Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells

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Mucins were extracted from the epithelial surface and the submucosal tissue of human trachea in order to enrich glycoproteins from the goblet cells and the submucosal glands respectively. The macromolecules were purified using density-gradient centrifugation, and the presence of the MUC5AC mucin was investigated using an antiserum raised against a synthetic peptide based on the sequence of the MUC5AC apoprotein. Mucins from the surface epithelium showed a higher reactivity with the antiserum relative to carbohydrate than those from the submucosa, and ion-exchange HPLC of reduced subunits revealed the presence of two distinct mucin populations in the samples. The predominant species from the surface epithelium was more acidic than the major population from the submucosa and showed a strong reactivity with the anti-MUC5AC antiserum. In contrast, the major portion of the submucosal mucins were less acidic and showed no MUC5AC reactivity, although a more acidic population did react with the antibody. Rate-zonal centrifugation showed that the MUC5AC mucin from the surface epithelium is smaller than the major submucosal mucin, and that both are composed of subunits. Immunolocalization confirmed that the MUC5AC mucin from human trachea originates from the goblet cells and that this glycoprotein is not a major product of the submucosal glands.

INTRODUCTION

Mucus is an essential component of the protective biofilm covering the mucosal surfaces of the body. The polymer matrix of mucus is provided by large glycoproteins which in the airways are produced by two different secretory cell populations: the surface epithelial goblet cells and the mucous cells in the submucosal glands. Airway diseases such as chronic bronchitis, cystic fibrosis and asthma are characterized by mucus hypersecretion, but it is not possible to evaluate the relative contributions to mucus secretion of the goblet cells and the submucosal glands. Since the two cell types are under distinctly different physiological control, such information might be vital for understanding the pathological processes.

The gel-forming mucins from the airways are large macromolecules [molecular masses of $(10–30) \times 10^6$ Da] composed of subunits linked end-to-end by disulphide bonds [1–3], and recent biochemical studies have indicated the presence of at least two different airway mucins [4]. Regions rich in serine, threonine and proline residues (STP-rich regions) alternate with stretches with a more 'conventional' amino acid composition. The STP-rich regions are densely substituted with O-linked oligosaccharides, and protease digestion gives rise to high-molecular-mass glycopeptides corresponding to these highly glycosylated domains [5]. cDNA cloning studies have revealed several different human mucin genes (*MUC1–MUC8*), and three of these (*MUC1*, *MUC2* and *MUC7*) have been sequenced completely (for a review, see [6]). In most mucins, the STP-rich regions contain characteristic tandemly repeated motifs.

MUC4, *MUC5AC* and *MUC5B* have been cloned from cDNA libraries from human tracheobronchial tissue [7–9]. Hybridization *in situ* shows that MUC1, MUC2, MUC4,

MUC5AC and MUC5B apoproteins [10,11] are expressed in the airways, but it is not known which mucins are the predominant ones in secretions or which secretory cells they originate from. Here we have isolated mucins from the surface epithelium and the submucosa of human airways and shown that the MUC5AC mucin is produced by the goblet cells. In addition, a major mucin population from the submucosal tissue has been identified.

EXPERIMENTAL

Materials

Di-isopropyl phosphorofluoridate (DFP), CHAPS and guanidinium chloride (practical grade) were purchased from Fluka. Stock solutions (approx. 8 M) of guanidinium chloride were treated with activated charcoal and filtered through a PM10 filter (Amicon) before use. N-Ethylmaleimide was bought from BDH Chemicals, dithiothreitol was from Merck, BSA was from Serva, and 3′,3′-diaminobenzidine hydrochloride and iodoacetamide were from Sigma Chemical Co. Alkaline-phosphatase-conjugated swine anti-rabbit serum was purchased from Dakopatts. The Mono Q HR 5/5 column was obtained from Pharmacia. All other reagents were of AnalaR or equivalent quality.

Analytical methods

Fractions from density gradients were analysed for sialic acid using an automated procedure [3]. Aliquots from fractions from density gradients, rate-zonal gradients and ion-exchange HPLC were blotted on to nitrocellulose membranes and assayed for
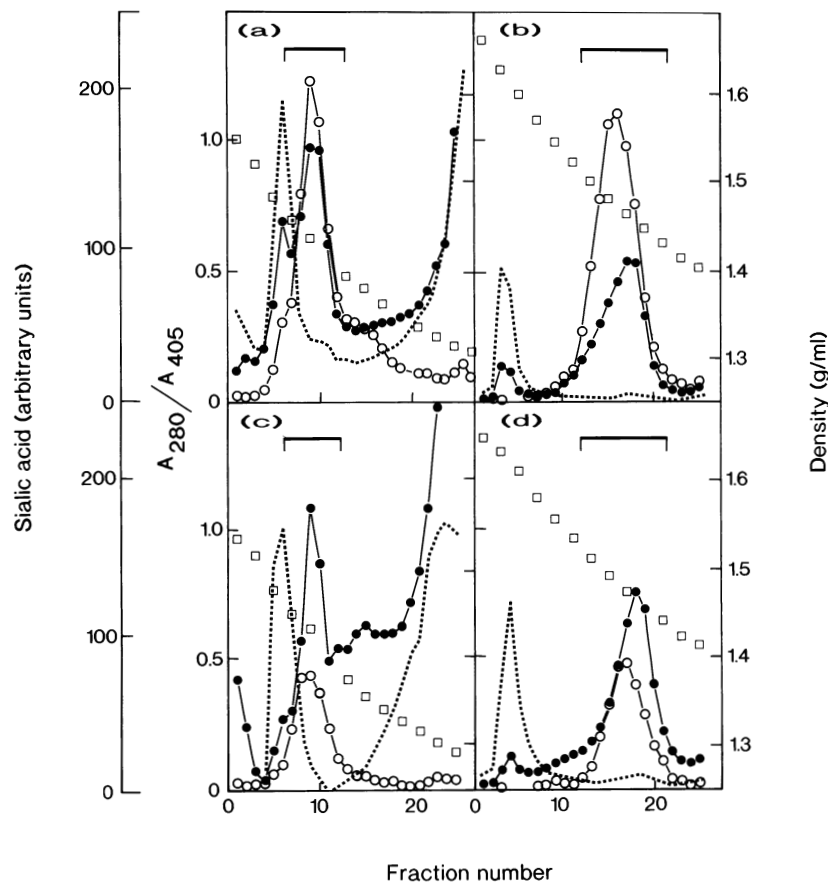

Figure 1   Isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride (a, c) and CsCl/0.5 M guanidinium chloride (b, d) of mucins extracted from the surface epithelium (a, b) and the submucosal tissue (c, d) of human trachea

Material extracted in 6 M guanidinium chloride, 10 mM sodium phosphate buffer, pH 6.5, containing 5 mM Na_2EDTA, 5 mM N-ethylmaleimide and 1 mM DFP from the surface epithelium (**a**, **b**) and the submucosal tissue (**c**, **d**) of human trachea was subjected to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride (36 000 rev./min, 15 °C, 80 h, Beckman 50.2Ti rotor, Beckman Optima L-70 ultracentrifuge). Fractions containing the major mucin populations were pooled as shown by the horizontal bars and re-centrifuged in CsCl/0.5 M guanidinium chloride containing 0.01 % CHAPS. After centrifugation, fractions were analysed for density (□), sialic acid (●), A_{280} (broken line) and reactivity with the LUM5-1 antiserum (○).

carbohydrate with the periodic acid/Schiff (PAS) reagent [12]. Density was measured using a Carlsberg pipette as a pycnometer.

Preparation of the anti-MUC5AC serum

A polyclonal antiserum (LUM5-1) was raised in rabbits against a synthetic peptide with the sequence RNQDQQGPFKMC present in the C-terminal portion [13,14] and in the two stretches flanking a tandem repeat region [15] of the MUC5AC apoprotein. The peptide was conjugated with keyhole limpet haemocyanin, and rabbits were initially injected intracutaneously with 370 μg of peptide mixed with Freund's complete adjuvant. After 3 weeks, a booster injection of 250 μg of peptide mixed with Freund's incomplete adjuvant was given, and the rabbits were bled 2–3 weeks later.

ELISA analysis

Fractions from isopycnic and rate-zonal gradients and from ion-exchange chromatography (maximum mucin concentration approx. 1 μg) were coated on to multiwell assay plates (Falcon) overnight at 4 °C. The plates were blocked with 0.15 M NaCl, 5 mM sodium phosphate buffer, pH 7.4, containing 0.05 %

Tween 20 and 0.5 % BSA (blocking solution), followed by incubation with LUM5-1 antiserum diluted 1:2000 in blocking solution. Reactivity with the LUM5-1 antiserum was detected with an alkaline-phosphatase-conjugated secondary antibody using nitrophenyl phosphate as a substrate. Reactivity is expressed as absorbance at 405 nm after 1 h.

Isolation of mucins from the surface epithelium and the submucosal tissue

Human tracheal tissue obtained at autopsy from a single individual was rinsed with 0.15 M NaCl/10 mM sodium phosphate buffer, pH 7.4 (PBS), and cut longitudinally into halves. The luminal surface was removed by scraping the mucosa with a glass microscope slide, after which the submucosal tissue was dissected away from the underlying cartilage. The samples, referred to below as 'surface epithelium' and 'submucosal tissue' respectively, were frozen immediately in liquid nitrogen and the submucosal tissue was disintegrated using a tissue pulverizer (Retsch) before extraction.

Samples were thawed slowly in the presence of DFP to give a final concentration of 1 mM prior to extraction with ice-cold 6 M guanidinium chloride, 10 mM sodium phosphate buffer, pH 6.5,

containing 5 mM Na_2EDTA and 10 mM N-ethylmaleimide (extraction buffer). The surface epithelium was suspended with a Dounce homogenizer (loose pestle; three strokes), whereas the submucosal tissue was dispersed by passing the material through a syringe. Both samples were then stirred gently overnight at 4 °C prior to being spun in a Beckman J2-MC 20 centrifuge (JA 20 rotor; 18000 rev./min; 4 °C; 45 min). The resulting pellets were re-extracted twice in extraction buffer and supernatants from sucessive extractions were pooled. Material which had not been solubilized after three extractions was treated with 6 M guanidinium chloride, 10 mM Tris/HCl buffer, pH 8.0, containing 5 mM Na_2EDTA and 10 mM dithiothreitol for 5 h at 37 °C, and alkylated with 25 mM iodoacetamide (15 h in the dark at room temperature). The samples were then centrifuged as outlined above and the supernatants retained.

Samples were subjected to isopycnic density-gradient centrifugation in a Beckman Optima L-70 ultracentrifuge in CsCl/4 M guanidinium chloride (36000 rev./min; 15 °C; 80 h; Beckman 50.2Ti rotor; initial density 1.39 g/ml). Fractions containing mucins (see Figures 1a and 1c) were pooled and subjected to a second isopycnic density-gradient step in CsCl/0.5 M guanidinium chloride as above, but containing 0.01 % CHAPS and with an initial density of 1.50 g/ml.

Ion-exchange HPLC of mucin subunits

Reduced subunits were prepared from whole mucins by treatment with 6 M guanidinium chloride, 10 mM Tris/HCl buffer, pH 8.0, containing 5 mM Na_2EDTA and 10 mM dithiotheitol for 5 h at 37 °C and subsequent alkylation with 25 mM iodoacetamide (15 h in the dark at room temperature). Subunits were then dialysed against 6 M urea, 10 mM piperazine/perchlorate buffer, pH 5.0, containing 0.1 % CHAPS (starting buffer) and subjected to ion-exchange HPLC on a Mono Q HR 5/5 column as described in [3]. The column was eluted at 0.5 ml/min with starting buffer for 10 min followed by a linear gradient of $LiClO_4$ to a final concentration of 0.4 M $LiClO_4$ in starting buffer. Fractions were analysed for carbohydrate using the PAS procedure and for reactivity with the LUM5-1 antiserum.

Rate-zonal centrifugation

Centrifuge tubes (12.5 ml) were loaded from the bottom with linear gradients of 6–8 M guanidinium chloride, and mucin solutions (100–200 μl) were layered on top of the gradients [16]. Centrifugation was performed in a Beckman Optima L-70 ultracentrifuge (SW40.1Ti rotor; 2 h 45 min; 40000 rev./min; 20 °C) and the tubes were emptied from the top to give 500 μl fractions. Fractions were analysed for carbohydrate using the PAS procedure and for reactivity with the LUM5-1 antiserum.

Immunohistochemistry

Small pieces of tissue were removed from the trachea prior to scraping and fixed in 10 % neutral buffered formol saline overnight. The pieces were dehydrated, embedded in paraffin and 4 μm sections cut. Sections were dewaxed in xylene, rehydrated and either stained with Alcian Blue at pH 2.5 followed by the PAS reagent, or treated with 10 mM citrate buffer, pH 6.0, at 100 °C for 10 min prior to treatment with LUM5-1 or the cognate preimmune antiserum (diluted 1:2000). Antibody binding was detected using the DAKO StreptABComplex/HRP kit (Dakopatts AB) with 3′,3′-diaminobenzidine tetrahydrochloride as the substrate.

RESULTS AND DISCUSSION

Mucins were isolated from the surface epithelium and the submucosa separately in order to enrich samples in glycoproteins from the goblet cells and the submucosal glands respectively. In CsCl/4 M guanidinium chloride, mucins from both the surface epithelium and the submucosa were found between the buoyant densities of 1.40 and 1.45 g/ml (Figures 1a and 1c), partially separated from material with a high absorbance at 280 nm at a density consistent with that of DNA [17]. Analysis for sialic acid coincided with reactivity against the LUM5-1 antiserum, and analysis for carbohydrate, as determined with the PAS method, followed that for sialic acid (results not shown). Although mucins from the surface epithelium and the submucosal tissue occurred at similar buoyant densities, the ratio of reactivity with the LUM5-1 antiserum to the amount of sialic acid was much greater for the former, suggesting that the MUC5AC mucin was more prevalent in the surface epithelium. In the surface epithelium, most of the material (80 %) was soluble in 6 M guanidinium chloride buffer, whereas in the submucosal tissue the proportion was lower (65 %). The subunits obtained after reduction/alkylation of the extraction residues from both sources did not differ in buoyant density or antibody reactivity from the mucins in the corresponding soluble material (results not shown).
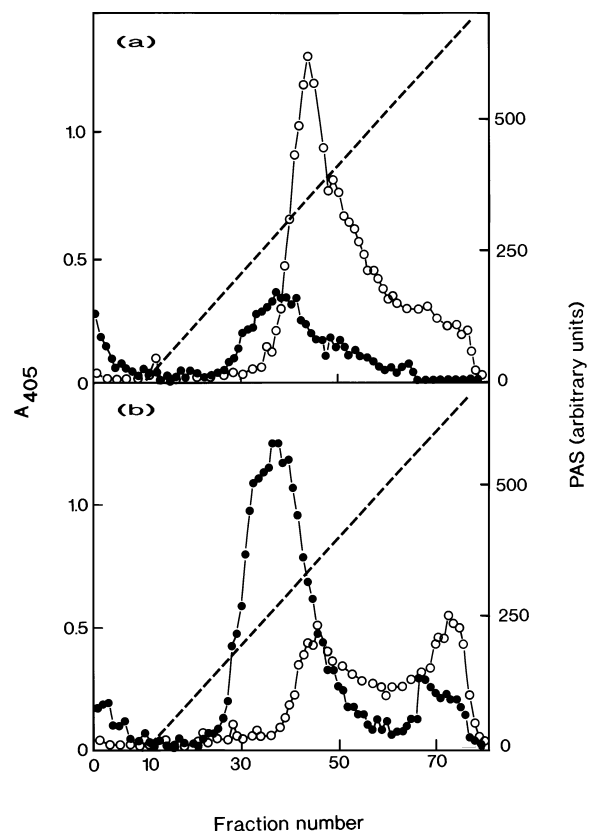

Figure 2   Ion-exchange HPLC of reduced subunits of mucins from the surface epithelium (a) and the submucosal tissue (b) of human trachea

Subunits prepared from whole mucins from (**a**) the surface epithelium and (**b**) the submucosal tissue of human trachea by reduction/alkylation were dialysed against 6 M urea, 10 mM piperazine/perchlorate buffer, pH 5.0, containing 0.1 % CHAPS (starting buffer) and loaded on to a Mono Q column. The column was eluted with a linear gradient of $LiClO_4$ (broken line; final concentration 0.4 M) in starting buffer. Fractions (500 μl) were analysed for PAS reactivity (●) and reactivity with the LUM5-1 antiserum (○).
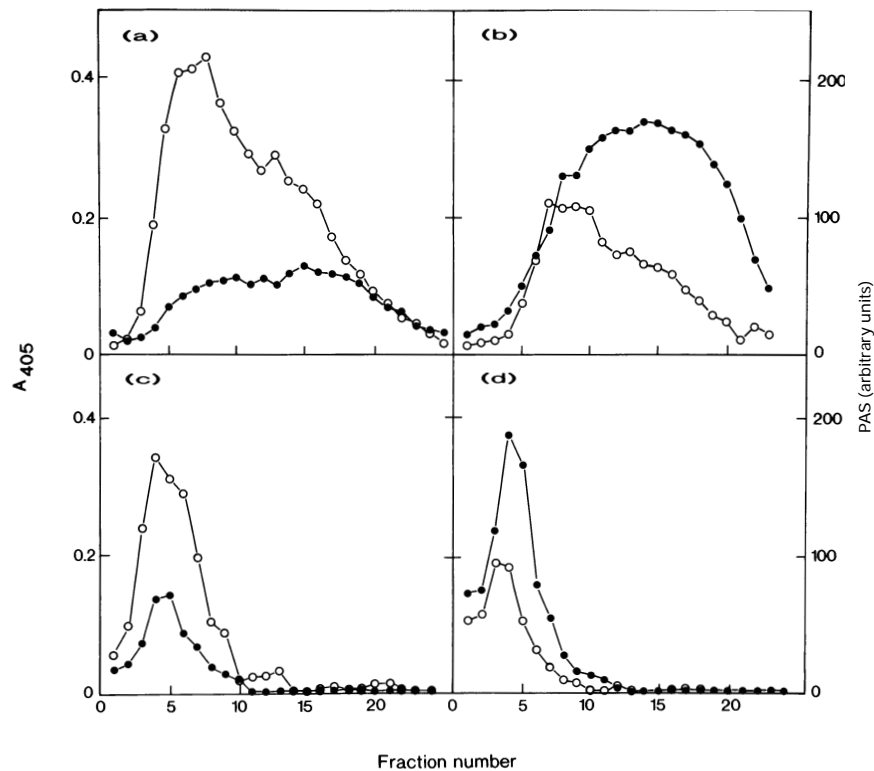
Figure 3 Rate-zonal centrifugation of whole mucins (a, b) and reduced subunits (c, d) from the surface epithelium (a, c) and the submucosal tissue (b, d) of human trachea

Aliquots (100–200 μl) of whole mucins and reduced subunits from the surface epithelium and the submucosal tissue of human trachea were subjected to rate-zonal centrifugation (40 000 rev./min, 20 °C, 2 h 45 min, Beckman SW40.1Ti rotor, Beckman Optima L-70 ultracentrifuge). The tubes were emptied from the top into 500 μl fractions and analysed for PAS reactivity (●) and reactivity with the LUM5-1 antiserum (○).

Mucins were pooled as shown by the horizontal bars in Figures 1(a) and 1(c) and re-centrifuged in CsCl/0.5 M guanidinium chloride. In both the surface epithelial and submucosal extracts, the major mucin peak was found between the densities of 1.43 and 1.50 g/ml, well separated from DNA at a density of 1.62 g/ml (Figures 1b and 1d). The ratio of reactivity with the LUM5-1 antiserum to the amount of sialic acid was again much higher in the surface epithelial than in the submucosal gland mucins. In both samples, the distribution of MUC5AC reactivity was shifted slightly towards a higher buoyant density with respect to sialic acid.

Ion-exchange HPLC of reduced subunits revealed distinct differences between mucins from the surface epithelium and the submucosal tissue (Figures 2a and 2b). LUM5-1 reactivity was greater relative to PAS in the surface epithelium than in the submucosal tissue, suggesting that the MUC5AC mucins are enriched in the goblet cells. In contrast, a less 'acidic' PAS-reactive population, eluting ahead of the MUC5AC mucins, predominated in the submucosal tissue. Furthermore, in the submucosal tissue, a small but distinct population of LUM5-1-reactive mucins appeared late in the chromatogram, suggesting the presence of a more acidic glycoform of MUC5AC.

Mucins from both the surface epithelium and the submucosa showed a broad range of molecular sizes, as studied with rate-zonal centrifugation (Figures 3a and 3b). Reactivity with the LUM5-1 antiserum was more pronounced at the lower end of the molecular size distribution and was much greater relative to carbohydrate in mucins from the surface epithelium than in

those from the submucosa. Reduced subunits were found as a narrow band at the lower end of the molecular mass distribution, indicating that mucins from both sources are composed of subunits held together by disulphide bonds.

Sections of human trachea were stained with Alcian Blue, pH 2.5/PAS or the LUM5AC-1 antiserum and viewed with Nomarski optics to enhance the delineation of the gland cells (Figure 4). The sections stained avidly with Alcian Blue, pH 2.5/PAS, revealing the presence of mucins both in the goblet cells in the surface epithelium and in the submucosal glands (Figure 4a). Staining with the antiserum was intense over the surface epithelial goblet cells (Figure 4b) and in goblet cells at the neck of submucosal gland ducts (results not shown). Within the goblet cells, staining was associated with the secretory granules. There was some weak staining over occasional submucosal glands (Figure 4c), but control sections treated with the pre-immune serum showed a similar reactivity (results not shown). The reactivity of the antiserum with the tissue sections was decreased, and finally abolished, by immunoprecipitation of the antiserum with increasing concentrations of purified respiratory mucins, suggesting that the antiserum reacted specifically with mucins (results not shown). The staining pattern thus indicates that the MUC5AC mucin originates mainly from the surface epithelial goblet cells and that this mucin is not a major secretory product of the submucosal glands. However, a distinct sub-population of MUC5AC mucins appearing as a retarded peak on ion-exchange HPLC was enriched in the submucosal tissue (Figure 2b), suggesting that a small amount of a highly acidic
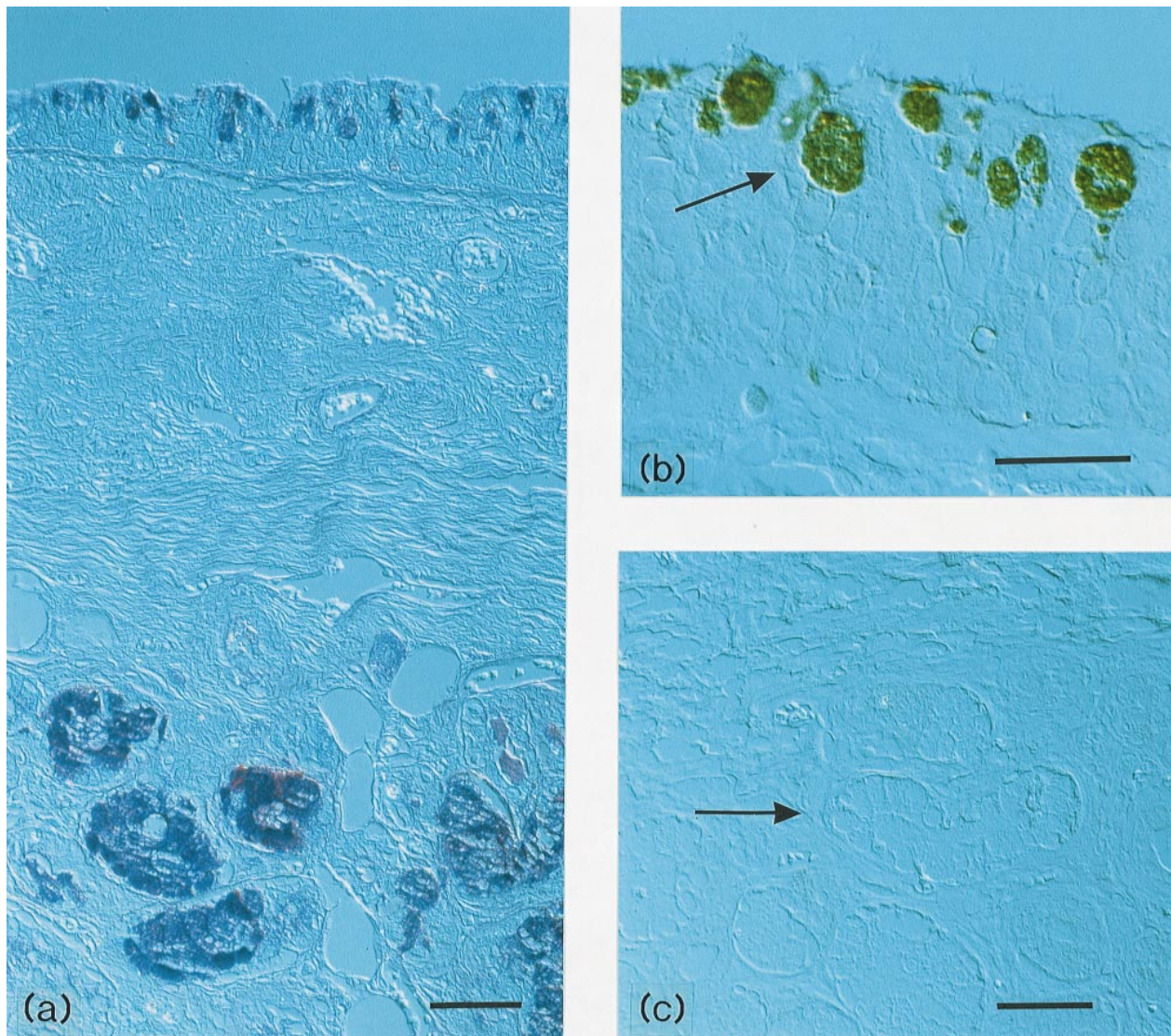
Figure 4   Sections of human trachea showing the surface epithelium and the submucosal glands stained with Alcian Blue, pH 2.5/PAS (a), and the surface epithelium (b) and submucosal tissue (c) stained with LUM5-1 antiserum

Sections (4 μm) of paraffin-embedded human trachea were stained with Alcian Blue, pH 2.5/PAS (**a**) or the LUM5-1 antiserum (**b**, **c**) as described in the text. Sections were viewed with a Nikon Optifot microscope using Nomarski optics. The bars represents 50 μm (**a**, **c**) and 25 μm (**b**). The arrows point at a goblet cell (**b**) and a submucosal gland (**c**).

glycoform of MUC5AC might be produced by the submucosal glands.

General discussion

Although previous studies have shown that several mucin genes are expressed in the human respiratory tract [10,11], there is currently little information about which apoproteins are present in the predominant mature mucins. Furthermore, it is not clear whether, for example, the goblet cells and submucosal glands produce the same, or different, mucin apoproteins. Finally, since secretory cells synthesizing the same apoprotein may express different glycosyltransferases, mucin glycoforms may be present. Since questions such as these can only be addressed by isolating mucins from the appropriate tissue and separating them into populations, mucins were prepared separately from the surface

epithelium and the submucosal tissue of human trachea so as to enrich the samples in secretory products from the goblet cells and the submucosal glands respectively.

Two major mucin populations were identified using ion-exchange HPLC. The more acidic population showed strong LUM5-1 reactivity and was enriched within the surface mucosa, whereas the other was less acidic, did not react with the antiserum and was the predominant mucin in the submucosal tissue. Both mucins were large and composed of subunits. Previous studies have also identified two major populations of mucins in respiratory tract secretions [4].

Immunolocalization showed that the MUC5AC mucin is produced by the goblet cells in the airways, and the presence of the mature MUC5AC mucin thus coincides with the major site of MUC5AC expression, as shown by *in situ* hybridization [18]. The identification of a small population of a more acidic

glycoform of the MUC5AC mucin in the submucosal tissue is compatible with a much lower expression level of MUC5AC in the glands. Furthermore, goblet cells may extend into the gland ducts, providing an additional possible source of MUC5AC mucins in the submucosal extract. In addition to MUC5AC, MUC1, MUC2, MUC4 and MUC5B are also expressed in airway tissue. MUC1 is a glycoprotein associated with the cell membrane [19] and does not belong to the family of large secreted mucins being studied here. An antiserum raised against a sequence in the MUC2 apoprotein [20] showed no reactivity with mucins from either the surface epithelium or the submucosal tissue (results not shown). cDNA probes for MUC4 have been shown to label the epithelium and the gland ducts but not the glandular acini, whereas a MUC5B probe labelled the mucous cells in the submucosal glands [11]. Thus MUC5B is a possible candidate for being the major mucin from the submucosal glands.

The goblet cells and the submucosal glands are regulated by different physiological mechanisms. The former secrete mainly in response to direct irritation by gases, bacterial proteinases, inflammatory mediators and luminal ATP [21–24], whereas discharge from the glands is under neural control [25]. The ability to identify mucins from the goblet cells and the submucosal gands in secreted mucus will allow us to categorize hypersecretory diseases such as chronic bronchitis, cystic fibrosis and asthma in terms of the malfunction of particular secretory cell types. This would be a major advance in our understanding of these diseases and thus in the quest for more specific treatments.

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