaCl/10 mM Na₂EDTA/10 mM *N*-ethylmaleimide/2 mM di-isopropyl phosphofluoridate/10 mM sodium phosphate buffer, pH 6.5. The gel and sol phases were then separated by centrifugation in an MSE 65 ultracentrifuge (45000 rev./min for 45 min at 4 °C in an 8 × 25 ml rotor). Mucins were purified from the chronic bronchitic gel and sol material as described by Thornton et al. [25]. Briefly, samples were solubilized by slow stirring overnight at 4 °C in 6 M guanidinium chloride/10 mM sodium phosphate buffer, pH 6.5, and subjected to isopycnic density-gradient centrifugation in an MSE 65 ultracentrifuge (36000 rev./min for approx. 65 h at 15 °C in an 8 × 25 ml rotor), first in 4 M guanidinium chloride/CsCl (initial density 1.39 g/ml) and then in 0.2 M guanidinium chloride/CsCl (initial density 1.50 g/ml). The mucin-containing fractions were pooled as indicated in Figure 1. Subunits were prepared by treatment with 10 mM dithiothreitol in 6 M guanidinium chloride/10 mM Tris/HCl buffer, pH 8.0, for 5 h at 37 °C and subsequent alkylation with a 2.5 molar excess of iodoacetamide over dithiothreitol for 12 h in the dark. Finally, high-molecular-mass glycopeptides were obtained from subunits by trypsin digestion (100 µg of trypsin per ml) in 0.1 M Tris/HCl buffer, pH 8.0, for 5 h at 37 °C.

Whole mucins and subunits from the gel phases of the chronic bronchitic mucins were chromatographed on a Sepharose CL-2B column (1.6 cm × 90 cm) in 4 M guanidinium chloride/10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 12 ml/h and fractions analysed for sialic acid. Subunits and high-molecular-mass glycopeptides from the same samples were chromatographed on a Sephacryl S-500 column (1.6 cm × 50 cm) with a 2150 LKB titanium-head pump and a Pharmacia V-7 injector. The latter column was eluted with 4 M guanidinium chloride, pH 7.0, at a flow rate of 9 ml/h and fractions (1.5 ml) were analysed for sialic acid. The high-molecular-mass glycopeptides were resolved into two peaks, which were pooled separately, dialysed against 0.1 M ammonium acetate, freeze-dried and subjected to ion-exchange HPLC on a Mono Q column as described below.

Isolation of whole mucins, subunits and high-molecular-mass glycopeptides from secretions from 'normal' individuals

Tracheal secretions were collected from ten healthy non-smoking children (aged 2–16 years) with no signs of respiratory tract infection. All were undergoing minor surgery for which they required tracheal intubation but no premedication with atropine or other antimuscarinic drugs. Anaesthesia was induced with intravenous infusion of thiopentone or inhalation of halothane in O₂, and maintained by spontaneous breathing of N₂O, O₂ and halothane. Secretions were aspirated from the tracheal surface into a catheter passed through the tracheal tube, washed out immediately with ice-cold 6 M guanidinium chloride/5 mM Na₂EDTA/10 mM sodium phosphate buffer, pH 6.5, containing 5 mM *N*-ethylmaleimide and 0.1 mM PMSF and stored at 4 °C. Samples were then subjected to isopycnic density-gradient centrifugation in 4 M guanidinium chloride/CsCl in a Beckman L-8 ultracentrifuge (40000 rev./min for approx. 65 h at 15 °C in a 70.1Ti rotor). Fractions with buoyant densities consistent with mucins (Figure 1b) in each sample were pooled and subjected to density-gradient centrifugation in 0.2 M guanidinium chloride/CsCl as outlined above. In four of the ten samples the amount of material was insufficient for a second density-gradient step.

The 'normal' mucins were reduced to yield subunits by

treatment with 10 mM dithiothreitol in 6 M guanidinium chloride/10 mM Tris/HCl buffer, pH 8.0, for 5 h at 37 °C and subsequent alkylation with a 2.5 molar excess of iodoacetamide over dithiothreitol for 12 h in the dark. This was followed by digestion with trypsin (100 µg of trypsin per ml) in 0.1 M Tris/HCl buffer, pH 8.0, for 5 h at 37 °C. The resultant high-molecular-mass glycopeptides were chromatographed on a Superose 6 column connected to a 2150 LKB titanium-head pump and a Pharmacia V-7 injector and eluted with 0.1 M ammonium acetate at a flow rate of 0.25 ml/min. The glycopeptide peak eluting in the void volume was dialysed against 0.1 M ammonium acetate, freeze-dried and subjected to ion-exchange HPLC on a Mono Q column as described below.

Investigation of the presence of proteoglycans in secretions from 'normal' individuals

To investigate the presence of proteoglycans, fractions with a buoyant density greater than 1.50 g/ml in the 4 M guanidinium chloride/CsCl gradient (Figure 1b) were pooled from all 10 'normal' samples to give one large pool of high-density material. This pool was dialysed against 50 mM sodium phosphate buffer, pH 7, containing 5 mM MgCl₂ and digested with deoxyribonuclease (100 µg) and ribonuclease (200 µg) for 6 h at 37 °C. After dialysis against 50 mM sodium phosphate buffer, pH 7, containing 5 mM Na₂EDTA and 5 mM cysteine hydrochloride, the sample was digested with papain (30 µg) for 8 h at 65 °C, dialysed against 0.1 M ammonium acetate, chromatographed on a Superose 6 column at a flow rate of 0.25 ml/min and fractions analysed for absorbance at 280 nm and uronic acid.

Ion-exchange HPLC of high-molecular-mass glycopeptides from chronic bronchitic and 'normal' mucins

The freeze-dried high-molecular-mass glycopeptides from chronic bronchitic and 'normal' mucins were subjected to ion-exchange HPLC on a Mono Q HR 5/5 column connected by Teflon tubing to a 2150 LKB titanium-head pump with a 2152 LKB controller and a Pharmacia V-7 injector. Samples were dissolved in 250 µl of 10 mM piperazine/perchlorate buffer, pH 5.0. Gradients were formed on the low-pressure side with a 2040-203 LKB mixing valve and the column was eluted at 0.5 ml/min with 10 mM piperazine/perchlorate buffer, pH 5.0 (starting buffer), for 10 min, followed by a linear gradient (60 min) to a final concentration of 0.25 M LiClO₄ in starting buffer.

Light scattering

Light scattering was performed as described in detail by Sheehan and Carlstedt [26]. In brief, aliquots of the gel-phase mucins from the chronic bronchitic samples (1.5 ml) were clarified by centrifugation in a bench-top centrifuge (10000 rev./min for 1 h at 20 °C). The photomultiplier of a Malvern Molecular Analyser System 4300 (20 mW He/Ne laser, 64-channel K7027 correlator) coupled to a photon-count rate meter was used both for total intensity and quasi-elastic measurements at angles between 20 ° and 90 °. Total intensity data were evaluated by the method of Zimm [27] and linear least-squares analysis was used to extrapolate $c/\Delta R_\theta$ to $c = 0$ and to $\theta = 0$ with the angles 20 °, 30 °, 40 ° and 50 °. Quasi-elastic measurements were evaluated with the autocorrelation function $[g^2(\tau)]$ as obtained by the K7023 correlator. The $\ln[g^2(\tau)]$ versus t functions were fitted to a second-order polynomial by least-squares analysis and the diffusion

coefficient was calculated from the linear term of the quadratic fit. Concentrations of mucin solutions were assessed after the physical measurements by sialic acid determination with the cognate mucin stock solution as a standard. The concentration of this standard was determined gravimetrically. The refractive index increment was determined with a Waters R401 differential refractometer as described previously [28].

Analytical ultracentrifugation

Sedimentation-velocity centrifugations were performed in an MSE 75 Centriscan analytical ultracentrifuge at 20000 rev./min and 20 °C using the Schlieren optics. Samples of chronic bronchitic gel-phase mucins were dialysed against 6 M guanidinium chloride/1 mM Na_2EDTA /1 mM sodium phosphate buffer, pH 6.5. The apparent sedimentation coefficients were determined from the peak positions, and values of s were extrapolated to zero concentration and corrected to standard conditions. A value of 0.65 ml/g was used for the partial specific volume [28]. The weight-average molecular mass was determined from the Svedberg equation by using the diffusion coefficient obtained from the light-scattering experiments.

RESULTS

Preparation of mucins

Whole mucins were isolated from the gel and sol phases of sputum from eight chronic bronchitic patients, by means of isopycnic density-gradient centrifugation in $\text{CsCl}/4\text{ M}$ guanidinium chloride. In all samples the major mucin population from the gel banded between 1.38 and 1.47 g/ml and hexose co-distributed with sialic acid. A typical example is shown in Figure 1(a). Most of the UV-absorbing material that was voided at the top of the gradient probably comprised lipids and proteins, and some absorbance was found at a position expected for DNA [29]. In all samples the sol phase gave rise to a major mucin population with a buoyant density in $\text{CsCl}/4\text{ M}$ guanidinium chloride similar to the dominating fraction from the gel phase. The sol-phase mucins were partly separated from sialic acid-containing material with a lower buoyant density and from large amounts of UV-absorbing material at the top of the gradients (results not shown). The amount of mucin in the sol phase was generally well below 10% of the total in the sputum, although two samples contained 12% and 17%. The sol-phase mucins were not investigated further.

The mucin-containing fractions from the gel phase were subjected to a second density-gradient centrifugation step in $\text{CsCl}/4\text{ M}$ guanidinium chloride to ensure complete removal of non-mucin proteins (results not shown). A third density-gradient step was then performed in $\text{CsCl}/0.2\text{ M}$ guanidinium chloride to remove DNA. In all samples the major mucin appeared as a unimodal peak with a buoyant density between 1.45 and 1.51 g/ml, somewhat higher than in $\text{CsCl}/4\text{ M}$ guanidinium chloride and in agreement with previous observations [29]. Within each sample, these fractions were pooled to give a 'major mucin' pool (Figure 1c). To investigate whether a denser mucin population such as that seen in CF sputum was present, material from the high-density side of the distribution was studied separately as a 'high-density' pool (Figure 1c). The total amount of material in these fractions was, however, very small compared with the major population.

In all ten samples of 'normal' respiratory secretions, sialic acid-containing material was present at a buoyant density in the $\text{CsCl}/4\text{ M}$ guanidinium chloride density gradient, consistent with

Figure 2 Gel filtration on Superose 6 of material pooled from secretions from ten 'normal' individuals

Material with a buoyant density higher than mucins in $\text{CsCl}/4\text{ M}$ guanidinium chloride from all ten 'normal' samples was pooled (see text) and chromatographed on a Superose 6 HPLC column (30 cm $\times$ 1.0 cm) in 4 M guanidinium chloride/10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 15 ml/h. Fractions (0.25 ml) were analysed for uronic acid (solid line) and absorbance at 280 nm (broken line).

Figure 3 Zimm plot of total-intensity light-scattering data from solutions of mucins from chronic bronchitic gel phase in 6 M guanidinium chloride

The following concentrations were used: 0.091, 0.182, 0.278 and 0.375 mg/ml. Readings were taken at 20°, 30°, 40°, 50°, 60°, 75° and 90°.

Figure 4 Angular dependence of the translational diffusion coefficient (D) (a), concentration dependence of the translational diffusion coefficient (b) and concentration dependence of the sedimentation coefficient (c) for whole mucins from chronic bronchitic gel phase

The translational diffusion coefficient was determined by using quasi-elastic light scattering and the sedimentation coefficient with analytical ultracentrifugation. All measurements were performed in 6 M guanidinium chloride/1 mM Na_2EDTA /1 mM sodium phosphate buffer, pH 6.5.

that of mucins. Most of the UV-absorbing material was voided at the top of the gradient although some absorbance was found at a position expected for DNA (Figure 1b). In six samples, the amount of mucin was sufficient to allow a second density-gradient step, which was performed in CsCl/0.2 M guanidinium chloride. In each sample, this revealed a major mucin band with a buoyant density between 1.45 and 1.53 g/ml (Figure 1d), in agreement with previous observations [14]. However, in one sample some material was present on the high-density side of the distribution.

Investigation of the presence of proteoglycans in secretions from 'normal' individuals

Fractions with a buoyant density greater than 1.50 g/ml in the CsCl/4 M guanidinium chloride gradient were pooled (Figure 1b) and material from all ten 'normal' samples was then combined before investigation for the presence of proteoglycans. The sample was chromatographed on a Superose 6 column after digestion with deoxyribonuclease, ribonuclease and papain (Figure 2). A peak of carbazole-reactive and UV-absorbing material eluted in the void volume, separated from a large peak of UV-absorbing material and a peak of carbazole-reactive material that were included on the column. Glycosaminoglycans released from proteoglycans by papain digestion were eluted in the void volume of a Superose 6 column whereas oligonucleotides, which have a high absorbance at 280 nm (and also show some reactivity with carbazole), were well included. The amount of uronic acid-containing material in the void volume was taken as a measure of the amount of glycosaminoglycan in the sample and was estimated with glucuronolactone as a standard. The total amount of uronic acid in the ten samples, estimated in this way, was approx. 30 μg , corresponding to approx. 100 μg of chondroitin sulphate. With a similar analysis, the total amount of mucin in the same samples was estimated as the area under the mucin band in the CsCl/4 M guanidinium chloride gradients of the ten samples given by sialic acid. With the assumption that respiratory mucins contain approx. 10 % sialic acid [25], this would correspond to approx. 2 mg. The relative amount of mucin was therefore, on average, about 20 times greater (w/w) than that of glycosaminoglycan in the ten samples. This is likely to be an underestimate because molecules other than glycosaminoglycans giving 'unspecific' reaction with the carbazole assay could also be eluted in the void volume of a Superose 6 column.

Macromolecular properties of chronic bronchitic mucins

Absolute intensity light-scattering measurements were made on six chronic bronchitic preparations and their molecular masses obtained by Zimm plot analysis (Table 1). A typical Zimm plot (Figure 3) indicates a large asymmetry in the scattered light with angle, and the radius of gyration (R_g) was measured from the limiting slope of the $\theta \rightarrow 0$ line at $c = 0$. Translational diffusion coefficients (D_t) for all samples showed a marked angular dependence (Figure 4a) and measurements were extrapolated to zero angle. The concentration dependence was low (Figure 4b). In contrast, sedimentation velocity measurements showed a marked concentration dependence that gave a linear dependence when plotted as $1/s$ against c (Figure 4c). Sedimentation con-

Table 1 Summary of physical data for six samples of chronic bronchitic mucins

Values of molecular mass and the radius of gyration (R_g) were determined by using total intensity light-scattering. The translational diffusion coefficient (D_t) was obtained with quasi-elastic light scattering and the sedimentation coefficient with analytical ultracentrifugation. Values of relative molecular mass (M) (s^0/D^0) were also calculated from sedimentation and diffusion data with the Svedberg equation. All measurements were made in 6 M guanidinium chloride/1 mM Na_2EDTA /1 mM sodium phosphate buffer, pH 6.5. The values in parentheses are corrected to standard conditions (water at 20 °C).

Preparation	Molecular mass (MDa)	R_g (nm)	$10^8 \times D_t$ ($\text{cm}^2 \cdot \text{s}^{-1}$)	$10^{13} \times s^0$	$10^{-6} \times M$ (s^0/D^0)
A	19	270	0.98 (1.57)	20.1 (48.9)	21.5
B	14	240			
C	29	329	0.78 (1.25)	22.5 (54.8)	30.3
D	21	290	0.87 (1.40)	22.9 (55.8)	27.6
E	20	300		19.8 (48.2)	
F	13	217	1.09 (1.75)		

Figure 5 Plot of $\log R_g$ against $\log(\text{molecular mass})$

$\log R_g$ was plotted against $\log(\text{molecular mass})$ and the slope (0.51) was obtained by linear regression with the use of least-squares analysis.

stants were obtained after extrapolation to zero concentration. Values of molecular mass were calculated with the Svedberg equation, where s and D_t were determined on cognate samples. There was good agreement between these values of molecular mass and those obtained with total intensity light-scattering. For a summary see Table 1. A plot of $\log R_g$ against $\log(\text{molecular mass})$ gave a straight line with a slope of 0.51, which is in keeping with the behaviour expected for linear, flexible threads in a good solvent (Figure 5).

Whole mucins from both the 'low-density' major mucin and the minor 'high-density' populations (Figure 1b) of the gel phase of chronic bronchitic sputum eluted in the void volume of a Sepharose CL-2B column, whereas, after reduction, 'subunits' were partly included (results not shown). 'Subunits' from the major mucin were excluded on sephacryl S-500 but trypsin digestion revealed the presence of two different glycopeptides, both of which were smaller than 'subunits' when chromatographed on Sephacryl S-500 (Figure 6). The relative amount of the smaller glycopeptide always appeared to be less than that of the larger.

Ion-exchange HPLC of glycopeptides from mucins from 'normal' secretions and chronic bronchitic sputum

Glycopeptides obtained after reduction/alkylation and trypsin digestion of mucins from the gel phase of chronic bronchitic sputum and 'normal' human mucins were subjected to ion-exchange HPLC. The high-molecular-mass glycopeptides isolated from the major chronic bronchitic mucin population eluted earlier (peak position around fraction 40) than those from the 'high-density' pool (peak position around fraction 60) (Figure 7). Sialic acid coincided with hexose. The elution profiles for the smaller glycopeptides closely resembled those for the cognate large glycopeptides (results not shown). In one 'normal' sample, which contained a 'high-density' and a 'low-density' population (as in the chronic bronchitic samples), the 'high-density' population eluted later than the 'low-density' one (results not shown).

The elution positions of high-molecular-mass glycopeptides from the major mucin pools from the chronic bronchitic samples were compared with those from the 'normal' mucins; Figure 8 shows the results obtained from five chronic bronchitic (Figures 8a–8e) and five 'normal' samples (Figures 8f–8j). Most of the

Figure 6 Gel filtration on Sephacryl S-500 of reduced subunits and high-molecular-mass glycopeptides from chronic bronchitic mucins

Subunits were prepared from the major mucins from the gel phase of chronic bronchitic sputum by reduction/alkylation. High-molecular-mass glycopeptides were then prepared from the subunits by trypsin digestion. Chromatography of reduced subunits (solid line) and high-molecular-mass glycopeptides (broken line) was performed on a Sephacryl S-500 HR column (50 cm $\times$ 1.6 cm) in 4 M guanidinium chloride/10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 9 ml/h. Fractions (1.5 ml) were analysed for sialic acid.

chronic bronchitic glycopeptide samples were eluted with a peak position around fraction 40, whereas the peak position of most of the 'normal' ones was at fraction 45. However, a substantial overlap between the distributions was noted for all samples. Furthermore, one chronic bronchitic sample eluted with a peak position at fraction 45 (as did most of the 'normal' samples) and one 'normal' sample had a peak position at around fraction 40 (as did most of the bronchitic samples).

DISCUSSION

The major mucin populations from the gel phase of chronic bronchitic sputum and the individual samples of mucins from the 'normal' airways had a buoyant density in CsCl/0.2 M guanidinium chloride similar to that of pooled 'normal' airway mucins [14] and mucins from CF sputum [25,30,31]. The proportion of mucins found in the sol phase was small (usually less than 10% of the total), in contrast with sputum samples from patients with CF, in which the sol phase contained large amounts of apparently degraded mucins [25]. All ten samples of 'normal' human respiratory secretions contained mucus glycoproteins with a buoyant density similar to that found previously for pooled 'normal' secretions [14]. It is improbable that all subjects were suffering from disease of the airways and it is therefore likely that mucins are a component of respiratory secretions in the non-diseased trachea. The finding is in agreement with those previous studies in which pooled secretions have been used

Figure 7 Ion-exchange HPLC of high-molecular-mass glycopeptides from (a) the major population and (b) the 'high-density' fraction of chronic bronchitic mucins

The major mucin band and the 'high-density' shoulder from one sample of chronic bronchitic sputum obtained by isopycnic density-gradient centrifugation in CsCl were subjected to reduction/alkylation followed by trypsin digestion. The resultant high-molecular-mass glycopeptides were chromatographed on a Mono Q column eluted with a gradient of 0–0.25 M LiClO₄ in 10 mM piperazine buffer, pH 5. Fractions (0.5 ml) were analysed for sialic acid (broken line) and hexose (solid line).

[14,15] although is at variance with the findings of Bhaskar et al. [16] in which mucus glycoproteins seemed to be absent from 'normal' secretions. In our study, the relative amount of proteoglycan material in the samples was smaller than that of the predominant macromolecular species, which had a buoyant density and fragmentation pattern consistent with mucins. Possible explanations for the discrepancies between the results of Bhaskar et al. [16] and those of our and other groups are put forward by Rose [32]. Mucins therefore seem to be the major macromolecular component in secretions collected from healthy respiratory tract. However, because the samples were obtained from children, the existence of age-related differences in composition cannot be ruled out. The absence of large amounts of proteoglycans in secretions from healthy airways is consistent with the finding that the presence of chondroitin sulphate in sputum from chronic bronchitic and CF patients is related to infection [17] and the presence of inflammatory cells and sloughed epithelial cells in the secretions.

Mucins from chronic bronchitic sputum had molecular masses consistent with those obtained previously for cervical, gastric and respiratory mucins from 'normal' subjects and patients with cystic fibrosis [5,14,25,28]. The physical data presented here and electron microscopy studies performed previously on mucins from chronic bronchitic sputum [2] show that chronic bronchitic mucins are long, linear, flexible molecules that behave as random coils in solution.

Both the major mucin and high-density pools from the chronic bronchitic samples were fragmented into subunits on reduction. A multiple subunit structure has also been identified in other respiratory mucins isolated from the sputum of patients with CF [25,30,31] and from 'normal' subjects [14]. The subunits could be further fragmented into high-molecular-mass glycopeptides, corresponding to the oligosaccharide clusters, by proteolytic cleavage. The macromolecular architecture of mucins from the respiratory tract is thus similar to that of cervical [28] and gastric mucins [5]. Several mucin genes, including MUC-2, MUC-4

MUC-5B and MUC-5AC, have been shown to be expressed in the airways [8]. The mucins identified here, in which one subunit contains several highly glycosylated regions separated by sparsely glycosylated domains, is consistent with the structure predicted from the MUC-5AC gene sequence [33]. The presence of two glycopeptides in the major mucin from the chronic bronchitic sputum, as revealed by gel chromatography, suggests the presence of two tandem repeat regions of different lengths within mucins from the respiratory tract. It is not yet clear, however, whether these represent two highly glycosylated regions with different lengths within the same mucin, as seen in human intestinal mucin (MUC-2) [34], or whether they originate from different mucins.

In CF sputum, a pronounced 'shoulder' of denser mucins, which gives rise to more acidic glycopeptides than those from the major mucin, is often present in the high-density side of the distribution in the density gradients [25]. In the chronic bronchitic samples, this 'shoulder' was much less pronounced and was absent from all but one of the 'normal' samples. However, the presence of two chronic bronchitic samples that did not contain the 'shoulder,' and one 'normal' sample that did, suggests that there is some overlap between the groups. The elution position on ion-exchange HPLC showed the glycopeptides from the major chronic bronchitic mucin to be less charged than those from the 'dense' pool, consistent with the earlier findings from CF sputum [35].

Since the 'high-density' mucin represents a small proportion of the total mucins in the chronic bronchitic sputum, we compared the elution positions from the ion-exchange column of, on the one hand, high-molecular-mass glycopeptides from the major mucin from chronic bronchitic sputum and, on the other, mucins from the 'normal' subjects. Histological investigations have previously shown a change in the histochemical staining of the submucosal gland cells from a neutral pattern to a more 'acidic' one in chronic bronchitis [36] and CF [37]. However, the glycopeptides from the chronic bronchitic samples eluted somewhat earlier than those from the 'normal' samples. This

Figure 8 Ion-exchange HPLC of high-molecular-mass glycopeptides from gel-phase mucins from chronic bronchitic sputum (a–e) and ‘normal’ respiratory mucins (f–j)

Mucins from five patients with chronic bronchitis (a–e) and five normal subjects (f–j) obtained by isopycnic density-gradient centrifugation in CsCl were subjected to reduction/alkylation followed by trypsin digestion. The resultant high-molecular-mass glycopeptides were chromatographed on a Mono Q column eluted with a gradient of 0–0.25 M LiClO_4 in 10 mM piperazine buffer, pH 5. Fractions (0.5 ml) were analysed for sialic acid (dotted line) and hexose (solid line).

suggests that the changes seen in the histochemical staining patterns are not directly related to an increased acidity of the major secreted mucin species in chronic bronchitic sputum relative to those from normal secretions. The respiratory tract contains two major secretory cell populations, the goblet cells and the submucosal gland cells, and the results obtained here could be explained by, for example, a change in the relative contribution to airway secretion (sputum) from these two sources in chronic bronchitis. Chronic bronchitis is known to lead to submucosal gland hypertrophy, and if less-acidic mucins are produced by these glands than by the goblet cells, our observations could be interpreted as an increased contribution by the

submucosal glands to sputum compared with the ‘normal’ secretion. If this is so, changes in ‘acidity’ may be masked by changes in the relative contributions of the different sources to sputum.

In conclusion we have shown that mucins, and not proteoglycans, are the major glycoconjugates present in secretions from ‘normal’ subjects without disease of the airways. Mucins from chronic bronchitic sputum have macromolecular properties and an ‘architecture’ similar to that of cervical, gastric and other respiratory mucins. Finally, mucins from chronic bronchitic patients are less rather than more acidic than those from ‘normal’ individuals, suggesting that changes other than the increased

'acidity' observed by histochemical investigations may occur in the disease process.

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