# Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B

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Respiratory mucus contains a mixture of gel-forming mucins but the functional significance of these different mucin species is unknown. To help gain a better understanding of mucus in airways we therefore need to ascertain the concentration of each of the gel-forming mucins within respiratory secretions. Thus the aim of this study was to determine the amounts of specific gel-forming mucins directly from solubilized secretions of the airways and purified mucin preparations. We investigated the feasibility of using direct-binding ELISA employing mucin-specific antisera but were unable to obtain reliable data owing to interference with the immobilization of the mucins on the assay surface by 6 M urea and high levels of non-mucin proteins. We therefore developed an alternative approach based on quantitative Western blotting after agarose-gel electrophoresis, which was not subject to these problems. Here we demonstrate that this procedure provides reliable and reproducible data and have employed it to determine the amounts of the MUC2, MUC5AC and MUC5B mucins in saline-induced sputa from healthy airways and spontaneous sputa from asthmatic airways. Additionally we have used this procedure to analyse these glycoproteins in mucin preparations purified from cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) mucus. Our findings indicate that MUC5AC and MUC5B are the major oligomeric mucins and that airways mucus contains variable amounts of these glycoproteins. By contrast, the MUC2 mucin comprised, at most, only 2.5% of the weight of the gel-forming mucins, indicating that MUC2 is a minor component in sputum. Finally, we show that the amounts and glycosylated variants of the MUC5AC and MUC5B mucins can be altered significantly in diseased airways with, for instance, an increase in the low-charge form of the MUC5B mucin in CF and COPD mucus.

Key words: asthma, chronic obstructive pulmonary disease, cystic fibrosis.

## INTRODUCTION

Respiratory mucus is produced at a low level in healthy airways; however, in common diseases of the airways such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) mucus is overproduced, leading to impaired lung function. The physical properties of mucus are due in large part to the high-molecular-mass oligomeric gel-forming mucins. The mRNA species of three known members of this family (MUC2, MUC5AC and MUC5B) are expressed in the airways [1]. Airways mucus should therefore contain a mixture of these mucins but as yet there has been no information on the amounts of the individual mucins in secretions. However, biochemical analyses have indicated that the MUC5AC and MUC5B glycoproteins are major gel-forming mucins in both normal and pathological secretions [2–8] but, in contrast, only small amounts of MUC2 have been found, in particular in CF sputum [9].

In normal airways the syntheses of the two major oligomeric mucins (MUC5AC and MUC5B) are spatially separated. The MUC5AC mucin is produced by the goblet cells in the surface epithelium [2], whereas the MUC5B mucin, which is found in differently charged forms (previously termed the low-charge and high-charge glycoforms), is produced predominantly by mucous cells in the submucosal glands [5,6,8,10]. Limited immunohistochemical staining for MUC5B has also been reported in some surface epithelial goblet cells and furthermore a more sulphated form of this mucin, which might represent the high-charge MUC5B variant, has been identified in a subpopulation of submucosal gland cells [8]. The composition of mucus can therefore be altered depending on the relative contribution to the secretion from these different cellular sources, thus providing a structural basis for tuning the properties of the mucus to a particular need.

Key features that affect the physical properties of the respiratory mucus gel are the amounts and types of gel-forming mucins present in the secretion. In pathological conditions, mucus from airways has been shown to have altered properties [11] and the component mucins can be changed in amount and type. For example, the thick viscid mucus plugging the airways of an individual in *status asthmaticus* had an increased mucin concentration (approx. 40 mg/ml [5]) compared with healthy secretions (approx. 0.6 mg/ml [12]). Furthermore, the mucin type responsible for the physical properties of this gel was a low-charge form of the MUC5B mucin [5]. Expression studies have demonstrated the up-regulation of both MUC2 and MUC5AC mRNA species in response to several disease-related factors such as bacterial products, cytokines and neutrophil elastase [13–15]. However, because mucins can be stored inside cells before secretion, and because of potential post-transcriptional regulation, mRNA studies might not be a good indicator of the quantities of specific mucins in the mucus gel. It is therefore important to be able to measure the amount of each of the gel-forming mucins in airways mucus.

Previous studies have attempted to measure the total mucin content in airways secretions by both chemical and antibody detection methods [12,16–18]. However, to our knowledge this is

Abbreviations used: BCIP, 5-bromo-4-chloroindol-3-yl phosphate; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; GdmCl, guanidinium chloride; PAS, periodate–Schiff.

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the first study to measure the amounts of the individual mucin gene products and their glycosylated variants in respiratory mucus. Using quantitative Western blotting after the separation of reduced mucins by agarose-gel electrophoresis we have been able to develop a reproducible and relatively simple method of analysing the amounts of the airways gel-forming mucins in sputa and purified mucin preparations.

## EXPERIMENTAL

### Materials

Periodate–Schiff (PAS) reagent, Nitro Blue Tetrazolium, 5-bromo-4-chloroindol-3-yl phosphate, guanidinium chloride (GdmCl), poly(ethylene glycol), BSA fraction V and CHAPS were all from Sigma Chemical Co. (Poole, Dorset, U.K.). Urea was purchased from BDH Laboratory Supplies (Poole, Dorset, U.K.). GdmCl (8 M) and urea (6 M) were treated with charcoal before use. Agarose UltraPURE (electrophoresis grade) was from BRL Gibco (Paisley, Renfrewshire, U.K.). Aquacide II was purchased from Calbiochem-Novabiochem Corp. (LaJolla, CA, U.S.A.) and enhanced chemiluminescence (ECL®) Western detection reagent was from NEN Life Science Products (Boston, MA, U.S.A.).

### Sputum collection

Mucus from the lower airways was collected by expectoration from subjects with disease of the airways who could cough sputum spontaneously and by sputum induction from healthy individuals.

### Collection of spontaneous sputum samples

In some cases subjects came to the laboratory and, after rinsing their mouths with water to minimize salivary contamination, coughed sputum into a Petri dish on demand; the sputum was then weighed. Subjects who found collection on demand impossible were given a weighed 25 ml pot containing 10–20 ml of 6 M GdmCl, pH 6.5, to take away. They expectorated a single sample of sputum into the pot and returned it to the laboratory as soon as possible. The increase in the weight of the pot was taken as the weight of the sputum.

### Collection of induced sputum

Induced sputum was collected by the method of Pin et al. [19]. Subjects' peak expiratory flow was measured in triplicate with a handheld spirometer (Vitalograph Escort). Subjects then inhaled an aerosol of 5% (w/v) NaCl solution, generated by ultrasonic nebulizer (DeVilbiss Ultraneb 2000), for up to 4 min. Those ready to expectorate at this stage rinsed the saliva from their mouths with water and coughed sputum into a Petri dish. Subjects unable to produce sputum had their peak expiratory flow measured again and, provided that this was more than 80% of its initial value, repeated the inhalation of 5% NaCl. This continued until the subject could expectorate, their peak expiratory flow had fallen to less than 80% of its inital value or had inhaled the NaCl aerosol for a total of 20 min. Three of the latter subjects inhaled 200 $\mu$g of salbutamol from a metered-dose inhaler in an attempt to lessen bronchoconstriction. Sputum collections were approved by the St George's Hospital Ethical Committee. Induced sputum was weighed before transfer to a screw-topped pot for mixing with 6 M GdmCl.

### Preparation of MUC5AC and MUC5B mucin standards

#### MUC5B mucin purification

Saliva was used as the source of the MUC5B mucin, and extraction and purification were performed essentially as described by Thornton et al. [20] by a combination of gel-filtration chromatography and isopycnic density-gradient centrifugation. The MUC5B mucin-containing fractions from the density gradient (1.41–1.52 g/ml) were pooled and concentrated by dialysis (12–14 kDa cut-off) against 6 M urea containing poly(ethylene glycol) of molecular mass 15–20 kDa, decreasing the volume from approx. 70 ml to 20 ml.

#### MUC5AC mucin purification

The A1 subclone of the human intestinal cell line HT-29 was cultured in RPMI 1640 medium with L-glutamine (Gibco BRL, Paisley, Renfrewshire, U.K.) containing 10% (v/v) foetal calf serum. The cell culture medium from HT-29 cells in culture (approx. 1 litre) was concentrated to 50 ml, initially by dialysis against 4 M GdmCl containing poly(ethylene glycol), followed by further concentration with Aquacide. The MUC5AC mucins were then purified by a combination of gel-filtration chromatography and isopycnic density-gradient centrifugation as described previously [21]. The MUC5AC mucin-containing fractions from the density gradient (1.4–1.46 g/ml) were pooled and concentrated from 40 ml to 15 ml by dialysis against 6 M urea/poly(ethylene glycol) and then to approx. 3 ml with Aquacide.

### Determination of the concentrations of the standard solutions

The mucin concentrations of the stock solutions were determined by refractive index measurements as follows. A 500 $\mu$l aliquot of the stock solution was chromatographed on a Sepharose CL-2B column eluted with 4 M GdmCl; the column eluate was monitored with an in-line Wyatt 903 interferometric monitor. The concentration of the mucins was calculated by integrating the refractive index peak associated with the material eluted in the void volume of the column and employing a value for the refractive-index increment ($dn/dc$) of 0.12 previously determined for cervical gel-forming mucins [22]. The reproducibility of this procedure was within 5% and we estimate the accuracy at approx. 10%.

### Antisera

Mucin-specific antisera were raised against peptide sequences (coupled to keyhole-limpet haemocyanin) found in the non-tandem repeat regions of the MUC5AC (MAN-5ACI [6]) and MUC5B (MAN-5BI [7]) mucin polypeptides. The immunizing peptide sequences and their positions along the mucin polypeptides are shown in Figure 1 below. The antisera were used at the following dilutions: MAN-5ACI, 1:10000; MAN-5BI, 1:2000.

### ELISA-based assays

Mucins, in 0.2 M NaCl, 6 M urea or 4 M GdmCl, were immobilized on either nitrocellulose membranes (slot-blotting) or on plastic ELISA plates. In some experiments BSA was added to the mucin solution before immobilization. Mucins were detected with either the MAN-5ACI or the MAN-5BI antiserum. In the slot-blotting experiments, mucins were also detected with a PAS assay [23].

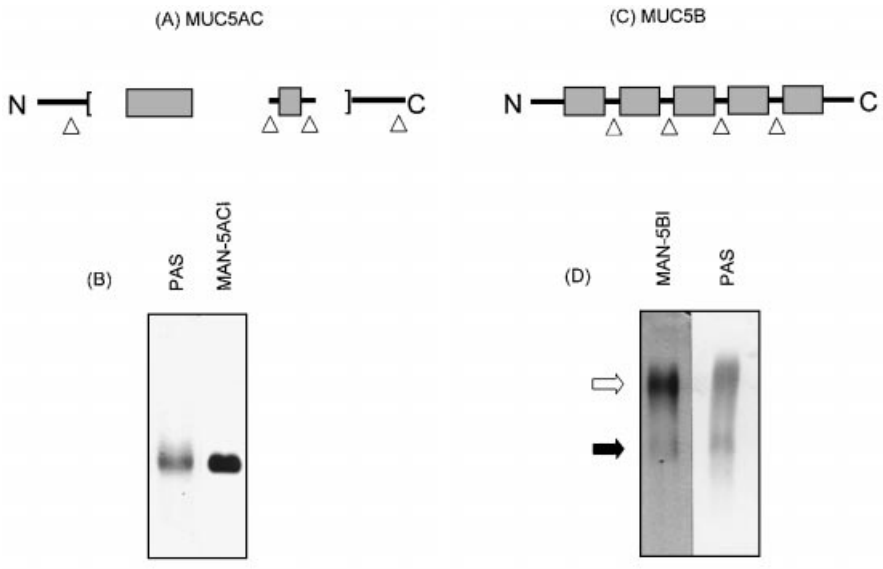


**Figure 1 Antisera directed against MUC5AC and MUC5B mucins**

Diagrammatic representations of the deduced structures of the MUC5AC (**A**) and the MUC5B (**C**) mucins, indicating the locations of the peptide sequences to which polyclonal antisera were raised. The shaded boxes represent regions of potential O-glycosylation and the triangles show the location of the peptide sequences against which the antisera were raised. The MUC5AC mucin is not yet completely sequenced and the structure is based on the model presented by Rose and Gendler [1]. The model for the MUC5B mucin is from results of Desseyn et al. [34–36]. The reduced and alkylated mucin standard preparations, MUC5AC (**B**) and MUC5B (**D**), were subjected to electrophoresis in 1% (w/v) agarose gels as described in the Experimental section. Mucins were transferred to nitrocellulose and then probed with either the PAS reagent or mucin-specific antisera. In (**D**) the low-charge (open arrow) and high-charge (filled arrow) populations of the MUC5B mucins are highlighted.

### Analytical agarose-gel electrophoresis

Reduced and alkylated mucins were separated by 1% (w/v) agarose-gel electrophoresis, which was performed at 30 V for 16 h as described previously [24]. After electrophoresis the molecules were vacuum-blotted on nitrocellulose membrane before the detection of mucins with antibodies [25].

### Quantitative Western blotting assay

This assay was performed on either purified mucin solutions stored in 6 M GdmCl or sputum samples solubilized in 6 M GdmCl. Before analysis, aliquots of the samples were dialysed into 6 M urea.

#### Standards

For each experiment a series of calibrants (five different concentrations) were prepared by dilution of the standard mucin solutions. The final amounts on the gel were 0.85, 1.28, 2.13, 3.20 and 5.12 ng for MUC5AC and 45, 69, 172, 229 and 344 ng for MUC5B. Quality-control standards (samples of known mucin concentrations that were analysed as unknowns) were included to assess the accuracy of the assay. These were prepared at either one or two concentrations, depending on the number of available lanes on the gel.

#### Sample preparation

Because the mucin concentrations in the sputum samples were in general much higher than the calibration range of the assay, a crude estimate of concentration was obtained by slot-blotting dilutions of the samples and detecting the mucins with the two antisera. On the basis of these estimates, aliquots were then taken from each sample and diluted to bring them approximately within the calibration range. To each 100 $\mu$l of sample solution was added 10 $\mu$l of 10 × sample buffer [400 mM Tris/acetate/10 mM EDTA (pH 8.0)/Bromophenol Blue]. Sample buffer also contained 1.0% (w/v) SDS, 1 M Tris, 50 mM EDTA and 50 mM dithiothreitol. The mixture was then heated for 15 min at 100 °C. The standard mucin solutions were treated in the same way.

#### Gel loading and electrophoresis

Duplicate standard solutions and diluted samples were loaded randomly into the wells of a 0.7% agarose gel with a Hamilton syringe. The loading volume of the standard solutions was 40 $\mu$l, whereas the samples were loaded at three from a possible five different volumes (either 80, 60, 40, 20 or 10 $\mu$l) to obtain at least one point within the calibration range. Samples were left to equilibrate in the wells for 20 min before electrophoresis, because this was found to increase the reproducibility of the data. The gel was subjected to electrophoresis for 2.5 h at 65 V in 40 mM Tris/acetate/1 mM EDTA (pH 8.0) containing 0.1% SDS.

#### Vacuum transfer and detection

After electrophoresis the gel was washed in 0.6 M NaCl/60 mM sodium citrate and the molecules were then transferred to nitrocellulose (pore size 0.45 $\mu$m) by vacuum blotting in the same buffer at 4.5 kPa for 1.5 h with a VacuGene XL (Pharmacia-LKB, St Albans, Herts., U.K.). Blots were probed as described previously with either the MAN-5ACI or the MAN-5BI antisera [25] except that the incubation with primary antiserum was

overnight at approx. 22 °C. Bands were revealed with the chromogenic substrate Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (3–4 min development time). Measurement of band intensity was performed with a Bio-Rad Model GS-700 imaging densitometer in reflectance mode.

### Data analysis

Calibration curves were constructed with a non-weighted least-squares linear regression analysis of the staining intensities of the mucin standards. The concentrations of the mucins in the quality-control and sample solutions were reported as averages of the duplicate results. If none of the three volumes of sample loaded produced results within the calibration range, a further dilution was produced and analysed in a separate experiment. Calibration curves were deemed to be acceptable if they complied with all the following criteria: at least four of the five calibration levels were used; no more than four individual calibrators were removed; relative S.D. values of the duplicates were within 20 %; correlation coefficients were over 0.95 and quality-control sample accuracies were within 20 %. The acceptance criteria for reporting sample concentrations were that the relative S.D. values of duplicate results were within 20 %. Additionally, if more than one sample replicate was within the calibration range, the relative S.D. values of these replicates had to be within 30 %; if this was so, the average of the values was calculated. Samples not obeying these criteria were re-analysed in a separate experiment. The mucin concentrations in the sputum samples from healthy and diseased airways were compared and the data were analysed for significance by the Mann–Whitney *U*-test with the SIMFIT program (www.biomed.man.ac.uk/simfit).

## RESULTS

### MUC5AC and MUC5B mucin-specific antisera and standard solutions

The antisera MAN-5ACI and MAN-5BI were raised against repeated peptide sequences located in cysteine-rich regions of the mucin polypeptides that are disulphide-bonded and folded in the native mucin (Figures 1A and 1C). To enhance the activity of these antisera it was necessary to reduce and alkylate the mucins to expose these 'cryptic' epitopes before their detection (results not shown). Similar observations have been reported for the detection of the MUC5B mucin with antisera comparable to those employed here [8].

As a prerequisite to developing an assay for the quantification of the MUC5AC and MUC5B mucins it was necessary to make preparations of these glycoproteins to use as standards. In mucus from airways these macromolecules are present in mixtures and we have been unable to separate and purify the intact mucins because of their chemical and physical similarities. By contrast, we have been successful in purifying and characterizing these glycoproteins from other sources [20,21]. We therefore prepared our mucin-standard solutions from either the medium of HT-29 cells in culture (MUC5AC) or saliva (MUC5B) by gel chromatography followed by isopycnic density-gradient centrifugation (results not shown). To check for the presence of other known oligomeric mucins or other glycoproteins, Western blots of an agarose-gel electrophoretic separation of the reduced and alkylated mucin preparations were probed with the general carbohydrate stain, the PAS reagent, and mucin-specific antisera (Figure 1). A single PAS-reactive species was present in the MUC5AC mucin preparation, which was also stained with MAN-5ACI (Figure 1B) but not with MAN-2I or MAN-5BI (results not shown), indicating that MUC5AC was the predominant glycoprotein. In contrast, two PAS-positive bands were detected in the MUC5B mucin preparation, which also stained with the MAN-5BI antiserum (Figure 1D) but not with MAN-2I or MAN-5ACI (results not shown). This suggests that MUC5B was present in two charged variants (high-charge and low-charge glycoforms); their relative PAS reactivities indicate that the lower-charge form was more abundant.

The concentration of the mucins was determined from on-line refractive index measurements after Sepharose CL-2B gel chromatography (results not shown). The material in both preparations was of high molecular mass and was eluted in the void volume of the column; integration of the total refractive index associated with this peak yielded concentrations of 900 $\mu$g/ml for MUC5AC and 340 $\mu$g/ml for MUC5B. In summary, the results presented here, together with previous biochemical characterizations of similar mucin preparations [21,22], demonstrate that the major high-molecular-mass glycoproteins in the standard solutions were either MUC5AC or MUC5B mucins.

### Mucin quantification

Our aim was to develop a quantitative mucin-specific assay employing the MAN-5ACI and MAN-5BI antisera that could be performed directly on solubilized sputum. Initially, quantification was attempted with direct binding ELISA-based protocols by using a dilution series of the solubilized sputum samples immobilized either on nitrocellulose membranes or plastic microtitre plates. However, we were unable to obtain reproducible data with these antisera because different dilutions yielded different values for the amount of mucin present in these samples. We therefore investigated whether this was due to the solvents used to solubilize and immobilize the mucins and/or to interference by other molecules in the secretion, in particular proteins.

To monitor the effect of different solvents on mucin detection, a range of quantities of the standard solutions were immobilized on the solid phase in 4 M GdmCl, 0.2 M NaCl or 6 M urea (solvents commonly used to extract and study mucins). Figure 2 shows the MAN-5BI antiserum detection of MUC5B mucins immobilized on plastic (Figure 2A) or nitrocellulose (Figure 2B). The antiserum response was lower in the samples immobilized in 6 M urea than in those immobilized in 4 M GdmCl and 0.2 M NaCl, which both gave similar data. This suggests that the amount of mucin bound to the solid phase was greatly decreased by 6 M urea. To test this further we assessed the amount of mucin immobilized on nitrocellulose by using a PAS assay (Figure 2C). Again, the mucins blotted in 6 M urea gave a lower response, which is consistent with a lower binding of the mucins to the membrane. Similar behaviour, on both nitrocellulose and plastic, was observed for the detection of the MUC5AC mucin with both MAN-5ACI and the PAS reagent (results not shown).

To monitor the effect of non-mucin protein concentration on detection we added different amounts of BSA to the mucin solutions before immobilization on the solid phase. The effect of this pretreatment on the detection of the MUC5AC mucin slot blotted on nitrocellulose is shown in Figure 2(D). In general the MAN-5ACI response was decreased with increasing concentration of BSA, as was the PAS reactivity (results not shown). Furthermore the decrease was inversely dependent on the amount of mucin. Similar observations were made for the detection of MUC5B mucins (results not shown).

It is clear from the above results that a different approach was required to quantify mucins directly from sputa. Previous studies have shown that purified mucins can be separated by agarose-gel electrophoresis and subsequently detected with antisera after

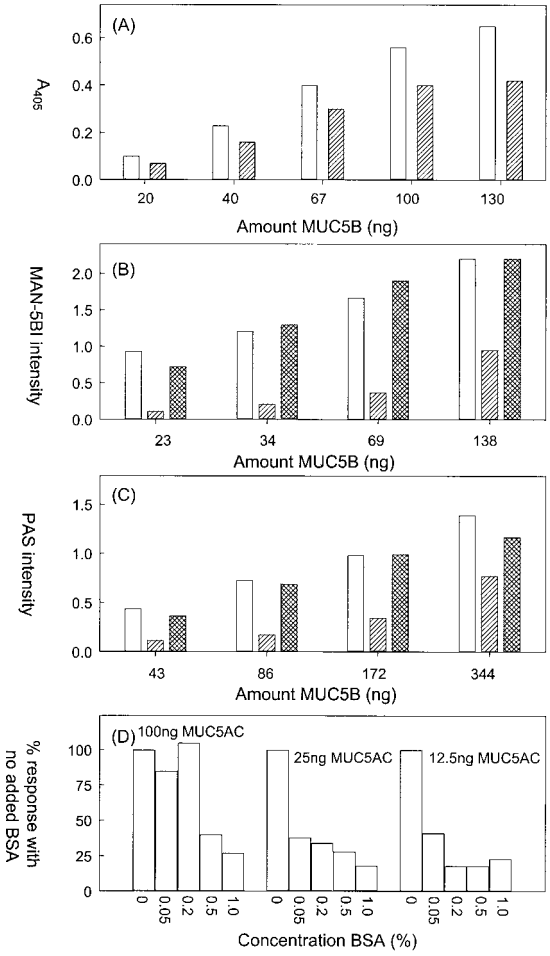


**Figure 2 Effect of solvent and non-mucin protein concentration on mucin detection**

(**A**, **B**) The reduced and alkylated standard MUC5B mucin preparation, in 4 M GdmCl (open bars), 6 M urea (hatched bars) or 0.2 M NaCl (cross-hatched bars) was immobilized by being coated on plastic microtitre plates (**A**) or being slot-blotted on nitrocellulose membranes (**B**) and the mucins were detected with the MAN5B-I antiserum. (**C**) Reduced mucins immobilized on nitrocellulose were also detected with the PAS reagent [23]. (**D**) The reduced and alkylated standard MUC5AC mucin preparation (100, 25 and 12.5 ng) in 0.2 M NaCl was mixed with BSA before being slot-blotted on nitrocellulose. BSA was added to final concentrations of 0%, 0.05%, 0.2%, 0.5% and 1% (w/v). The membrane was then probed with the MAN-5ACI antiserum. Results (at each mucin concentration) are presented as percentages of the staining intensity with no BSA added.

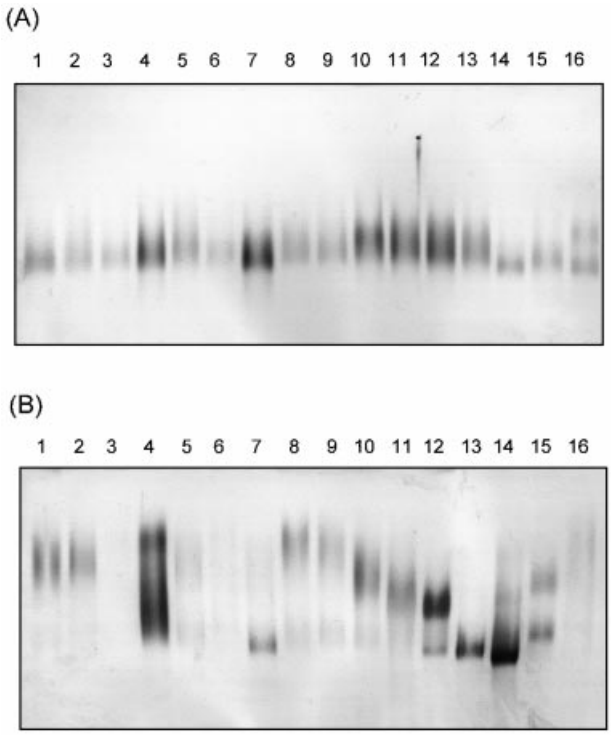


**Figure 3 Agarose-gel electrophoresis of reduced and alkylated MUC5AC and MUC5B mucins from airway secretions**

Sputum, NaCl-induced from healthy airways (lanes 4, 7, 8, 13, 14 and 16) and spontaneous secretions from pathological airways (lanes 1–3, 5, 6, 9–12 and 15), was solubilized in 6 M GdmCl and then dialysed against 6 M urea. Mucins were reduced, alkylated and subjected to electrophoresis on a 0.7% agarose gel as described in the Experimental section. After electrophoresis, gels were transferred to nitrocellulose and probed with MAN-5ACI (**A**) and with MAN-5BI (**B**).

Western blotting [5–7]. Furthermore, electrophoresis would be expected to separate the lower-molecular-mass proteins from the mucins. We therefore developed an assay employing quantitative Western blotting after agarose-gel electrophoresis. First we tested the feasibility of performing the electrophoresis directly on sputum samples. Sputa, NaCl-induced from normal airways and spontaneous from asthmatic airways, were extracted in 6 M GdmCl, reduced and alkylated, dialysed into 6 M urea and then subjected to 0.7% agarose-gel electrophoresis (Figure 3). A Western blot probed with the MAN-5ACI antiserum revealed, in most samples (15 out of 16), a single band with similar electrophoretic mobility (Figure 3A). In contrast, MAN-5BI detected one or two bands having considerable differences in electrophoretic mobility (Figure 3B). The relative amounts of the differently charged forms seemed to vary greatly between samples and it was apparent that the nature of high-charge and low-charge glycoforms was more complex than previously observed [7]. In the remainder of the study in samples in which two MUC5B bands were present, one was designated low charge and the other high charge. However, this assignment might not always be straightforward because in some samples only one band might be present.

To make the electrophoretic separation faster, rather than performing it overnight (as in the data presented in Figure 3), a higher voltage was used for a shorter time. Under these conditions proteins were separated from the mucins as assessed by staining with Coomassie Blue (results not shown) and it was still possible to discern charge variants of the MUC5B mucin (Figures 4A and 4B, inset). A range of amounts of the two mucin standards in quadruplicate (2–48 ng MUC5AC and 45–860 ng MUC5B) was subjected to electrophoresis; Western blots of the gels were probed with either MAN-5ACI or MAN-5BI (Figures 4A and 4B, inset). The intensity of staining in each track was determined by reflectance densitometry and response curves were generated (Figures 4A and 4B). The MUC5B data were generated from the total staining within each track (i.e. the combined intensity of the differently charged forms). The response curves show that the MAN-5ACI antiserum was able to detect less mucin than the MAN-5BI probe. Furthermore, the MAN-5ACI probe showed a non-linear response with increasing amount of MUC5AC mucin, whereas the MAN-5BI response was linear over the entire range but the results were less reproducible with larger quantities of MUC5B. Unlike the direct-binding ELISA-

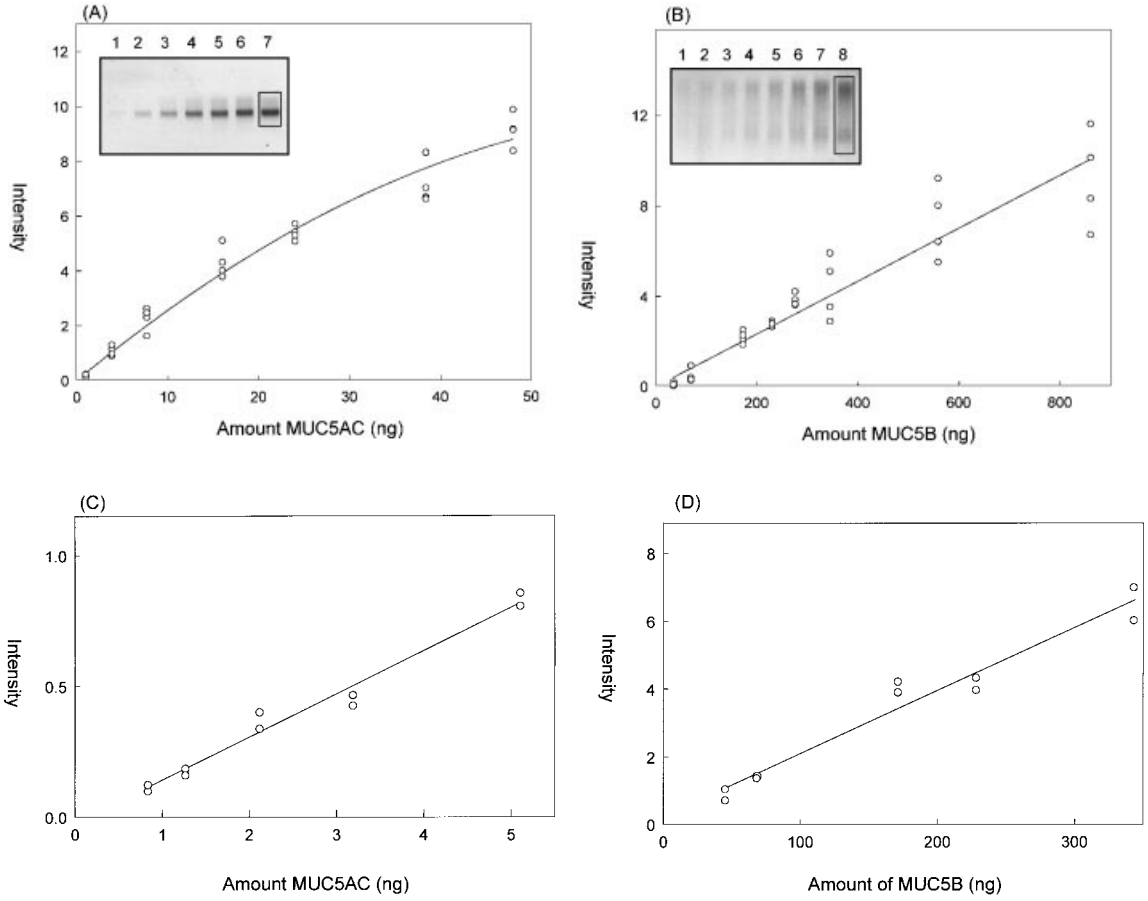


**Figure 4 Agarose-gel electrophoresis of mucin standard solutions and mucin calibration curves**

(**A**, **B**) A range of amounts of MUC5AC mucins (2–48 ng) (**A**) and MUC5B mucins (45–860 ng) (**B**) were subjected to electrophoresis on 0.7% agarose gels as described in the Experimental section. The mucins were then transferred to nitrocellulose membranes by vacuum blotting and the membranes were probed with MAN-5ACI [inset to (**A**)] and MAN-5BI [inset to (**B**)]. Bands were detected and their intensities were measured as described in the Experimental section. For MAN-5ACI the intensity within a rectangular area encompassing the largest band was measured in each track [inset to (**A**)]. For MAN-5BI a rectangular area that encompassed the two bands and any staining in between was measured [inset to (**B**)]. A local background was determined around each area measured and this was subtracted to yield the final intensity. The data presented in (**A**) and (**B**) are from four replicates at each concentration. (**C**, **D**) The final calibration ranges employed for the MUC5AC (**C**) and MUC5B (**D**) mucins are shown; duplicate samples were analysed.

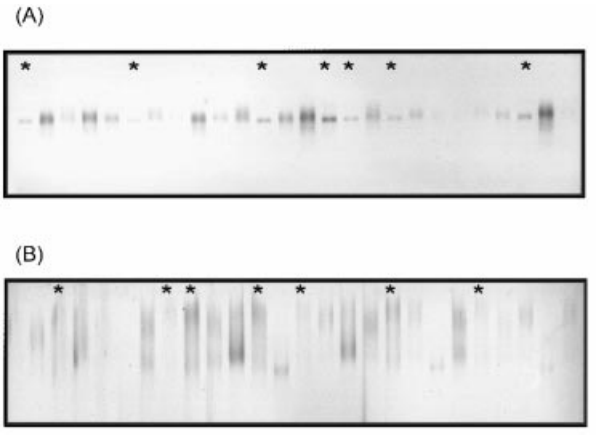


**Figure 5 Examples of Western blotting data**

Solubilized sputum samples and mucin calibrants were subjected to electrophoresis on 0.7% agarose gels as described in the Experimental section. After electrophoresis, mucins were transferred to nitrocellulose and probed with MAN-5ACI (**A**) and with MAN-5BI (**B**). Lanes containing calibrants are marked with an asterisk.

type assays, the addition of BSA to the standard solutions before electrophoresis caused no decrease in mucin detection because even at high concentrations of BSA (up to 2.0%) the standard curves were unchanged (results not shown).

We then used this method to obtain data on the amounts of the MUC5AC and MUC5B mucins in sputum samples and also in purified respiratory mucin preparations. The calibration ranges used for these determinations were 0.85–5.12 ng for MUC5AC and 45–344 ng for MUC5B, because over these ranges the data could be fitted to a straight line and were more precise (Figures 4C and 4D). In these experiments duplicate samples and standards were distributed randomly throughout the gel to minimize any non-uniform effects during electrophoresis, blotting, and probing or colour development. A typical gel is shown in Figure 5.

## Quantification of MUC5AC and MUC5B mucins directly from sputum

These experiments were performed on saline-induced secretions from healthy airways ($n = 15$) and on spontaneous sputum collected from asthmatic individuals ($n = 6$). All sputum samples

**Table 1 Quantities of mucins in induced healthy and spontaneous asthmatic sputa**

Data are expressed as $\mu$g/g of sputum. Total mucin is defined as the sum of the amounts of the MUC5AC and MUC5B mucins.

| | | | Mucin content ($\mu$g/g) | | | |
|---|---|---|---|---|---|---|
| Sputum type | *n* | Mucin | Mean | Median | S.D. | Range |
| Healthy | 15 | MUC5AC | 1328 | 291 | 1030 | 45–12000 |
| | | MUC5B | 269 | 156 | 276 | 2–1042 |
| | | Total | 1597 | 560 | 3101 | 108–12250 |
| Asthma | 6 | MUC5AC | 2955 | 2228 | 2720 | 690–8233 |
| | | MUC5B | 1798 | 503 | 3130 | 157–8164 |
| | | Total | 4753 | 2709 | 5785 | 1190–16400 |

were weighed before analysis. The MUC5AC and MUC5B mucin concentrations of these samples are presented in Table 1 and Figure 6. The induced healthy sputa showed a broader range of mucin concentration than the asthmatic samples. Analysis of the data with a Mann–Whitney *U*-test show significantly more mucin ($P < 0.005$) in the asthmatic samples than in those from healthy individuals (Figure 6A) owing to significant increases in the amounts of both MUC5AC ($P < 0.004$) and MUC5B ($P < 0.033$) mucins (Figures 6B and 6C). Also shown for these samples is the ratio of the amount of the MUC5B mucin to that of MUC5AC (Figure 7A). Four additional asthmatic samples were included in these determinations; however, the amount of sputum solubilized in these extra samples was not measured. No significant difference was observed between the two groups but there did seem to be a trend towards the presence of more MUC5AC than MUC5B in asthmatic sputa.

### Quantification of MUC5AC and MUC5B mucins in purified respiratory mucin preparations

Respiratory mucins were isolated from the sputum of individuals with CF and COPD after extraction with 6 M GdmCl and purification by density gradient centrifugation [2,3,5,8,9]. Unlike the above experiments, these analyses were performed on mucins from an unknown amount of sputum; the data are therefore presented as the ratio of the concentration of the two mucins per ml of purified mucin solution (Figure 7A). These data were compared with the ratios obtained for healthy and asthmatic secretions determined above. The CF and COPD mucin preparations showed a significant increase in the relative amount of the MUC5B mucin over the MUC5AC mucin in comparison with the asthma mucin ($P < 0.03$ for COPD and $P < 0.04$ for CF) but not in comparison with the healthy samples. However, there was less dispersion in this ratio in the CF and COPD samples in comparison with the healthy, with a trend towards more MUC5B than MUC5AC.

### MUC5B mucin glycoforms

Most samples studied contained two glycoforms of the MUC5B mucin; we therefore investigated whether the ratio of the two forms was different between the various sample groups by measuring the intensity of each band within a track and calculating the ratio of the high-charge to low-charge glycoform. Relative to the induced 'normal' sputa there was a significant increase in the amount of the low-charge MUC5B mucin in the asthmatic sputa ($P < 0.006$) and the CF ($P < 0.014$) and COPD ($P < 0.004$) mucin preparations (Figure 7B).

### Was the MUC2 mucin present in these samples?

The amount of MUC2 mucin present in the sputum samples and in the purified mucin preparations was determined by using the quantitative Western blotting assay in conjunction with the polyclonal antiserum MAN-2I [26]. A MUC2 mucin standard was purified from human adenocarcinoma cells in culture and the concentration range used in the assay was 30–170 ng/ml. Only 6 out of the 44 samples (1 healthy, 2 COPD and 3 asthma) showed detectable amounts of the MUC2 mucin (results not shown); this ranged from 0.2 % to 2.5 % of the total mucin content (MUC2 + MUC5AC + MUC5B).

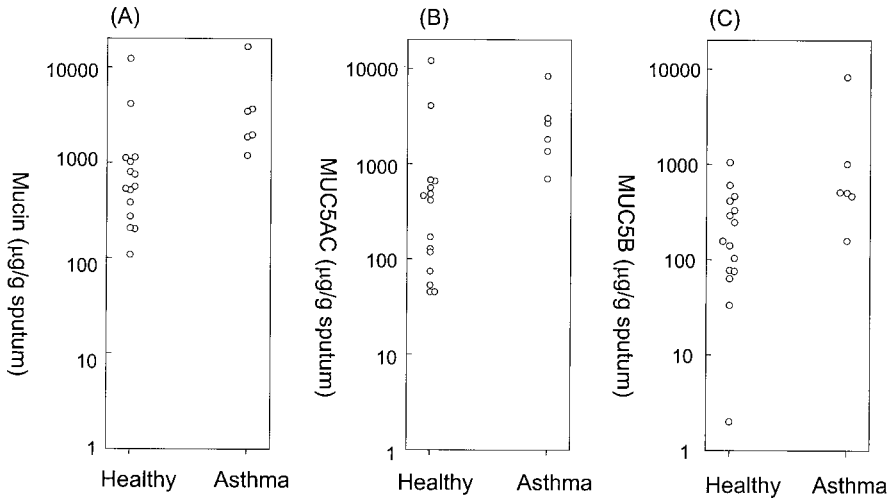


**Figure 6 Amounts of MUC5AC and MUC5B mucins in NaCl-induced healthy and spontaneous asthmatic sputum**

The concentration of the MUC5AC and MUC5B mucins in the NaCl-induced secretions from healthy airways and the spontaneous sputum from asthmatic airways was determined by quantitative Western blotting. Data are presented for concentrations of total mucin (MUC5AC + MUC5B) (**A**), MUC5AC mucin (**B**) and MUC5B mucin (**C**).

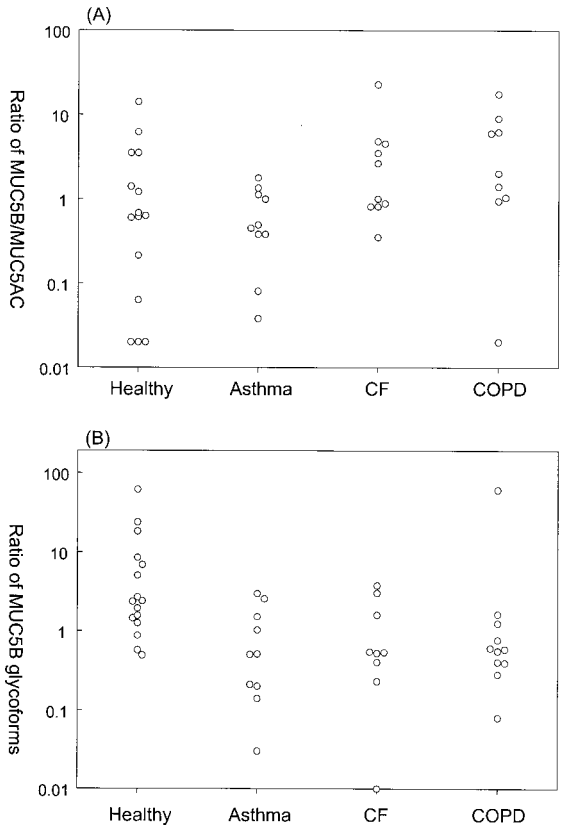


**Figure 7 MUC5AC and MUC5B mucins in NaCl-induced healthy and spontaneous asthmatic, COPD and CF sputum**

(**A**) The ratios of the amount of MUC5AC to MUC5B mucins in the NaCl-induced secretions from healthy airways and the spontaneous sputum from asthmatic, CF and COPD airways were determined by quantitative Western blotting. (**B**) The MAN-5BI antiserum highlighted two bands corresponding to the differently charged forms of this mucin (see Figures 3 and 5B). In all samples only one or two bands were revealed; when two were present we have defined the faster-migrating band as the high-charge form and the slower-migrating band as the low-charge form. Therefore for samples containing two bands we measured the intensity of staining of each band separately and have expressed the data as the ratio (high charge to low charge) of their intensities.

## DISCUSSION

We have developed a sensitive and reproducible quantitative Western blotting assay that can be used to measure the amounts of the different gel-forming mucins directly from mucus or in purified mucin preparations. Many factors need to be considered when trying to determine the amounts of these glycoproteins. One key issue is the solubilization of the mucins from the mucus gel. This requires high concentrations of chaotropic solvents (such as 6 M GdmCl or 6–8 M urea) with or without reducing agents. These reagents can interfere with the non-covalent binding of probes (i.e. antibodies) to the mucins and also, as we have demonstrated for 6 M urea, can inhibit the binding of the mucins to the solid phase. Caution should therefore be exercised when analysing mucins with assays in which immobilization of these glycoproteins is a prerequisite.

Besides their role in mucin solubilization these denaturing solvents are vital for protection against degradative enzymes. The polypeptides of intact mucins, and their reduced subunits in particular, are susceptible to cleavage by proteinases [21,27]. Unchecked proteolysis during mucus solubilization could result in loss of epitope and underestimation of the amount of mucin. Apart from non-specific proteolysis there is evidence for 'specific' cleavage of the mucin polypeptides [8,26,28]. As a consequence, potential post-translational modification needs to be taken into account when designing polypeptide-specific probes for the detection of mucins. The two antisera employed here potentially have multiple epitopes spread throughout the main body of the glycoproteins (see Figure 1); post-translational cleavage at the ends of the polypeptides, particularly for MUC5B, should therefore not cause a loss of epitopes.

Another important consideration is that mucus does not contain only oligomeric mucins but is a complex mixture of water, ions, lipids, proteins, monomeric mucins and glycoproteins and the concentrations of these components can vary, particularly in disease. For example the protein content of sputum can increase to as much as 0.5% of the dry weight [29]. This can cause problems for mucin detection because our study has shown that non-mucin proteins present in mucin solutions can cause an underestimation of the amount of the mucin with solid-phase binding assays. One should therefore be wary when employing these assays for samples with high protein content because this can give rise to misleading data. However, interference by high concentrations of contaminating proteins is not a problem in the Western blotting assay that we have developed because proteins are removed during electrophoresis.

Finally, the complex nature of the oligomeric gel-forming mucins must be taken into account when choosing probes for their detection. The structure of these glycoproteins, namely extensively glycosylated regions and domains that are potentially highly folded and stabilized by disulphide bonds, means that accessibility of probes to the polypeptide is limited and their effective use might require pretreatment of the mucins. Probes to the polypeptide underlying the glycosylated domains in the 'mature' mucins require harsh chemical treatments for their activity, which can result in epitope destruction. Probes to the 'naked' regions of the mucin polypeptides might therefore be preferable [6–8]. However, these antisera require reduction of the mucin for maximal reactivity and are therefore not without drawbacks of their own. For example, Wickstrom et al. [8] have shown that it might be necessary to block thiol groups after reduction to prevent 'refolding' and decrease in activity. For a more detailed discussion of factors to consider when assaying mucins with antibodies see [30].

The results presented here show that the electrophoretic behaviour of the mucins MUC5AC and MUC5B from airways is different. The former mucin was more homogeneous and in general a single species of MUC5AC mucin was found; by contrast, the MUC5B mucin was heterogeneous, with two forms present in many of the samples studied. This is consistent with earlier findings in which different glycoforms of the MUC5B mucin were described [7–9]; however, the range of forms is more complex than previously observed. Within two broad charge populations previously designated low-charge and high-charge glycoforms there are subpopulations of molecules; their relative amounts differ between individuals. It is interesting to note that some individuals secrete MUC5B species with similar electrophoretic behaviour to those synthesized by NHTBE cells in culture [26]. This provides further evidence that this cell culture secretes mucins with similar 'phenotypes' to those isolated from secretions *in vivo* and that it is therefore a valid model system with which to study airway mucins.

With the use of the quantitative Western blotting assay we have shown that, of the known gel-forming mucins expressed in the airways, MUC5AC and MUC5B are the predominant oligomeric species in mucus from airways. Furthermore our results demonstrate that mucus from airways is not a single

substance but is heterogeneous, containing a variable amount of each of these mucin species. We detected MUC2 in 6 out of 44 samples but, when present, MUC2 was found to comprise less than 2.5% of the mucins. This is consistent with previous findings in which MUC2 mucins were detected in CF sputum and accounted for less than 4% of the total weight of the gel-forming mucins [9]. Although the possibility has not been ruled out that substantial amounts of MUC2 are produced in the airways, studies performed on the mucins isolated from sputum and also from the apical secretions of human tracheobronchial cells in culture indicate that both sources contain very little of this mucin [3,9,26].

The NaCl-induced sputa from healthy airways contained a broad range of concentrations of MUC5AC (45–12000 $\mu$g/g of sputum) and MUC5B (2–1042 $\mu$g/g of sputum) glycoproteins and their relative levels varied between individuals. On the basis of their reported cellular distributions [10,26] our results indicate that induction with saline triggers secretion from both surface goblet cells (MUC5AC) and mucous cells in the submucosal glands (MUC5B). In comparison with diseased airways, in particular CF and COPD, the healthy secretions contained more of the acidic variants of MUC5B. Interestingly, a more sulphated form of MUC5B is synthesized by a subpopulation of submucosal gland cells [8,] suggesting that the MUC5B secretion elicited by induction with saline might come from a subgroup of glandular cells.

The asthmatic sputum samples contained more MUC5AC and MUC5B mucins than did the NaCl-induced healthy sputa, indicating an increase in secretion from both the surface epithelium and the glands. This is consistent with morphological investigations that report hypertrophy and hyperplasia of secretory cells in these locations in asthmatic airways [31–33]. The combined amounts of MUC5AC and MUC5B mucins reported here are in broad agreement with the data reported previously by Fahy et al. [12], who reported levels of mucin-like glycoproteins of $562.2 \pm 90.5$ and $2574 \pm 907.8$ $\mu$g/ml (means $\pm$ S.E.M.) in saline-induced healthy and asthmatic sputa respectively.

The results from the mucin preparations purified from CF and COPD sputum indicate an increase in the relative amount of MUC5B over MUC5AC mucins. Assuming no change in the cellular expression of the mucins, this suggests that these secretions might be more glandular in origin [8,10]. Moreover, the type of MUC5B is different from the 'healthy' and asthmatic airway sputa, with the low-charge MUC5B forms being increased in amount. This change in mucin type might have consequences for the properties of the mucus gel in CF and COPD airways and might give an insight into the altered physical nature of these secretions. In this context it is interesting to note that a low-charge population of the MUC5B mucin was responsible for the abnormal physical properties of a viscid gel plug removed from the airways of an individual in *status asthmaticus* [5].

In conclusion, our results demonstrate that respiratory mucus can contain different amounts and charged variants of gel-forming mucins, which are principally the products of the *MUC5AC* and *MUC5B* genes. The functional significance of the different mucin compositions, and in particular of the different charged forms of the MUC5B mucin, is at present unclear. In disease of the airways there are statistically significant changes in the mucin component of mucus and in particular a significant increase in low-charge forms of the MUC5B mucin in COPD and CF. It can be speculated that the goblet cells are slowly feeding the epithelial surface in the normal lung, whereas in disease there is a greater contribution from the submucosal glands. This could lead to the production of distinct mucus gels with different functional properties. By using the methodology developed here it will be important to extend our observations with larger studies of the quantity and type of the MUC5AC and MUC5B mucins in mucus from healthy and diseased airways.

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