Identification of MUC5B, MUC5AC and small amounts of MUC2 mucins in cystic fibrosis airway secretions

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To investigate the genetic identities of the mucins secreted in cystic fibrosis (CF) airways, sputum was collected from five individuals. Samples were separated into gel and sol phases by high-speed centrifugation and the gel phase was extracted in 6 M guanidinium chloride. The ‘insoluble’ residue remaining after extraction of the gel phase was brought into solution by reduction/alkylation. Density-gradient centrifugation in CsCl revealed polydisperse distributions of sialic acid-containing mucins in the gel phase, insoluble residue and sol phase fractions and the degree of variation between the different individuals was low. Antibodies recognizing MUC5AC and MUC5B identified these mucins in each of the fractions. MUC2, however, was present only as a component of the insoluble residue from the gel which accounted for less than 4% by mass of the total mucins. MUC5B and MUC5AC from the gel phase were large oligomeric species composed of disulphide-bond linked subunits and MUC5B was present as two populations with different charge densities which are likely to correspond to MUC5B glycoforms. The sol phase contained, in addition to MUC5AC and MUC5B, mainly smaller mucins which did not react with the antisera and which were probably degraded. MUC5AC appeared to be enriched in the sol, suggesting that this mucin may be more susceptible to proteolytic degradation than MUC5B. The mucins present in sputum remained broadly similar during acute exacerbation and following antibiotic treatment, although the relative amount of an acidic MUC5B glycoform was decreased during infection.

Key words: cystic fibrosis, mucin, MUC5B, MUC5AC, MUC2, respiratory tract.

INTRODUCTION

Cystic fibrosis, a disease caused by a range of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR, see [1] for a review), is associated with accumulation of mucus in the airways and impaired lung function. The polymer matrix of airway mucus is provided by oligomeric, gel-forming mucins with molecular masses of 10-40 million Da [2-5]. The native molecules are linear assemblies of subunits linked by disulphide bonds. Reduction of the mucins yields reduced subunits containing heavily glycosylated domains alternating along the peptide core with sparsely glycosylated or ‘naked’ regions. Airway mucins are produced by goblet cells in the surface epithelium and glands in the submucosal tissue. Of the eight different mucin genes (MUC1–4, MUC5AC, MUC5B, MUC6 and MUC7) now identified, all but MUC6 appear to be expressed in the respiratory tract (for reviews see [6,7]). Immunohistochemistry has shown MUC5AC to be produced by the epithelial goblet cells [8] and MUC5B and MUC7 to originate mainly from the submucosal glands [9,10]. In addition, MUC2 mRNA expression has been demonstrated in the goblet cells of nasal epithelium [11,12].

In secretions from normal and chronic bronchitic airways, both MUC5AC and MUC5B have been identified as major gel-forming mucins [8,10,13,14] but little or no MUC2 mucin was found [13]. Biochemical studies aimed at identifying differences in CF as compared to normal and chronic bronchitic secretions have shown CF mucins to be very heterogeneous [3,4,15]. In addition to oligomeric gel-forming mucins, large amounts of small species have been identified, a factor which has been attributed to the increased proteolytic activity associated with infection [3,4,16]. Differences in the glycosylation of mucins with an increase in sulphate and a decrease in sialic acid content, have also been reported in CF [17,18] but the genetic identities of the major mucins secreted in CF airways have not been determined. Recently it has been shown that exoproducts from bacteria such as Pseudomonas aeruginosa can upregulate the expression of the MUC2 and MUC5AC genes in bronchial tissue and epithelial cells in culture [19-21] suggesting that colonization may influence the mucins present in CF airways. In order to address the molecular basis of CF disease, murine models in which the CFTR gene is disrupted have now been developed (for a review see [22]). Mice which lack functional CFTR secrete large amounts of mucus in the intestine [23] but, although the animals show severe gastrointestinal symptoms, both the airway mucus hypersecretion and the heavy bacterial colonization with P. aeruginosa or Staphylococcus aureus seen in human CF are absent [24], suggesting that complementary approaches are required for studies of human CF airways disease.

In this study we have, for the first time, investigated the genetic identities of the major mucins which are present in human CF airway secretions using antisera raised against regions within the non-glycosylated domains of the MUC5AC, MUC5B and MUC2 mucins. In addition, the effects of heavy bacterial colonization have been investigated through study of the mucin gene products in sputum during acute exacerbation of infection through treatment with intravenous antibiotics into a ‘recovery’ phase.
EXPERIMENTAL

Materials

Guanidinium chloride (practical grade) and iodoacetamide were from ICN Biochemicals. Approximately 8 M stock solutions of guanidinium chloride were treated with activated charcoal and filtered (PM10 filter, Amicon) before use. N-Ethyl maleimide (NEM) and dianisobenzidine were bought from Sigma Chemical Co., nitrophenyl phosphate and bovine serum albumin (BSA) (Fraction V, pH 7.0) were from Serva and dithiothreitol (DTT) from Merck. Di-isopropyl phosphofluoridate (DFP) was purchased from Fluka. Alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins were bought from Dakopatts. Sephacryl S-500 HR and the Mono-Q HR 5/5 column were from Pharmacia. All other reagents were of A.R. or equivalent quality.

Collection of respiratory secretions

Sputum was collected on several occasions during physiotherapy sessions from five ‘stable’ cystic fibrosis patients (patient 5 who was ΔF508/T338I, all of whom were homozygous for the ΔF508 mutation except patient 5 who was ΔF508/T338I). All patients were infected with mucoid strains of *P. aeruginosa* and received mucolytic therapy (patients 3–5, N-acetyl cysteine; patient 2, N-acetyl cysteine and bromhexine hydrochloride and patient 1, bromhexine hydrochloride alone), inhaled steroid treatment and antibiotic therapy in conjunction with physiotherapy. The sputum samples were pooled and stored at −20 °C until use. In addition, secretions were collected at later dates from one of the individuals (patient 2) at intervals following administration of intravenous antibiotics for acute exacerbation [Nebcin® (Lilly) and Fortum® (Glaxo Wellcome)]. Sputum was pooled on the day of admission, as well as after 7 days and after a total of 14 days of treatment and stored at −20 °C until use.

Isolation of mucins

Samples were thawed after mixing with an equal volume of 0.3 M NaCl/10 mM sodium phosphate buffer, pH 7.4, containing 10 mM NEM and 0.2 mM DFP as described previously [3, 5]. Secretions were separated into a gel and a sol phase by centrifugation (Beckman L70 Optima centrifuge, Beckman 70.1Ti rotor, 4 °C, 45 min, 36000 rev./min) and the sol phase mixed with an equal volume of 6 M guanidinium chloride/10 mM sodium phosphate buffer, pH 7, containing 5 mM sodium EDTA and 5 mM NEM extraction buffer) prior to dialysis against this buffer. The gel phase samples were stirred overnight in extraction buffer and centrifuged (Beckman J2-MC centrifuge, Beckman JA20 rotor, 4 °C, 45 min, 23000 g). Supernatants from three such extractions of each sample were pooled. The ‘insoluble’ mucins remaining in the extraction residue were solubilized by the addition of 6 M guanidinium chloride/100 mM Tris/HCl buffer, pH 8, containing 5 mM sodium EDTA and 10 mM DDT (5 h, 37 °C) followed by iodoacetamide (2.5 molar excess over DTT), 15 h in the dark at room temperature.

Mucins were isolated from the gel and sol phases, as well as the reduced/alkylated extraction residue, by isopycnic density-gradient centrifugation in 4 M guanidinium chloride/CsCl containing 5 mM sodium EDTA and 10 mM sodium phosphate buffer, pH 7 (Beckman L70 Optima centrifuge; Beckman 50.2Ti rotor, 36000 rev./min 15 °C 90 h, initial density 1.39 g/ml). Mucin-containing fractions were pooled (Figure 1b, d, f) and subjected to a second density-gradient centrifugation step in 0.5 M guanidinium chloride/CsCl containing 5 mM sodium EDTA, 10 mM sodium phosphate buffer, pH 7 and 0.01 % CHAPS (initial density 1.50 g/ml). For preparation of reduced mucin subunits, fractions were pooled, dialysed against reduction buffer and reduced/alkylated as described above.

Analytical methods

Densities were measured by weighing aliquots. Sialic acid was detected with an automated procedure according to Davies et al. [5]. Carbohydrate was detected as periodate-oxidizable structures using a glycan detection kit from Roche Molecular Biochemicals [25].

ELISA

ELISA was carried out using the LUM5-1, LUM5B-2 and LUM2-3 antisera recognizing the fully glycosylated forms of the MUC5AC, MUC5B and MUC2 mucins respectively [8, 10]. After dilution in 4 M guanidinium chloride/10 mM sodium phosphate buffer, pH 7, aliquots (100 μl) from samples were subjected to ELISA. For analysis with the LUM5B-2 and LUM2-3 antisera, fractions were reduced in Sero-Wel dilution plates (Bibby Sterilin, U.K.) for 1 h at room temperature with 1 mM DTT (10 μl of a 10 mM solution in 6 M guanidinium chloride/1 M Tris/HCl buffer, pH 8, containing 5 mM sodium EDTA) and then alkylated for 1 h with 2.5 mM iodoacetamide (10 μl of a 25 mM solution in 6 M guanidinium chloride/1 M Tris/HCl buffer, pH 8, containing 5 mM sodium EDTA). Fractions were then coated onto Microtest III multiiwell assay plates (Falcon 3912) overnight at 4 °C. Fractions for analysis with the LUM5-1 antisera were coated directly onto Microtest III multiwell assay plates overnight at 4 °C. All plates were blocked for 1 h with 0.15 M NaCl/5 mM sodium phosphate buffer, pH 7.4, containing 0.05 % (v/v) Tween-20 and 0.5 %, (w/v) BSA (blocking solution) and then incubated for 1 h with the LUM5-1, LUM5B-2 or the LUM2-3 antisera diluted 1:2000, 1:2000 and 1:1000, respectively in blocking solution. Reactivity with the antisera was detected with an alkaline phosphatase-conjugated swine anti-rabbit antisem using nitrophenyl phosphate (2 mg/ml in 5 mM MgCl2/1 M diethanolamine/HCl buffer, pH 9.8) as a substrate. Reactivity is expressed as absorbance at 405 nm after 1 h.

GeL chromatography and ion-exchange HPLC

Whole mucins from the gel and sol phases of sputum were pooled as fractions between 1.4–1.58 g/ml (see Figure 3a, c) and subjected to gel chromatography on a Sephacryl S-500 HR column. The column (100 ml) was connected to an FPLC system with an LKB model 2152 control unit, a Pharmacia V-7 injector and an LKB model 2150 titanium-head pump using Teflon tubing for the solvent stream. Ion-exchange HPLC on a Mono-Q 5/5HR column was performed as outlined by [8]. Reduced mucin subunits were dialysed against 6 M urea/10 mM piperezine perchlorate buffer, pH 5, containing 0.1 % CHAPS (starting buffer). The column was eluted with starting buffer for 10 min with a flow of 0.5 ml/min followed by a linear gradient of LiClO4 in starting buffer up to a final concentration of 0.4 M LiClO4 over 60 min.

Rate-zonal centrifugation

Mucins from the gel phases corresponding to fractions between densities 1.4–1.58 g/ml (Figure 3a) and reduced subunits prepared from them were subjected to rate-zonal centrifugation. Tubes (12 ml) were loaded from the bottom with a linear gradient (6–8 M) of guanidinium chloride using a Hoefer gradient maker.
and an LKB 2232 microperpex pump with a flow of 50 ml/h [26]. Mucin samples in 5 M guanidinium chloride (400 μl) were layered onto the gradients and centrifugation was performed in a Beckman SW 40.1Ti rotor (40 000 rev./min, 20 °C, 2 h 45 min). Tubes were emptied from the top and fractions (500 μl) analysed for reactivity with the LUM5-1 and LUM5B-2 antisera.

RESULTS

Identification of gel-forming MUC5B, MUC5AC and MUC2 mucins in sputum

Mucin samples from five patients were analysed and the results from one individual (patient 2) are shown in Figures 1 and 2. The gel phase mucins which were soluble in guanidinium chloride, contained two partially separated populations in CsCl/4 M guanidinium chloride reacting with the assays for sialic acid and carbohydrate (Figure 1a). The population at a higher density coincided with a peak of absorbance at 280 nm corresponding to DNA [27]. Reactivity with the LUM5B-2 antiserum showed the presence of two MUC5B populations which coincided with those identified using the chemical analyses (Figure 1b). The LUM5-1 antiserum also showed a bimodal reactivity that was shifted towards a lower density than that of LUM5B-2 (Figure 1b). No reactivity was seen with the LUM2-3 antiserum. Extraction of the gel phase in 6 M guanidinium chloride left a residue of ‘insoluble’ mucins. After reduction/alkylation of this material, density-gradient centrifugation revealed a major mucin peak reacting with the assays for sialic acid and carbohydrate and partially separated from DNA (Figure 1c). Reactivity with the LUM5B-2 antiserum showed the presence of a MUC5B population at 1.43 g/ml whereas MUC5AC, as revealed by the LUM5-1 antiserum, appeared at a slightly lower density (Figure 1d). Reactivity with the LUM2-3 antiserum indicated the presence of MUC2 mucins at a density of 1.40 g/ml. Fractions were pooled as shown in Figure 1(b, d) and subjected to a second density-gradient centrifugation step in CsCl/0.5 M guanidinium chloride.

In the gel phase, the mucins were well separated from DNA and the assays for sialic acid and carbohydrate revealed a major mucin peak at 1.46 g/ml which was partially separated from a population present on the high-density side of the distribution (Figure 2a). Reactivity with the LUM5B-2 antiserum showed the presence of two MUC5B mucin populations corresponding to the dominant mucin peak and the ‘shoulder’ respectively (Figure 2b). Thus two populations of MUC5B mucins, which are partially separated in the gradient, were present in the gel phase. MUC5AC mucins were present as two partially separated species which banded on the low-density side of the MUC5B distribution. The ‘insoluble’ mucins from the gel phase gave rise to one major
Isopycnic density-gradient centrifugation in CsCl/0.5 M guanidinium chloride of mucins of one CF individual

Fractions from the CsCl/4 M guanidinium chloride gradients were pooled as shown in Figure 1(b, d, f) and dialysed against 0.5 M guanidinium chloride. After the addition of CsCl and 0.01% (w/v) CHAPS, the material was centrifuged in a Beckman L8-60M centrifuge (50.2Ti rotor, 36000 rev./min, 90 h, 15°C, starting density 1.50 g/ml): (a, b) mucins from the gel phase; (c, d) mucins from the ‘insoluble’ mucin fraction and (e, f) mucins from the sol phase. Tubes were emptied from the bottom and the fractions analysed for (a, c, e): sialic acid (*), carbohydrate (glycan detection method) (D), A280 (––), density (+) and (b, d, f): reactivity with the LUM5-1 (.), LUM5B-2 (v) and LUM2-3 (▲) antisera as described in the text. Fractions from the gel phase (a, b) were also pooled as ‘high-density’ (pool A) and ‘low-density’ (pool B) populations for ion-exchange HPLC shown in Figure 7.

Presence of MUC5AC and MUC5B mucins in the sol phase

In the sol phase, a population of mucins reacting with the assay for sialic acid was present between 1.50 and 1.40 g/ml in CsCl/4 M guanidinium chloride (Figure 1e). The assay for carbohydrate also showed a broad distribution which largely followed that of sialic acid. The LUM5B-2 antiserum revealed a peak of sialic acid and carbohydrate-containing mucins, although, as in the gel phase, a ‘shoulder’ was discerned on the high-density side of the distribution (Figure 2c). Reactivity with the LUM5B-2 antiserum revealed the presence of two distinct species of MUC5B at densities corresponding to those of the major peak and the high-density ‘shoulder’ from the gel phase (Figure 2d). However, the relative proportions of the two species were different between the gel phase and the insoluble fraction with the high-density species dominating in the latter. MUC5AC in the insoluble fraction was again present at a density similar to that of the low-density MUC5B species. Reactivity with the LUM2-3 antiserum indicated that MUC2 was present at 1–46 g/ml and was, under these conditions, not separated from MUC5AC or MUC5B. Thus, the soluble fraction from the gel phase contained MUC5B (present as two populations), as well as MUC5AC, while the insoluble fraction contained, in addition to MUC5B and MUC5AC, some MUC2.

Comparison between secretions from five individual patients

To assess the degree of variation between the five samples, the distribution of sialic acid in the CsCl/0.5 M guanidinium chloride gradients of the gel phase, insoluble fraction and the sol phase.
MUC5AC, MUC5B and MUC2 in cystic fibrosis airway secretions

Figure 3  Isopycnic density-gradient centrifugation in CsCl/0.5 M guanidinium chloride of mucins of five CF individuals

Fractions pooled from the CsCl/4 M guanidinium chloride gradients were centrifuged in CsCl/0.5 M guanidinium chloride as described in the legend to Figure 2 and analysed for sialic acid. The sialic acid distributions from the five subjects were normalized by plotting against density to allow comparison between them. Data for the five patients are plotted in order with patient 1 on the uppermost panel: (a) mucins from the gel phase; (b) mucins from the ‘insoluble’ mucin fraction and (c) mucins from the sol phase.

From each individual was compared after plotting the data against density to normalize them (Figure 3). In the gel phase, all samples contained a dominant population at 1.46 g/ml (Figure 3a). In all cases, the major mucin species was partially separated from a second population present as a ‘shoulder’ on the high-density side of the distribution. Reactivity with the LUM5B-2 antiserum indicated the presence of two populations of MUC5B in all the gel-phase samples (data not shown). In the insoluble fraction, the sialic acid assay revealed the presence of a single mucin species at a density of 1.46 g/ml in 4 of the 5 individuals (Figure 3b). In one patient (patient 4), however, the high-density shoulder found in the gel phase was clearly visible. As in the gel phase, the LUM5B-2 antiserum indicated the presence of two partially resolved populations of MUC5B (data not shown).

Each of the sol phases showed a broad distribution of mucins with a major peak around 1.46 g/ml. As in the gel phase, this was partially resolved from a second population on the high-density side of the distribution and in one individual (patient 4) the separation was distinct. Thus the mucins isolated from five different individuals showed similar patterns in the density-gradients, with two sialic acid-containing mucins and two MUC5B populations present. However, the relative amounts of these varied between the gel phase, insoluble gel fraction and the sol phase as well as between individuals.

Proportion of mucins in the gel phase, insoluble fraction and sol

To investigate how much mucin was present in the gel as opposed to the sol phase of the secretions, the relative amounts of mucins in the guanidinium chloride ‘soluble’ and ‘insoluble’ fractions from the gel as well as in the sol were estimated gravimetrically after pooling, dialysis against water and lyophilization of the mucin-containing fractions (densities 1.40–1.58 g/ml) from the CsCl/0.5 M guanidinium chloride gradients (see Figure 3a–c). The distribution of mucins between the fractions varied amongst individuals (Table 1). In three patients (1, 2, 5) most of the mucins were present in the gel phase with the majority found in the sol phase in two patients (3, 4). In all cases, the insoluble fraction from the gel, which contained the LUM2-3 reactivity, represented only a small amount of the total mucins (< 4 %).

Table 1 Percentage of mucins in the gel and sol phases of CF sputum

Mucins pooled from the CsCl/0.5 M guanidinium chloride gradients of five individuals were dialysed against water, lyophilized and weighed. The relative amount of mucins present in each fraction is expressed as a percentage of the total.

<table>
<thead>
<tr>
<th>Patient</th>
<th>% of total mucins</th>
<th>Gel (GuHCl) soluble</th>
<th>Gel (GuHCl) insoluble</th>
<th>Sol</th>
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<tbody>
<tr>
<td>1</td>
<td>163</td>
<td>4</td>
<td>13</td>
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<tr>
<td>2</td>
<td>63</td>
<td>1</td>
<td>36</td>
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<td>3</td>
<td>40</td>
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<td>4</td>
<td>43</td>
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<tr>
<td>5</td>
<td>60</td>
<td>3</td>
<td>37</td>
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Size distributions of mucins in the gel and sol phases

To investigate the size distributions of the mucins in CF sputum, the gel and sol fractions were subjected to gel chromatography on a Sephacryl S-500HR column after pooling material between the densities of 1.40–1.58 g/ml from the CsCl/0.5 M guanidinium chloride density-gradients (Figure 3a, c). In the gel phase from each individual, all sialic-acid reactivity, as well as all reactivity with the LUM5-1 and LUM5B-2 antiseras, was present in the void volume, suggesting that the molecules are large (Figure 4a). In the sol phase, however, most of the material was smaller with only a small proportion of the sialic acid and LUM5-1 and LUM5B-2 reactivity occurring in the void volume (Figure 4b). Both the MUC5B and MUC5AC mucins were present as several populations which were eluted in the partially included volume. In addition, several major peaks of sialic acid-containing molecules which lacked antibody reactivity were further included on the column. The size distribution of the large mucins from the gel phase, which were excluded on the Sephacryl S-500HR column, was further studied using rate-zonal centrifugation. Reactivity with the LUM5B-2 antiserum revealed a broad distribution of MUC5B across the gradient with a partial separation into three populations, indicating that MUC5B mucins of different molecular sizes are present (Figure 5a). This pattern was repeated in 3 of the 5 individuals (data not shown). MUC5AC mucins showed a much narrower distribution closer to the top of the gradient and are thus apparently smaller than MUC5B mucins. After reduction and alkylation, both MUC5B and MUC5AC banded close to the top of the gradient indicating that they had become smaller (Figure 5b). Thus, while the gel-phase mucins were large oligomeric species composed of subunits linked by disulphide bonds, most of the molecules in the sol phase were smaller.

Charge densities of mucins in the gel phase

Investigation of the charge densities of the gel phase mucins from each of the five individuals, using ion-exchange HPLC of reduced subunits, revealed a complex pattern (Figure 6a–e). Sialic acid reactivity was found between fractions 30 and 50 and indicated the presence of two partially separated populations. In one individual (patient 2), two populations of MUC5B mucins were clearly distinguished with the earlier eluting species showing a
Figure 6  Ion-exchange HPLC of reduced subunits from the gel phase of five individuals

Mucin subunits prepared by reduction/alkylation of mucins pooled from the gel phase were subjected to ion-exchange HPLC on a Mono-Q 5/5 HR column as described in the text. Panels (a–e) correspond to patients 1–5, respectively. Fractions (0.5 ml) were analysed for sialic acid (●) and reactivity with the LUM5-1 (▲) and LUM5B-2 (▼) antisera. Greater reactivity with the LUM5B-2 antiserum than the later one (Figure 6b). In three other individuals (patients 1, 3, 5), the reactivity with the LUM5B-2 antiserum closely followed that of sialic acid with one dominating MUC5B species partially separated from later-eluting species (Figure 6a, c, e). In patient 4, only a single population of MUC5B appeared to be present (Figure 6d). MUC5AC mucins eluted at a position intermediate to that of the two MUC5B populations (Figure 6a–c, e). The data indicate that the pattern of elution of the gel-forming mucins from each of the individuals was quite similar although the actual elution positions differed slightly. To investigate whether the partially separated MUC5B populations seen in the density-gradients of the gel-phase mucins corresponded to the two peaks seen on the ion-exchange column, the two populations were pooled separately (pools A and B) from the CsCl/0.5 M guanidinium chloride gradient (Figure 2b) and chromatographed individually (Figure 7a, b). This indicated that the high-density population from the density-gradient corresponded to the highly charged, later-eluting MUC5B population whereas the low-density mucins were less charged and eluted earlier from the column.

Figure 7  Ion-exchange HPLC of reduced subunits from (a) ‘high-density’ and (b) ‘low-density’ mucin populations from the gel phase.

Mucin subunits prepared by reduction/alkylation of ‘high-density’ and ‘low-density’ mucins (pools A and B respectively – see Figure 2b) from patient 2 were subjected to ion-exchange HPLC on a Mono-Q 5/5 HR column as described in the text. Fractions (0.5 ml) were analysed for reactivity with the LUM5B-2 (▼) antiserum.

Effects of exacerbation upon the mucins present in sputum

In order to study whether infection affected the mucin secretion pattern, sputum was collected from patient 2 on the first day of intravenous antibiotic treatment (day 1), after 7 days (day 7) and finally after ‘recovery’ (following 14 days of treatment – day 14). After separation into a gel and a sol phase and extraction of the gel, the mucins in the gel phase, insoluble extraction residue from the gel and the sol were investigated with density-gradient centrifugation as described above. In the gel phase from day 1, a peak of sialic acid and carbohydrate occurred at 1–45 g/ml in the CsCl/0.5 M guanidinium chloride gradient together with a ‘shoulder’ at 1.50 g/ml (Figure 8a). The LUM5B-2 antiserum showed the presence of one major MUC5B mucin population corresponding to the dominant sialic acid-containing mucin peak although reactivity was also associated with the high-density ‘shoulder’ (Figure 8b). These secretions appeared to contain less of the high-density MUC5B mucins relative to those from the same individual under ‘stable’ conditions (Figure 2b). The MUC5AC mucins, however, appeared to be similar to the stable condition and were again present as two partially separated populations which banded towards the low-density side of the MUC5B distribution. After 7 days of treatment, the total amount of mucins in the sample was increased as compared to day 1. The
Figure 8  Isopycnic density-gradient centrifugation in CsCl/0.5 M guanidinium chloride of mucins from the gel phase of sputum from patient 2 before, during and after treatment with intravenous antibiotics

Chemical analyses showed a much broader distribution of mucins in the density-gradient with a relative increase in the material at a higher density (Figure 8c). Reactivity with the LUM5B-2 antiserum showed a clear separation into two peaks and the relative amount of high-density MUC5B mucins was increased although there was still less than under ‘stable’ conditions (Figure 8d). MUC5AC was found as one peak at a density similar to that of the major MUC5B population. Following 14 days of treatment, the mucins were again found widely distributed over the density-gradient (Figure 8e) and the distribution of MUC5B was similar to that on day 1 with a major population at 1.45 g/ml and a high-density population present as a ‘shoulder’ rather than a distinct peak (Figure 8f). The distribution of MUC5AC was also very similar to that on day 1. Thus, with respect to the gel-forming mucins, the pattern seen during exacerbation was very similar to that after the antibiotic treatment. The data were also broadly in keeping with those obtained from the same individual under ‘stable’ conditions although there appeared to be a relative decrease in the amount of the more highly charged, high density ‘glycoform’ of MUC5B (Figure 2b). During the resolution of the exacerbation, the mucins also appeared to have a greater range of buoyant densities. The patterns obtained for mucins from the insoluble gel fraction and the sol phases (data not shown) were also similar to those seen for the patient under ‘stable’ conditions (Figure 2d–f) although a relative decrease in the amount of the high-density MUC5B population, as compared to the ‘stable’ condition, was seen. These observations suggest that the more acidic ‘glycoform’ of MUC5B was decreased in all fractions of the sputum both in acute exacerbation and immediately following cessation of treatment.

DISCUSSION

Several different mucin genes (MUC1–MUC5B and MUC7) are expressed in the respiratory tract but the genetic identities of the mucins present in human CF airway secretions have not previously been described. The gel phase was separated from the sol to allow identification of the gel-forming mucin species and polyclonal antisera recognizing the fully glycosylated forms of MUC5B, MUC5AC and MUC2 were then used to investigate the presence of these mucins in sputum. Density-gradient centrifugation revealed the presence of one major mucin species as well as a population which appeared as a shoulder on the high-density side of the distribution, and the relative proportions of these two varied between the gel phase, insoluble residue from the gel and
the sol phase. In all of five individuals, however, the patterns of mucins present were broadly similar although close comparison revealed slight differences in the relative proportions of the two components. Analysis with the antisera revealed the presence of gel-forming MUC5B and MUC5AC and CF airway secretions were thus similar to those from normal and asthmatic individuals, as well as patients with chronic bronchitis, where MUC5B and MUC5AC have been identified as major components [10,13,14,28]. Since MUC5B is mainly produced by the mucous cells of the submucosal glands [9,10], whereas MUC5AC originates from the surface epithelial goblet cells [8], both secretory cell types appear to contribute to CF secretions. In several individuals, two populations of MUC5B mucins from the gel phase could be separated in the density-gradients, as well as after ion-exchange HPLC, suggesting that they correspond to two differently glycosylated MUC5B mucins or ‘glycoforms’ such as those identified previously in respiratory secretions from asthmatic [14] and chronic bronchitic subjects [10]. Both MUC5B and MUC5AC in the gel were oligomeric mucins composed of subunits linked by disulphide bonds. Rate-zonal centrifugation indicated that they were of similar size to respiratory mucins in other disease states as well as from salivary and cervical secretions [10,13,14,28]. Three partially separated populations of MUC5B were discerned in some samples most likely corresponding to differently sized mucins, but in this study it was not possible to decide whether these MUC5B variants arise during biosynthesis or from proteolytic modification after secretion. However, the presence of such populations in 3 of the 5 individuals makes it unlikely that they result from random proteolytic degradation.

MUC5B and MUC5AC mucins were found in the sol phase which represents a mixture of non gel-forming and degraded mucins as well as gel-forming mucins in equilibrium with the gel. In agreement with previous studies, the sol contained predominantly mucins which were smaller than those in the gel [3]. The relative distribution of mucins between the gel and sol phases varied widely between individuals despite the fact that all patients had received mucolytic drugs which would be expected to fragment the large gel-forming mucins causing them to lose their gel-forming properties and increase the relative proportion of material present in the sol phase. The patients in this study were, as usual for CF patients, heavily colonized with Pseudomonas aeruginosa, making it likely that their airway secretions contained high levels of bacteria- and neutrophil-derived proteases which cause degradation of mucins [3,4,16]. In all cases, the relative amount of MUC5AC compared to that of MUC5B was greater in the sol than in the gel and it is possible that MUC5AC mucins are more susceptible to proteolytic degradation than MUC5B. However, a large proportion of the mucins in the sol, especially the smaller species, did not react with any of the antisera after gel chromatography suggesting the presence of a different mucin species and/or fragments of MUC5AC and MUC5B from which the epitopes recognized by the antibodies have been cleaved.

Studies using in situ hybridization [29,30], immunohistochemical staining [31] and Northern blot analysis [11,12,32] have indicated that MUC2 is expressed in airway epithelium. It has been suggested that MUC2 is upregulated in CF airways [30], while others have found no differences between normal and CF individuals [12]. In our study, MUC2 was not found in the gel or sol phases which corresponded to at least 95% by mass of the total material but was present in the ‘insoluble’ extraction residue from the gel. In total, this fraction comprised less than 4% of the total mucins by mass. Even if MUC2 had been the only component of this fraction, the amount would have been small but since MUC5B and MUC5AC were also present, MUC2 must represent a small part of the total mucins by weight. The fact that the MUC2 which is present is insoluble suggests that these mucins from the airway have similar properties to those from human colon where virtually all MUC2 occurs as an ‘insoluble’ complex [33]. Varying amounts of such ‘insoluble’ material have also previously been identified in respiratory secretions from bovine trachea, human submandibular/sublingual saliva and rat small intestine [34]. One reason for the low level of the mucin in sputum could be that the presence of ‘insoluble’ MUC2 changes the physical properties of the mucous gel such that material containing MUC2 is not removed from the airways by mucociliary transport or coughing. In previous studies, however, MUC2 was also largely absent from normal and chronic bronchitic airway secretions [13].

To determine whether the mucins present in the airways change during infection, the pattern from one individual was obtained at acute exacerbation, during antibiotic treatment and at ‘recovery’. Comparison with the ‘stable’ pattern for this patient suggested that there were no major differences in the patterns of MUC5B and MUC5AC secretion during exacerbation and recovery. However, during the exacerbation, there was an apparent decrease in the relative amount of the high-density population which was shown to correspond to the acidic glycoform of MUC5B. A sulphated glycoform of MUC5B with a similar charge density to the acidic glycoform has previously been shown to originate from a subpopulation of mucous cells within the submucosal glands [10]. The reduction in the amount of this material may thus suggest that certain mucous cells are less active during heavy infection. However, since sputum samples probably also contain saliva in which several acidic glycoforms of MUC5B are present [10], it cannot be ruled out that some of the forms of MUC5B identified here originate from this secretion.

Mucins in the gel phase of CF sputum had similar charge densities to those from chronic bronchitic and normal airways [2,5] although it has been reported that CF airway mucins are more heavily sulphated [17,18]. However, in such investigations, an increase in the $^{35}$S-sulphate labelled material eluting in the void volume of Sepharose CL-2B was used to demonstrate increased mucin sulphation. We have recently shown that in tracheal tissue in culture, $^{35}$S-sulphate is mainly incorporated into a high-turnover, monomeric mucin rather than the large gel-forming species [35]. Since the monomeric glycoproteins also elute in the void volume of Sepharose CL-2B, a rise in $^{35}$S-sulphate is likely to reflect an increase in the amount of this mucin rather than changes in sulphation of the oligomeric gel-forming ones. Other monomeric mucins have been proposed to play an important role in the pathogenesis of CF [36]. Mice which lack functional CFTR suffer from intestinal obstruction due to large amounts of mucus in the lumen, an effect which is abolished in double knockout mice which also lack the monomeric MUC1 mucin. This suggests that mucins traditionally regarded as cell-associated species may be involved in the pathogenesis of hypersecretion in CF, although further studies are required to determine their possible roles.

In conclusion, CF sputum contains predominantly MUC5B and MUC5AC which originate from the submucosal glands and epithelial goblet cells respectively. No major differences in the macromolecular properties of MUC5AC and MUC5B mucins as compared with normal airway secretions were identified and no major differences associated with age, CF genotype or infection could be demonstrated. Small amounts of MUC2 mucin were present within the insoluble fraction from the gel but it is unlikely that the small amounts present would be sufficient to explain the viscous nature of CF secretions.
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