Respiratory mucins: identification of core proteins and glycoforms

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At least eight mucin apoproteins are expressed by the tracheo-bronchial epithelium, but it is not known which, if any, of these are major constituents of the respiratory secretions responsible for the formation of the mucus gel. To address this we have isolated mucins from normal, asthmatic and chronic bronchitic secretions. The asthmatic mucin reduced subunits were fractionated into four populations (I–IV) by anion-exchange HPLC. Amino acid and monosaccharide compositional analysis, as well as M_r and size measurements, indicate that two of these populations (I and II) are glycoforms of the same or related apoprotein(s) and that the other populations contain two different apoproteins. A panel of antibodies and antisera recognizing the variable number tandem repeat (VNTR) of specific mucin apoproteins did not, as predicted, react with the glycosylated molecules, but after deglycosylation the majority of these probes (with the

exception of those to MUC2, which were negative) reacted at a low level with each of the subunit populations. In contrast, an antiserum against a non-VNTR sequence of MUC5AC identified one of the populations (III) as the MUC5AC mucin. The MUC5AC reduced subunit had an M_r of 2.2×10^6 and an R_g (radius of gyration) of 57 nm. The genetic identities of the major mucin (populations I and II) and a minor component (population IV) were not established. The MUC5AC mucin was also identified as a major component in the pooled normal secretions from 20 individuals, whereas in a chronic bronchitic sample it was only a minor constituent. Furthermore, in all these different respiratory secretions the MUC5AC mucin appears as a similar biochemical entity, as assessed by Mono Q chromatography and agarose electrophoresis, suggesting that it may have a well-defined pattern of glycosylation in the respiratory tract.

INTRODUCTION

Mucus produced by cells in the epithelial surface layer and glands in the underlying submucosa protects and cleanses the respiratory tract. The molecules responsible for the formation of this biofilm are high- M_r O-linked glycoproteins referred to as mucins. These complex glycoproteins are polydisperse, linear polymers [M_r (5–30) $\times 10^6$] that upon reduction are fragmented into monomers [M_r (2–3) $\times 10^6$] that we term reduced subunits [1–4]. The protein cores of the monomers represent the primary gene product (apomucin). Histochemical investigations have shown both neutral and acidic mucins in the respiratory tract [5,6], demonstrating that differently glycosylated populations are present. These findings have been substantiated by ion-exchange chromatography of glycopeptides prepared from mucins isolated from asthmatic, chronic bronchitic, cystic fibrotic and normal respiratory secretions [1,4,7,8]. It is not known whether these populations are due to differential glycosylation of a mixture of apomucins and/or to glycoforms of single or multiple apomucins.

By using gel electrophoresis of reduced respiratory mucin subunits, we have identified two distinct mucin populations that comprise the majority of the mucins in respiratory tract secretions [4]. Northern blot analysis and *in situ* hybridization have indicated that at least eight mucin genes (*MUC1*, 2, 3, 4, 5AC, 5B, 7 and 8) are expressed in human tracheobronchial epithelia [9–17]. However, it has not been established which, if any, of these genes encode the apoproteins of the two identified populations [4]. Macromolecular size and fragmentation data for asthmatic,

chronic bronchitic, cystic fibrotic and normal respiratory mucins rule out MUC1 and MUC2 as major components of the gel [1–4,7,8].

Here we extend our studies on respiratory mucins by using ion-exchange chromatography and agarose gel electrophoresis to further analyse the different populations previously described [4]. We identify one of them as a MUC5AC mucin while the genetic identity of the other(s) remains to be established. The level of the MUC5AC mucin varies greatly between respiratory samples but the molecule is in all cases similar with regard to charge density and electrophoretic mobility.

EXPERIMENTAL

Materials

Agarose UltraPURE (electrophoresis grade) was from BRL (Paisley, Scotland, U.K.). The enhanced chemiluminescence (ECL) Western detection kit was from Amersham International Plc. (Amersham, Bucks., U.K.). Immobilon-P [poly(vinylidene difluoride); PVDF] membranes were from Millipore (Milton Keynes, Bucks., U.K.). Trifluoromethanesulphonic acid (TFMSA) was from Aldrich (Gillingham, U.K.). Neuraminidase (EC 3.2.1.18; type X from *Clostridium perfringens*), Tween 20, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) (CHAPS), *N*-ethylmaleimide, ϵ -amino-*n*-hexanoic acid, benzamide hydrochloride, Schiff's reagent, NitroBlue Tetrazolium,

Abbreviations used: ECL, enhanced chemiluminescence; PAS, periodate/Schiff; VNTR, variable number tandem repeat; TFMSA, trifluoromethanesulphonic acid; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate); PVDF, poly(vinylidene difluoride).

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5-bromo-4-chloro-3-indolyl phosphate and guanidinium chloride (practical grade) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Stock solutions of guanidinium chloride (approx. 8 M) were treated with charcoal before use. Goat anti-(rabbit IgG) horseradish peroxidase conjugate, goat anti-(mouse IgG) horseradish peroxidase conjugate (whole molecule) and goat anti-(mouse IgM) horseradish peroxidase conjugate (μ -chain specific) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Goat anti-(rabbit IgG) alkaline phosphatase conjugate was from Promega (Southampton, U.K.).

Preparation of reduced mucin subunits

Respiratory secretions were collected *post mortem* from the lungs of an asthmatic individual, as sputum from an individual with chronic bronchitis or by tracheal intubation from 20 'normal' individuals. Data on these samples have been reported previously [1,4]. Mucins were extracted with 6 M guanidinium chloride/10 mM sodium phosphate buffer, pH 6.5, containing proteinase inhibitors (10 mM EDTA/10 mM *N*-ethylmaleimide/100 mM ϵ -amino-*n*-hexanoic acid/5 mM benzamide hydrochloride) and purified by three rounds of density-gradient centrifugation in CsCl [1,2]. Reduced mucin subunits were obtained by treatment of the purified mucins in 6 M guanidinium chloride/0.1 M Tris, pH 8.0, with 10 mM dithiothreitol for 5 h at 37 °C. Iodoacetamide was then added to a final concentration of 25 mM and the mixture left in the dark overnight at room temperature [4]. The asthmatic mucin subunits were further purified by density-gradient centrifugation in CsCl/4 M guanidinium chloride (initial density of 1.42 g/ml) containing 0.1% (w/v) CHAPS using a Beckman Ti 70 rotor at 40000 rev./min and 15 °C for 70 h to remove a low-density protein-rich fraction generated by the reduction process [4]. Reduced mucin subunits were dialysed into 6 M guanidinium chloride and stored at 4 °C.

Anion-exchange HPLC

Reduced mucins were transferred from 6 M guanidinium chloride into 6 M urea/10 mM piperazine, pH 5.0, containing 0.02% CHAPS by chromatography on a Pharmacia PD-10 column prior to separation by anion-exchange HPLC. Reduced mucins were then chromatographed on a Pharmacia Mono Q HR 5/5 column eluted with a linear gradient of 0 to 0.4 M lithium perchlorate/10 mM piperazine, pH 5.0, in 6 M urea containing 0.02% CHAPS [18]. The gradient program and chromatographic system have been described previously [4].

Agarose gel electrophoresis

Reduced mucin subunits were either dialysed or diluted into 40 mM Tris-acetate/1 mM EDTA, pH 8.0, containing 0.1% (w/v) SDS. One-tenth of a volume of 30% (v/v) glycerol in 40 mM Tris-acetate/1 mM EDTA, pH 8.0, containing 0.002% (w/v) Bromophenol Blue was added to the samples prior to electrophoresis [18]. Samples (1–5 μ g/lane) were electrophoresed in 1.0% (w/v) agarose gels (15 cm $\times$ 15 cm) prepared in 40 mM Tris-acetate/1 mM EDTA, pH 8.0, containing 0.1% (w/v) SDS. Electrophoresis was performed in a horizontal gel apparatus using the Bio-Rad DNA sub cell [160 ml of gel, 1900 ml of 40 mM Tris-acetate/1 mM EDTA, pH 8.0, containing 0.1% (w/v) SDS, for 30 V and 18 h at room temperature]. After electrophoresis subunits were transferred to nitrocellulose or PVDF by vacuum blotting in 0.6 M sodium chloride/0.06 M sodium citrate using a Pharmacia LKB VacuGene XL at a

suction pressure of 40 mbar (4000 Pa) for 1 h prior to detection of mucins using antibodies [18].

Deglycosylation

Mucins or reduced mucin subunits were immobilized on to PVDF membranes by slot or Western blotting and O-glycans removed by treatment with TFMSA [18]. Briefly mucins or reduced mucin subunits transferred to PVDF were incubated with neuraminidase (0.001 unit/cm² of membrane) in 50 mM sodium acetate/150 mM sodium chloride/100 mM calcium chloride, pH 5.5, for 30 min at 37 °C. The membrane was washed extensively in water, thoroughly dried in a warm airstream and then transferred to a precooled stoppered glass tube (5.5 cm $\times$ 16 cm). Ice-cold TFMSA was added (50 μ l/cm² of membrane) and the tube placed on a rolling platform for 15 min at 4 °C. After this the membrane was removed and extensively washed in water. Blots were probed with antibodies and antisera raised against or reactive with synthetic peptides corresponding to amino acid sequences identified from cDNA cloning of the mucins MUC1 to MUC5.

Monoclonal and polyclonal antibodies

MUC1

The murine monoclonal antibody BC2 (IgG₁ ascites, 1:1000) was raised against human milk fat globule membranes and recognizes the protein core epitope APDTR [19]. The monoclonal antibody C595 (IgM, affinity-purified tissue culture supernatant 1:100) was produced against human urinary MUC1 mucin and defines the determinant RPAP [20].

MUC2

The murine monoclonal antibody 996 (IgM, affinity-purified tissue culture supernatant 1:10) was prepared against the hydrophilic region of the MUC2 variable number tandem repeat (VNTR) and it recognizes the motif TGTQ [21]. The monoclonal antibody 4F1 (IgM ascites, 1:1000) was raised to a synthetic peptide corresponding to a single repeat of the MUC2 VNTR and recognizes two different sites, TPTP and PTTT [22]. A rabbit polyclonal antiserum (LUM2-3) was prepared using a synthetic peptide NGLQPVRVEDPDGC outside the VNTR of MUC2.

MUC3

The murine monoclonal antibody 1143/B7 (IgM, affinity-purified tissue culture supernatant 1:10) was prepared against the hydrophilic region of the MUC3 VNTR (L. Durrant and M. R. Price, unpublished work). Two monoclonal antibodies M 3.2 (IgG ascites, 1:500) and M3.3 (IgG ascites, 1:500), a kind gift from Drs. Pei-Xiang Xing and Vasso Apostolopoulos, were raised against a synthetic peptide corresponding to two repeat units of the MUC3 VNTR [23].

MUC4

Two polyclonal antisera, one rabbit (LUM4) and one murine (PDM4), were prepared using a synthetic peptide corresponding to the VNTR of MUC4.

MUC5AC

Two polyclonal antisera were prepared against synthetic peptides from MUC5AC: one, a murine antiserum (PDM5), was raised

against a synthetic peptide corresponding to the VNTR of MUC5AC and the other, a rabbit antiserum (LUM5-1), was against a non-VNTR synthetic peptide RNQDQQGPFCMC [24]. In a standard ELISA, using a coating concentration of the synthetic peptide of 1 µg/ml, the LUM5-1 antiserum exhibited saturable binding at a dilution of 1:1000 which was decreased by 60% at a dilution of 1:10000.

As a general non-specific probe a rabbit polyclonal antiserum raised against cervical mucin reduced subunits that recognizes protein epitopes on a range of mucin subunits was employed [25]. Finally monoclonal antibody AM3, whose epitope is the carbohydrate structure sialyl Le^x [26], was a kind gift from Dr. Chris Hanski.

Secondary antibodies were used at the following dilutions: goat anti-(rabbit IgG) horseradish peroxidase conjugate (1:10000), goat anti-(rabbit IgG) alkaline phosphatase conjugate (1:7500), goat anti-(mouse IgG) horseradish peroxidase conjugate (1:10000), and goat anti-(mouse IgM) horseradish peroxidase conjugate (1:10000).

Amino acid analysis

Samples were subjected to vapour-phase acid hydrolysis (6 M HCl at 105 °C for 16 h). The resulting amino acids were derivatized with phenyl isothiocyanate and the derivatives separated by reverse-phase HPLC using a Spherisorb 3 µm ODS2 column with gradient elution (0–50% acetonitrile in 12 mM sodium phosphate buffer, pH 6.4).

Monosaccharide analysis

Samples were hydrolysed in 2 M trifluoroacetic acid at 100 °C for 5 h then rotary-evaporated to dryness, re-dissolved in water and the monosaccharides separated on a Dionex CarboPac PA1 column eluted at 1 ml/min with 21 mM sodium hydroxide. Monosaccharides were detected using a Dionex PAD II detector.

Light scattering

Reduced mucin subunits (200–400 µg/ml) were chromatographed on a Pharmacia Superose 6 HR 10/30 column eluted with PBS, pH 7.4, at a flow rate of 0.5 ml/min. The column effluent was passed through an in-line Dawn[®] DSP laser photometer and a Wyatt/Optilab 903 interferometric refractometer (Optichem, Clywd, U.K.) to measure light scattering and sample concentration respectively. Light scattering measurements were taken continuously at 18 angles between 15° and 151° and the data analysed according to Zimm [27].

RESULTS

Mucin subunit fractionation

Reduced asthmatic respiratory mucin subunits were examined by anion-exchange HPLC on Mono Q and the periodate/Schiff (PAS) profile (Figure 1a) shows evidence of two major populations. Fractions were also assayed with a number of carbohydrate probes and data for the lectins *Tetragonolobus purpureas* (fucose) and *Sambucus nigra* (sialic acid α2,6-linked *N*-acetylgalactosamine) and the monoclonal antibody AM3 (sialyl-Le^x) show evidence for further subpopulations within both main species (Figures 1b–1d). Agarose electrophoresis across the Mono Q profile verifies the presence of the two major populations (Figure 2) and also indicates the presence of subpopulations. In general, there is an increase in electrophoretic mobility with increasing charge density, but the most charged molecules (Mono Q fractions 39–45) are not electrophoretically the most mobile ones.

Figure 1 Anion-exchange HPLC of asthmatic reduced mucin subunits

Asthmatic reduced mucin subunits (approx. 13 mg) were chromatographed on a Mono Q HR 5/5 column as described in the Experimental section. Column fractions (0.5 ml) were assayed, after immobilization on to nitrocellulose by slot blotting, for (a) carbohydrate (solid line) with the PAS assay [30] and the nominal gradient is indicated (broken line). Fractions were also analysed, after immobilization on to nitrocellulose by slot blotting, for binding to (b) the lectin *Tetragonolobus purpureas*, (c) the monoclonal antibody AM3 and (d) the lectin *Sambucus nigra* [31]. Data were obtained using NitroBlue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the chromogenic substrate and the integrated intensity is defined as in Thornton et al. [30]. Fractions from the column were pooled as follows; 18–25 (I), 26–32 (II), 33–38 (III) and 39–45 (IV) and these poolings are indicated in (a) by the bars.

Figure 2 Agarose gel electrophoresis of fractions from across the Mono Q chromatogram of the asthmatic reduced mucin subunits

Aliquots (1–3 µg/lane) of selected fractions were subjected to 1% (w/v) agarose gel electrophoresis and subsequently blotted on to nitrocellulose as described in the Experimental section. The blot was blocked for 30 min with 1% (w/v) skimmed milk powder in 10 mM Tris/HCl, 150 mM sodium chloride, pH 8.0, containing 0.1% (v/v) Tween 20 (TBST) and then probed for 1 h with an antiserum raised against reduced cervical mucin subunits (1:1000) in TBST. The blot was washed three times in TBST, incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min and finally washed again with TBST. Bands were visualized using the ECL Western detection kit.

Figure 3 Anion-exchange HPLC of pooled reduced mucin fractions

The four pooled asthmatic reduced mucin subunit fractions (a) I, (b) II, (c) III and (d) IV (pooled as shown in Figure 1) were chromatographed on a Mono Q HR 5/5 column as described in the Experimental section. Column fractions (0.5 ml) were assayed, after immobilization on to nitrocellulose by slot blotting, for carbohydrate with the PAS assay (solid line) and the nominal gradient indicated (broken line). The integrated intensity is defined as in Thornton et al. [30]. Aliquots (2–3 μ g/lane) of fractions from each distribution (e) I, (f) II, (g) III and (h) IV were subjected to 1% (w/v) agarose gel electrophoresis and subsequently blotted on to nitrocellulose. The blot was probed with an antiserum raised against reduced cervical mucin subunits as described in the legend to Figure 2. Fractions from each chromatogram were pooled on the basis of their electrophoretic mobilities into four populations (I–IV) as indicated by the bars. The amount of material in each population (I–IV), determined after dialysis and lyophilization, was 46%, 33%, 14% and 7% (w/w) respectively.

Fractions from the Mono Q column were pooled (Figure 1a) on the basis of their reactivity with the carbohydrate probes (Figures 1b–1d) and their electrophoretic mobilities (Figure 2). The four resultant populations were rechromatographed on the Mono Q column under identical conditions (Figures 3a–3d) and

fractions across the chromatograms were again analysed by agarose gel electrophoresis (Figures 3e–3h). Fractions were finally pooled (Figures 3a–3d) to yield four populations (I–IV) of reduced mucin subunits which after electrophoresis appeared as single bands of different electrophoretic mobilities (Figure 4).

Figure 4 Agarose gel electrophoresis of the four reduced mucin subunit populations

The four reduced mucin subunit populations (I–IV) were electrophoresed (2 µg/lane) in a 1% (w/v) agarose gel and subsequently blotted on to nitrocellulose as described in the Experimental section. The blot was probed with an antiserum raised against reduced mucin subunits as described in the legend to Figure 2.

These four electrophoretically homogeneous populations of subunits were then subjected to compositional analyses and their M_r values and sizes determined.

Mucin subunit characterization

The monosaccharide composition of the four mucin subunit populations is presented in Table 1(a). Populations I and II have similar compositions that differ from those of III and IV, which are also similar to each other. Populations I and II are significantly richer in fucose and galactose and relative to *N*-acetylgalactosamine have a higher monosaccharide content, suggesting they have longer oligosaccharide chains. Populations III and IV have a lower neutral sugar content but more sialic acid, consistent with their higher charge density. Amino acid analysis (Table 1b) shows that populations I and II are similar, indicating they share a common apoprotein. Populations III and IV have a different composition from populations I and II and are less alike than the former subunits, suggesting that they may contain

Table 1 Compositional and physical data on the reduced mucin subunit populations

Abbreviations: nd, not detected; R_G , radius of gyration.

(a) Monosaccharide composition (per mol of GalNAc)								
Monosaccharide	Subunit population ... I	II	III	IV				
GalNAc	1.0	1.0	1.0	1.0				
Fucose	4.0	5.9	1.6	1.8				
GlcNAc	4.1	5.1	1.9	2.5				
Gal	5.2	9.1	3.4	3.6				
Sialic acid ^a	2.0	2.8	4.0	5.0				
(b) Amino acid composition (residues/1000)								
Amino acid	Subunit population ... I	II	III	IV	Sequenced mucins ...	MUC1 ^b	MUC2 ^b	MUC7 (11)
Asx	57	50	65	68		73	46	56
Glx	55	11	60	20		52	61	73
Ser	108	112	138	94		224	47	123
Gly	50	49	48	47		52	55	8
Thr	305	335	253	218		122	349	171
Ala	104	93	72	68		78	23	118
Pro	117	124	116	95		92	158	210
His	21	23	20	18		27	15	34
Arg	13	19	18	15		21	17	31
Tyr	8	9	15	23		27	17	8
Val	32	32	36	45		75	53	31
Met	13	9	nd	17		8	7	6
Cys	7	15	nd	nd		5	41	6
Ile	19	20	32	34		28	36	22
Leu	33	33	33	44		62	32	48
Phe	17	17	19	25		33	15	17
Lys	41	47	78	168		19	21	39
Trp	nd	nd	nd	nd		6	2	0
(c) Physical data								
	Subunit population ... I	II	III	IV				
M_r ($\times 10^{-6}$)	2.5	2.5	2.2	2.8				
R_s (nm)	57	56	57	69				

^a Expressed as % dry weight.

^b Data obtained from the SWISSPROT database.

Table 2 Analysis of reduced mucin subunit populations with antisera and antibodies recognizing mucin apoproteins

Mucin subunits (250 ng/well in duplicate) were transferred to PVDF by slot blotting. The membrane was treated with TFMSA as described in the Experimental section and then incubated in (a) for 2 h and (b) 6 h with the above antisera or antibodies as described in the legend to Figure 2. The blots were visualized using a horseradish peroxidase-conjugated secondary antibody and the ECL Western detection kit with a 15 min exposure (—, no signal; +, weak signal; + + + + +, strongest signal).

Mucin	Antiserum	Untreated subunit				TFMSA-treated subunit			
		I	II	III	IV	I	II	III	IV
(a) Non-VNTR probes	MUC2								
	LUM2-3	+	+	+	+	+	+	+	+
MUC5AC	LUM5-1	—	—	++++	+++	—	—	++++	+++
(b) VNTR probes									
MUC1									
	BC2	—	—	—	—	+	+	+	+
MUC2	C595	—	—	—	—	—	—	—	—
	4F1	—	—	—	—	—	—	—	—
	996	—	—	—	—	—	—	—	—
MUC3									
	M3.2	±	±	±	±	+	+	+	+
	M3.3	±	±	±	±	+	+	+	+
MUC4	1143/B7	—	—	—	—	—	—	—	—
	PDM4	—	—	—	—	+	+	+	+
	LUM4	—	—	—	—	+	+	+	+
MUC5AC									
	PDM5	—	—	—	—	+	+	+	+

Figure 5 Identification of mucin apoproteins

Mucin subunits from the four populations (I–IV) were electrophoresed (2 µg/lane) in 1% (w/v) agarose gels, blotted on to nitrocellulose and the blots probed in (a) with the anti-(non-VNTR MUC5AC) serum (LUM5-1; 1:2000 for 2 h) and (b) with the anti-(non-VNTR MUC2) serum (LUM2-3; 1:1000 for 5 h). The blots were treated as described in the legend to Figure 2 and bands visualized with the ECL Western detection kit with a 5 min exposure in (a) and a 15 min exposure in (b).

different mucin apoproteins. M_r and size data (Table 1c) show that subunit populations I and II are comparable with regard to these parameters. In contrast the molecules in populations III and IV have a difference in both size and M_r . In summary, the data indicate that the populations contain three different apoproteins and that the major component (populations I and II) occurs in different glycoforms.

Identification of mucin core proteins

Non-VNTR probes

Slot blots of the four subunit populations were analysed with two polyclonal antisera that recognized protein epitopes outside the

Figure 6 Comparison of reactivity of the two non-VNTR antisera

Reduced mucin subunits from the MUC5AC-rich population III (●) and human colon (○) and BSA (■) were slot blotted on to nitrocellulose (500, 250, 100, 50, 25, 10 and 1 ng/well). The blots were probed for 2 h in (a) with the anti-(non-VNTR MUC5AC) serum (LUM5-1, 1:2000) and (b) with the anti-(non-VNTR MUC2) serum (LUM2-3, 1:1000) as described in the legend to Figure 2 and bands were visualized using the ECL Western detection kit with a 3 min exposure. The integrated intensity data were obtained by densitometry as described in Thornton et al. [30].

VNTRs of MUC2 and MUC5AC. In both cases removal of O-glycans was not a pre-requisite for their reactivity (Table 2a). The anti-MUC2 serum was reactive at a low level with all four

Figure 7 Anion-exchange HPLC of reduced mucin subunits

Reduced subunits prepared from (a) asthmatic (approx. 13 mg), (b) pooled normal (approx. 750 µg) and (c) chronic bronchitic mucins (approx. 800 µg) were chromatographed on a Mono Q HR 5/5 column as described in the Experimental section. The nominal gradient is indicated (dotted line). Column fractions were assayed, after immobilization on to nitrocellulose by slot blotting, for carbohydrate with the PAS assay (solid line) and probed with the anti-(non-VNTR MUC5AC) serum (LUM5-1; 1:2000, broken line) as described in the legend to Figure 2 with the exception that the secondary antibody was alkaline phosphatase-conjugated, and NitroBlue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were employed as the chromogenic substrate. The integrated intensity is defined as in Thornton et al. [30].

populations. In contrast, the anti-(non-VNTR MUC5AC) serum gave a strong positive signal with subunit population III and to a lesser extent with population IV, but no reaction was observed with either subunit populations I or II (Table 2a).

VNTR probes

Slot blots of the four populations were also analysed with a range of antibodies and antisera recognizing the VNTR of MUC1–5 (Table 2b). Before removal of O-glycans the majority of these reagents, with the exception of the MUC3 probes (M3.2 and 3.3), were unreactive. However, after removing the O-glycans a weak reactivity was observed for all four populations with probes for MUC1, 3, 4 and 5AC. In contrast, none of the MUC2 probes was reactive.

To determine whether the observed reactivities were with the major electrophoretic bands, this panel of probes were used to analyse Western blots of agarose gel electrophoretic separations of the four populations. The strongly reactive MUC5AC probe visualized a band corresponding to the position of subunit III

Table 3 Analysis of respiratory mucin preparations with antisera and antibodies recognizing mucin apoproteins

For experimental details see legend to Table 2 (—, no signal; +, weak signal; + + + + +, strongest signal).

Mucin	Antisera/antibodies	Normal	Chronic bronchitic
MUC1	BC2	+	+
	C595	—	—
MUC2	4F1	—	—
	996	—	—
	LUM2-3	+	+
MUC3	M3.2	+	+
	M3.3	+	+
	1143/B7	—	—
MUC4	PDM4	+	+
	LUM4	+	+
MUC5AC	PDM5	+	+
	LUM5-1	+ + + + +	+ + + + +

and this was observed in both populations III and IV (Figure 5a). The reactivity observed in population IV is likely to be a consequence of cross-contamination at the final pooling. In contrast, the weakly reactive probes (Table 2) detected all four of the subunit bands and in addition the bands were of similar intensity. A typical example of the results obtained, in this case using the anti-(non-VNTR MUC2) serum (LUM2-3), is shown in Figure 5(b).

Because of the cross-reactivity we observed with the different mucin antibodies we compared the response of one of the weakly reactive probes, the anti-(non-VNTR MUC2) serum (LUM2-3), with the highly reactive anti-MUC5AC serum (LUM5-1) using the MUC5AC subunits (population III), human colonic mucin subunits and BSA as antigens (Figure 6). The results demonstrate that MUC5AC subunits were primarily reactive with the MUC5AC probe while the colonic mucin subunits showed a similar behaviour with the MUC2 probe. Under the conditions of the experiment similar amounts of BSA gave no response (Figure 6a). A low level of reactivity was observed for the MUC2 probe with the MUC5AC-rich population III (Figure 6b) and the other subunit populations I, II and IV (results not shown). These experiments were performed using an ECL detection kit and it is important to note that the level of response could be amplified by a longer exposure of the blot to X-ray film (results not shown). Interestingly, under these circumstances the signal obtained with the mucin protein was significantly higher than that of a similar amount of BSA on the membrane (results not shown).

The anti-(non-VNTR MUC5AC) serum was then used to probe the original Mono Q chromatogram shown in Figure 1. A single peak of reactivity was eluted between fractions 32 and 41 (Figure 7a) and, as observed with the Western blot (Figure 5a), the major population of molecules did not react with this antiserum.

The MUC5AC mucin in respiratory secretions

Reduced mucin subunits, prepared from the respiratory secretions of 20 normal individuals and from an individual with

Figure 8 Agarose (1%) gel electrophoresis of respiratory mucin subunits

Reduced subunits (approx. 2.5 μ g/lane) prepared from chronic bronchitic (lanes 1 and 3) and pooled normal mucins (lanes 2 and 4) were electrophoresed in a 1% (w/v) agarose gel, subsequently blotted on to nitrocellulose and probed as described in the legend to Figure 2 with a polyclonal antiserum recognizing reduced mucin subunits (1:1000; lanes 1 and 2) and the anti-(non-VNTR MUC5AC) serum (LUM5-1, 1:2000; lanes 3 and 4).

chronic bronchitis, were examined for reactivity with the panel of anti-mucin probes (Table 3). As was the case with the asthmatic sample the most reactive one was the anti-(non-VNTR MUC5AC) serum (LUM5-1). However, while this antiserum was highly reactive with the normal mucins, a much weaker reactivity was observed with the chronic bronchitic mucins. The two subunit preparations were fractionated by ion-exchange chromatography and the distributions analysed with the PAS assay and the non-VNTR MUC5AC probe (Figure 7). As expected little MUC5AC reactivity was observed with the chronic bronchitic mucin subunits (Figure 7c), whereas a significant MUC5AC response was present with the pooled normal mucin subunits (Figure 7b). Furthermore, in both cases, the MUC5AC-reactive component was observed as a single peak in a similar elution position to that observed for the asthmatic subunits (Figure 7a). The major PAS-reactive peak in both samples showed no MUC5AC reactivity. A Western blot of these two reduced subunit preparations indicates that the faster-migrating of the two bands is the MUC5AC-positive subunit and no reactivity is observed with the slower migrating band (Figure 8) as was the case with the asthmatic mucin subunit preparation (Figure 5a).

DISCUSSION

By using rate-zonal centrifugation and agarose electrophoresis we have previously identified two major populations of mucins dominating respiratory secretions [4]. Using a combination of anion-exchange HPLC on Mono Q and agarose electrophoresis we have shown here that there are at least three populations of mucins contributing to the secretions, and in addition the major component (79% by weight) exists in different glycoforms.

The reduced subunits of these three mucins have values for size and M_r that are in good agreement with those determined previously for reduced subunits from cervical mucins [28]. There is a substantial difference in M_r (compare 2.2×10^6 with 2.8×10^6) and size (compare 57 with 69 nm) between the smallest and largest populations but gel chromatography cannot distinguish between them (results not shown). Recently we have shown that agarose gel electrophoretic separation of reduced mucin subunits is largely dependent on their charge density [18]. However, in the present study the most anionic subunit population (IV) is not the most mobile in electrophoresis. This apparently anomalous electrophoretic behaviour could be explained by the larger size of these subunits (R_g 69 nm) compared with the others (R_g 57 nm).

In order to establish the genetic identity of the apoproteins of the three different mucins, the subunit populations were screened

with mucin-specific antibodies or antisera. Some of the probes, particularly those to the VNTR of MUC2, proved negative in our assays. The lack of reactivity with the MUC2 probes was not surprising since our amino acid composition data would not implicate MUC2 as a major component in the preparation. However, many of the other antibodies and antisera raised against, or reactive with, synthetic peptides from the VNTR of MUC1, 3, 4 and 5AC and also from the non-VNTR of MUC2 were weakly reactive with all subunit populations, and Western blotting indicated that these probes did not discriminate between them. It is noteworthy that the response obtained with these latter probes could be amplified above that of a non-mucin protein control by extending the time of the ECL development. The conclusion from these results is either that each of these populations contains multiple apoproteins, or, as seems more likely, that there is a low level of cross-reactivity with the antibodies and antisera after TFMSA treatment, even though these probes appear to be stringent in their reactivity against synthetic peptides. In contrast, the polyclonal antiserum (LUM5-1) raised against a non-VNTR sequence of the MUC5AC apoprotein did discriminate between the subunit populations and identified one of them (population III) as the MUC5AC mucin.

In the asthmatic sample the major mucin (populations I and II, 79% by weight) and the minor component IV (> 7% by weight) were not recognized by the anti-MUC5AC serum. Further evidence that these populations comprise different apoproteins is indicated by their distinct amino acid compositions. At present we are unable to establish if these apoproteins are the products of known or as yet unidentified mucin genes. Population IV contains two apoproteins, one of which is the MUC5AC polypeptide. In comparison with the MUC5AC subunits the other component is more retarded on Mono Q, has a higher M_r and size, a slightly lower electrophoretic mobility and a higher buoyant density in CsCl (D. J. Thornton, M. Howard and J. K. Sheehan, unpublished work).

Preparations of reduced mucin subunits isolated from respiratory secretions of 20 normal individuals and from an individual with chronic bronchitis gave similar data to that of the asthmatic sample when analysed with the anti-VNTR probes. The anti-(non-VNTR MUC5AC) serum was again the only discriminatory probe and suggests that the MUC5AC mucin is a major component in 'normal' secretions, whereas in this particular chronic bronchitic patient it is only a minor component. In both cases, the genetic identity of the other major component(s) in the preparation was not determined. Interestingly, the MUC5AC reduced subunit from the normal, asthmatic and chronic bronchitic secretions appears as a similar biochemical entity, as assessed by Mono Q chromatography and agarose electrophoresis. This suggests that the MUC5AC apoprotein may have a defined pattern of glycosylation associated with it in the respiratory tract. However, the functional relevance of this finding is unknown.

Light scattering measurements on the MUC5AC reduced subunit yields an M_r of approx. 2.2×10^6 . Assuming a carbohydrate content of 80% (w/w) we would predict a polypeptide of M_r about 440 000. This M_r is consistent with the size of MUC5AC mRNA (> 12 kb) estimated by Northern blot analysis [29].

In conclusion, we have identified the MUC5AC mucin as one of the major components in normal respiratory secretions. Biochemically the MUC5AC mucin appears to be similar in different respiratory secretions, but its level varies. It will be of great interest, in the future, to determine, for instance, whether the level of the MUC5AC mucin in secretions can be linked to

any pathological process and what effect its level has on the properties of the protective biofilm. The genetic identity of the other major mucins in the secretions has yet to be established. However, amino acid composition and molecular size data suggest they are not products of the *MUC1*, *MUC2* or *MUC7* genes.

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