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Heterogeneity of mucus glycoproteins from cystic fibrotic sputum

Are there different families of mucins?

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High-M_r mucin glycopeptides prepared from sputum of an individual with cystic fibrosis (CF) were studied by ion-exchange h.p.l.c. The glycopeptides were heterogeneous and a number of partially resolved populations were identified. Whole mucins from the gel phase were separated into four fractions by isopycnic density-gradient centrifugation in CsCl, and high-M_r glycopeptides from these fractions were examined by ion-exchange h.p.l.c. The acidic nature of the high-M_r glycopeptides increased with increasing buoyant density of the intact mucins, and a periodate–Schiff (PAS)-rich and an extremely high-iron diamine (HID)-reactive component were present in the lowest and highest density fractions respectively. The various glycopeptide populations were identified in different proportions in mucins from four other individuals with CF. CF sputum thus seems to contain distinct mucin populations containing different oligosaccharide clusters corresponding to these high-M_r glycopeptides.

INTRODUCTION

Histochemical studies on the respiratory tract indicate that the mucous cells in the submucosal glands and the goblet cells at the surface epithelium contain both neutral and acidic mucins (Jeffrey & Reid, 1977; Spicer *et al.*, 1983). These observations are supported by lectin histochemistry (Mazzuca *et al.*, 1982), and suggest that respiratory mucus may contain more than one distinct population of mucus glycoproteins. However, there is no biochemical evidence to answer the question as to whether goblet cells and submucosal glands produce structurally different mucins.

Mucus glycoproteins contain numerous oligosaccharides, most of which are packed into proteinase-resistant clusters flanked by unsubstituted, naked segments of the protein core (Carlstedt & Sheehan, 1989). Bronchial mucins are substituted with a diverse range of oligosaccharide structures (Van Halbeek *et al.*, 1982; Lamblin *et al.*, 1984*a*,*b*; Breg *et al.*, 1987, 1988; Klein *et al.*, 1988), but it is not known whether (1) discrete families of bronchial mucins contain different oligosaccharides, (2) certain glycans are enriched in different oligosaccharide clusters within one single mucin, or (3) all species are randomly distributed over the entire length of a single glycoprotein population. In the previous paper (Thornton *et al.*, 1991) we showed that cystic fibrosis (CF) respiratory mucins are heterogeneous in buoyant density, suggesting the presence of a number of distinct mucin populations. Here we have examined high-M_r mucin glycopeptides from CF mucins by ion-exchange h.p.l.c. and demonstrated that these structures can be separated into a number of discrete populations. Furthermore, distinct populations of whole mucins obtained after density-gradient centrifugation were enriched in a single type of glycopeptide, in keeping with the presence of different families of CF respiratory mucins.

EXPERIMENTAL

Materials

Guanidinium chloride (practical grade) was purchased from Riedel-de-Haen, Seelze, Germany. Stock solutions (8 M) were treated with charcoal before use. Sephacryl S-200 and S-500 were products of Pharmacia. Trypsin (EC 3.4.21.4; type XIII; TPCK-treated), deoxyribonuclease I (EC 3.1.21.1; type IV), ribonuclease A (EC 3.1.27.5; type I-A), *NN*-dimethyl-*p*-phenylenediamine and *NN*-dimethyl-*m*-phenylenediamine were purchased from Sigma. Alcian Blue 8XG was from BDH; PBS tablets (Dulbecco's medium) from Oxoid and di-isopropyl phosphofluoridate from Fluka. The latter was used as a solution (0.1 M) in propanol.

Isolation of high-M_r mucin glycopeptides from sputum

Sputum was obtained at sessions of physiotherapy from a female CF patient (16 years old, blood group O, *Pseudomonas*-infected) and frozen immediately after collection. Pooled samples were thawed by gentle mixing into cold 0.2 M-NaCl/10 mM-sodium phosphate buffer, pH 6.5 (30 ml) containing 5 mM-Na_2EDTA/5 mM-*N*-ethylmaleimide and 1.2 ml of 0.1 M-di-isopropyl phosphofluoridate. The sol and gel phases were separated by ultracentrifugation (Beckman 50.2 Ti rotor, 30 min, 145000 $g_{av.}$ at 4 °C), and cold ethanol (4 vol.) was added to both samples. The sol was left to precipitate and the gel was gently stirred at 4 °C overnight. After centrifugation (4500 $g_{max.}$, 20 min, room temperature, Kita bench-top centrifuge), the precipitates were dissolved and reduced with 6 M-guanidinium chloride/0.1 M-Tris/HCl buffer, pH 8.0, containing 5 mM-Na_2EDTA and 10 mM-dithiothreitol, for 5 h at 37 °C. The samples were then alkylated with iodoacetamide (2.5-fold molar excess over dithiothreitol) overnight, dialysed into 5 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-$MgCl_2$ and digested with deoxyribonuclease (1 mg for the gel; 0.5 mg for the sol) and ribonuclease (2 mg for the gel; 1 mg for the sol) for 6 h at 37 °C. After dialysis against 0.1 M-Tris/HCl buffer, pH 8.0, the samples were digested with trypsin for 5 h at 37 °C (4 mg and 2 mg for the gel and sol respectively). Cold ethanol (4 vol.) was added and both samples were left to precipitate for 2 h at 4 °C. The precipitates were recovered by centrifugation as described above, dissolved in 4 M-guanidinium chloride and chromatographed on

Abbreviations used: CF, cystic fibrosis; PAS, periodic acid–Schiff; HID, high-iron diamine.
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a Sephacryl S-200 column (4.5 cm × 88 cm) eluted with 4 M-guanidinium chloride, pH 7, at a flow rate of 50 ml/h. The high-M_r glycopeptides were recovered from the void volume after extensive dialysis against water and freeze-drying. The sequence of reduction/alkylation, nuclease and trypsin digestion as well as the gel chromatography step were repeated once to ensure complete breakdown of the macromolecules into oligosaccharide clusters and total removal of nucleic acid fragments. The yields of the pure glycopeptides were 212 mg and 163 mg from the gel and sol respectively. The glycopeptides (2 mg/ml) were chromatographed on a column of Sephacryl S-500 (97 cm × 1.5 cm) eluted with 0.2 M-ammonium acetate at a flow rate of 6 ml/h, and fractions were analysed for hexose as described by Goa (1955) and for sialic acid by using an automated procedure (Lohmander *et al.*, 1980). The material was pooled as indicated (Fig. 1), dialysed against water and lyophilized.

Isolation of mucus glycoproteins and high-M_r glycopeptides thereof

The gel phase of the sputum from five individuals with CF was extracted with 6 M-guanidinium chloride containing proteinase inhibitors, and the mucus glycoproteins were purified by three-step isopycnic density-gradient centrifugation as described (Thornton *et al.*, 1991). The mucins in the second gradient (CsCl/0.2 M-guanidinium chloride) from patients CF-I to CF-IV were pooled as indicated into light and heavy fractions where appropriate (see Fig. 1 of Thornton *et al.*, 1991), whereas those from patient CF-V were re-centrifuged (as described in the legend to Fig. 4) and pooled into four fractions (CF-V, A–D) as shown in Fig. 4. All pooled mucin fractions were dialysed into 4 M-guanidinium chloride and chromatographed on Sepharose CL-2B, and samples of the mucins eluting with the void volume of the column were reduced and alkylated, digested with trypsin as described by Carlstedt *et al.* (1983), dialysed against water and subjected to ion-exchange h.p.l.c.

Ion-exchange h.p.l.c.

The high-M_r glycopeptides were chromatographed on a Pharmacia Mono Q HR 5/5 ion-exchange column using a 2150 LKB titanium-head pump connected to a 2152 LKB controller and a 2040-203 LKB mixing valve. Gradients were formed at the low-pressure side and samples were loaded with a Pharmacia V-7 injector. The column was eluted at a flow rate of 0.5 ml/min, first isocratically for 10 min with 10 mM-piperazine/perchlorate buffer, pH 5.0 (buffer A), then with a linear gradient (60 min) to 0.25 M-lithium perchlorate/10 mM-piperazine/perchlorate buffer, pH 5.0 (buffer B). Fractions (0.5 ml) were analysed by an automated procedure for sialic acid (Lohmander *et al.*, 1980) and neutral sugars were analysed using a solution PAS assay (Mantle & Allen, 1978). Recovery of high-M_r glycopeptides from the column was at least 80%.

Slot-blotting and histochemical staining

Samples of fractions from ion-exchange h.p.l.c. were blotted on to nitrocellulose membranes (0.20 μm pore size) using a Schleicher and Schuell Minifold II slot-blot apparatus as described by Thornton *et al.* (1989). Blotted membranes were stained with either the PAS procedure (Thornton *et al.*, 1989), Alcian Blue at pH 1.0 and at 2.5 (Pearse, 1985) or the high-iron diamine (HID) stain (Lev & Spicer, 1965) and measurements of the colour intensity were performed as described by Thornton *et al.* (1989). The glycopeptide preparation had a hyperbolic standard curve with all the stains used, and the data presented

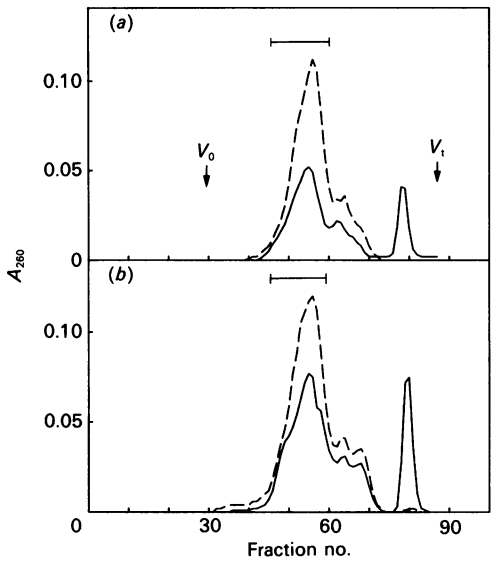

Fig. 1. Gel chromatography on Sephacryl S-500 of high-M_r glycopeptides from the gel (*a*) and sol (*b*) phases of CF sputum

Glycopeptides (2 mg/ml) were chromatographed on a column of Sephacryl S-500 (97 cm × 1.5 cm) eluted with 0.2 M-ammonium acetate at a flow rate of 6 ml/h. Fractions (2 ml) were collected and assayed for hexose (---) and sialic acid (——). Material was pooled as indicated

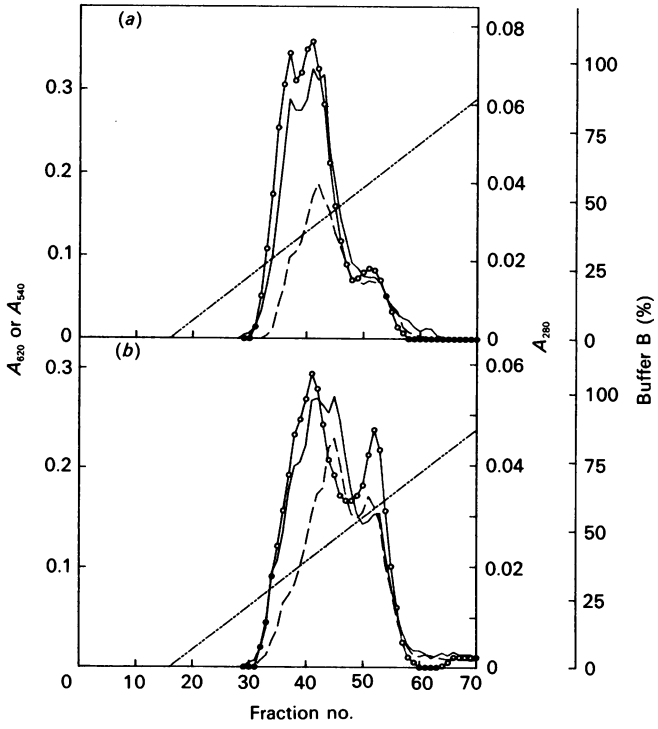

Fig. 2. Ion-exchange h.p.l.c. of high-M_r glycopeptides from the gel (*a*) and sol (*b*) phases of CF sputum

Glycopeptides were chromatographed on a Mono Q HR 5/5 column and the fractions were assayed for sialic acid (---), hexose (——) and absorbance at 280 nm (○). The percentage of buffer B is indicated (-·-·-).

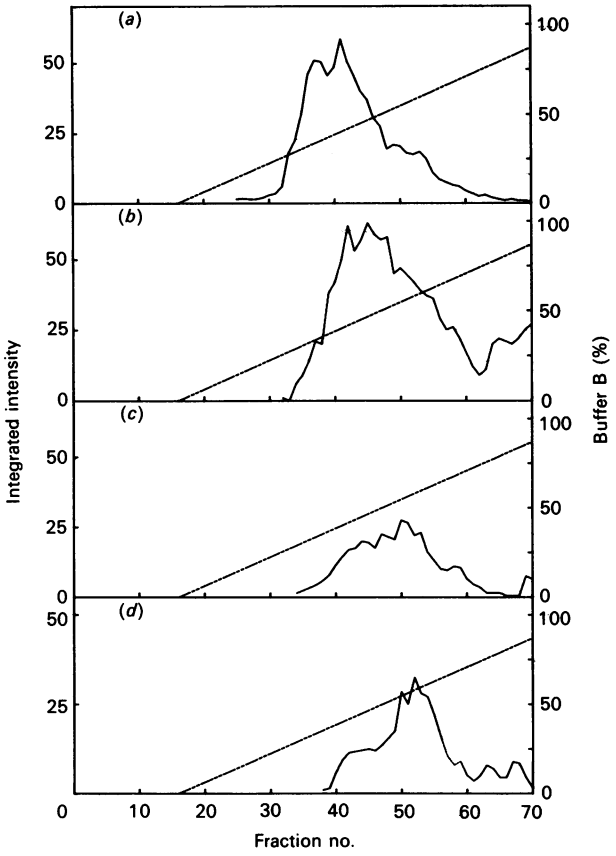

Fig. 3. Ion-exchange h.p.l.c. profiles of high-M_r glycopeptides from the gel phase of CF sputum

Samples from each fraction were slot-blotted on to polylysine-treated nitrocellulose membranes and stained with (*a*) the PAS procedure, (*b*) Alcian Blue at pH 2.5, (*c*) Alcian Blue at pH 1.0 and (*d*) the HID procedure. The percentage of buffer B is indicated (–···–). The PAS profile is in good agreement with those obtained for the sialic acid determination and the solution assay for PAS. This shows that immobilization of the glycopeptides on the nitrocellulose membranes is representative across the distribution.

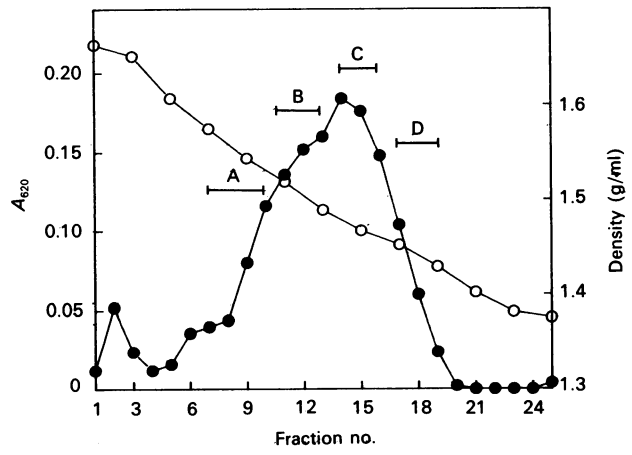

Fig. 4. Isopycnic density-gradient centrifugation of CF-V in CsCl/0.2 M-guanidinium chloride

Purified mucus glycoproteins (patient V) were centrifuged on a Beckman L5 ultracentrifuge for 70 h at 38000 rev./min in a Ti 70 rotor at 15 °C. Fractions were collected from the bottom of the tube and analysed for sialic acid (●) and density (○). The mucins were pooled into four fractions (CF-V-A to CF-V-D) as indicated.

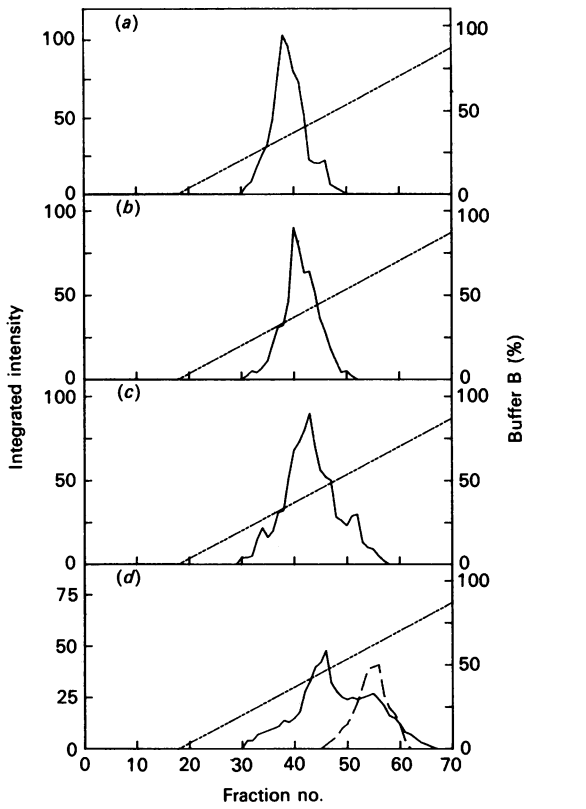

Fig. 5. Ion-exchange h.p.l.c. of high-M_r glycopeptides from CF-V mucin fractions

High-M_r glycopeptides prepared from (*a*) CF-V-D, (*b*) CF-V-C, (*c*) CF-V-B and (*d*) CF-V-A were chromatographed on a Mono Q HR 5/5 column. Samples from each fraction were slot-blotted on to polylysine-treated nitrocellulose membranes and stained with the PAS (——) and HID (– – –) procedures. The percentage of buffer B is indicated (–···–).

were obtained within the non-saturation region (up to approx. 600 ng of glycopeptide per band).

RESULTS AND DISCUSSION

CF sputum was separated into sol and gel phases and high-M_r glycopeptides were isolated from both sources after reduction/alkylation followed by nuclease and trypsin digestion and gel chromatography on Sephacryl S-200. The glycopeptides (T-domains), corresponding to the oligosaccharide clusters in the whole mucins, which were eluted with the void volume of the column, were then chromatographed on Sephacryl S-500 and the major anthrone- and sialic-acid-containing peaks were pooled (Fig. 1). Ion-exchange h.p.l.c. of these high-M_r glycopeptides (Fig. 2) showed that the A_{280}, neutral sugar and sialic acid profiles are not coincident, indicating that the samples are heterogeneous, i.e. they contain a number of partially resolved glycopeptide populations. The chromatographic profiles of the glycopeptides from the gel and sol phases (Figs. 2*a* and 2*b*) are different, the sol containing a greater proportion of acidic species. In the previous paper (Thornton *et al.*, 1991), it was shown that mucins from the sol are smaller than those in the gel, and it was suggested that they may be proteolytic fragments of the larger species in the gel. The results obtained here suggest that, if this is so, then the most acidic mucins are more prone to degradation.

A much more complex picture emerged when fractions from the Mono Q column were immobilized on to nitrocellulose

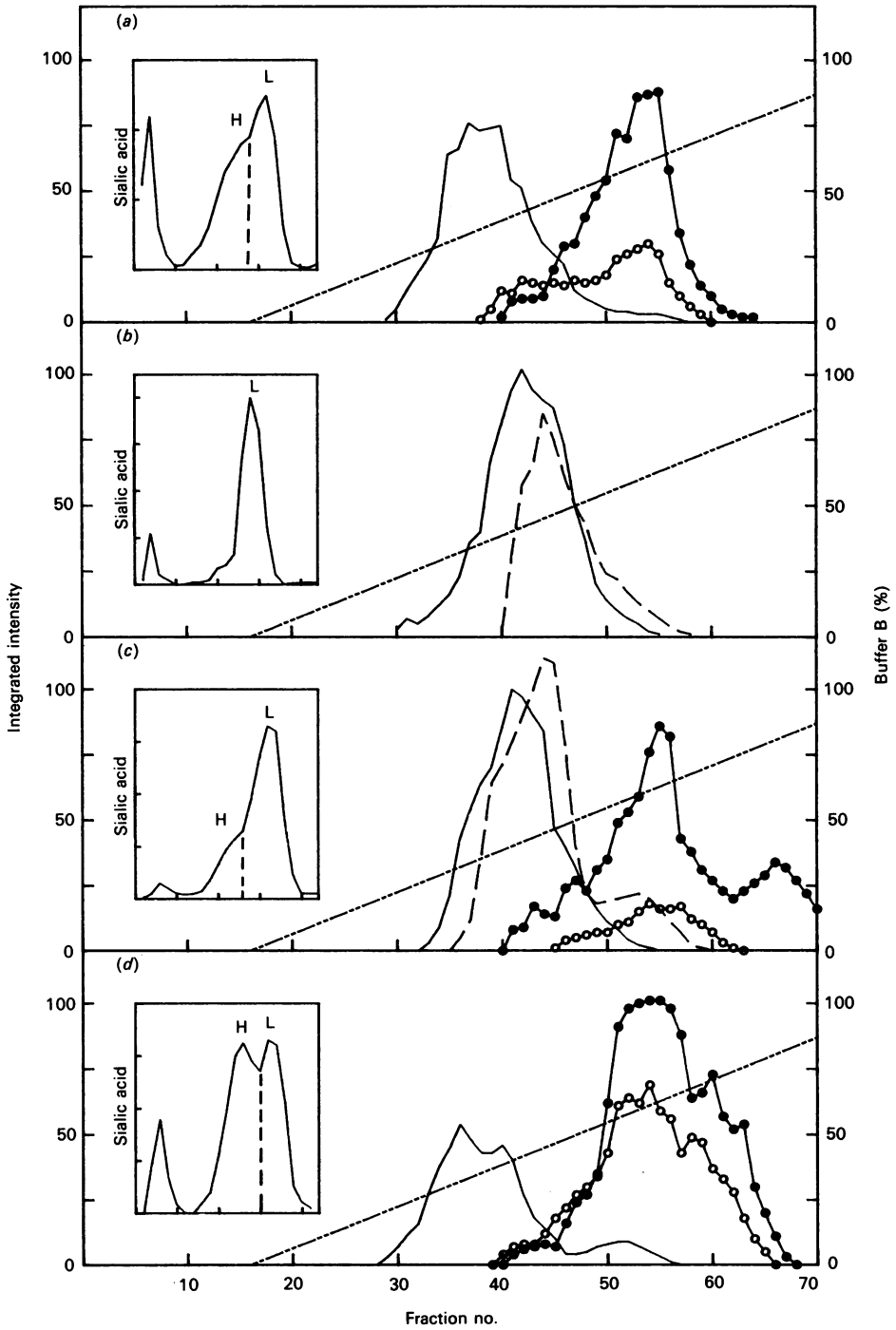

Fig. 6. Ion-exchange h.p.l.c. of high-M_r glycopeptides from heavy and light CF mucin populations

High-M_r glycopeptides prepared from the following CF mucin fractions: (*a*) CF-IV-light and -heavy, (*b*) CF-III-light, (*c*) CF-II-light and -heavy and (*d*) CF-I-light and -heavy, were chromatographed on Mono Q. Samples of each fraction were slot-blotted on to polylysine-treated nitrocellulose membranes and stained with the PAS (——, light fraction; ○, heavy fraction) and HID (– – –, light fraction; ●, heavy fraction) procedures. The percentage of buffer B is indicated (– · –). The insets show the density-gradient profile in CsCl/0.2 M-guanidinium chloride (sialic acid assay) for each mucin preparation, and the pooling of heavy (H) and light (L) mucin fractions is indicated.

membranes and subjected to histochemical staining procedures expected to reveal different types of mucins (Fig. 3). Glycopeptides eluting early from the column (fractions 30–40) were visualized by the PAS procedure but were only weakly stained with Alcian Blue both at pH 2.5 and at pH 1.0. In contrast, Alcian Blue at pH 2.5 highlighted a PAS-reactive population of glycopeptides eluting between fractions 40 and 50. At pH 1.0, the carboxylate groups on the sialic acid residues are protonated and Alcian Blue is supposed to bind to sulphate groups. This is supported by the finding that HID, a stain selective for sulphate, and Alcian Blue at pH 1.0 give similar distributions, although the former reacts relatively more strongly with the late-eluting component (fractions 50–60). There is no HID staining of the glycopeptides eluting early in the chromatogram. Glycopeptides from the sol fraction gave similar staining profiles with a relatively higher proportion of the late-eluting HID-positive material. The

glycopeptide populations identified by the slot-blot analyses clearly correspond to the partially resolved components observed with A_{280}, the solution PAS procedure and sialic acid determinations (Fig. 2*a*). However, the histochemical staining demonstrates the heterogeneity more clearly and, in particular, the profiles obtained with the PAS and HID procedures are very different. These two stains were thus used to examine the heterogeneity of the mucins in greater detail. In the previous paper we showed evidence of distinct populations of intact mucins by using density-gradient centrifugation. Thus the relationship between solution density of the whole mucins and the charge density of the cognate glycopeptides was investigated by using ion-exchange h.p.l.c.

Whole mucins from the gel phase of sputum from the same patient (CF-V) were fractionated by using density-gradient centrifugation in CsCl/0.2 M-guanidinium chloride, and the mucin peak was separated into four fractions (Fig. 4). After chromatography on Sepharose CL-2B, material eluting with the void volume of the column was used to prepare high-M_r glycopeptides for ion-exchange h.p.l.c. The data presented in Fig. 5 show that there is a gradual increase in acidity of the glycopeptides with increasing buoyant density of the intact parent mucins. The early-eluting PAS-rich component is enriched in the mucin fraction (CF-V-D) of lowest buoyant density, whereas the late-eluting HID-rich component arises from the mucins (CF-V-A) of highest buoyant density. Under the conditions of the experiment, glycopeptides from CF-V-(B–D) did not stain for sulphate with HID.

The above data indicate that mucins of different buoyant densities contain different oligosaccharide clusters, and this is well demonstrated in the ion-exchange h.p.l.c. profiles of glycopeptides prepared from the heavy and light CF mucin fractions of the four other individuals described in the previous paper (Thornton *et al.*, 1991) (Fig. 6). Three distinct populations of glycopeptides are observed which seem to correspond to the glycopeptides described above. However, in two samples (CF-II-light and CF-III-light), the component eluting between fractions 40 and 50 also gives a large HID response (Figs. 6*b* and 6*c*). The glycopeptides from the light mucin fractions are clearly different from those in the heavy fractions and, in all cases, the light fraction mucin glycopeptides are less acidic.

GENERAL DISCUSSION

Histochemical investigations of the respiratory epithelium, using PAS, Alcian Blue at different pH values and HID, have identified so-called neutral and acidic mucus glycoproteins, the latter being further sub-divided into sialo- and sulpho-mucins (Jeffrey & Reid, 1977; Jones & Reid, 1978; Reid & Clamp, 1978; Spicer *et al.*, 1983). Various combinations of staining patterns have been observed in different cells, and changes have been shown to occur in disease. In the previous paper (Thornton *et al.*, 1991) we demonstrated that CF respiratory mucins may be partially resolved into populations by density-gradient centrifugation. The heterogeneity is not due to size or to differences in the polymeric structure of the molecules. Because of the large size of the whole mucins, there are few techniques available for resolving them into components, and other approaches must be developed to explore this issue further. The oligosaccharide clusters in the mucins can be isolated as high-M_r glycopeptides which are smaller, less polydisperse in size and thus more amenable to other fractionation techniques (Carlstedt & Sheehan, 1989). We show that such structures from CF sputum are partially resolved by ion-exchange chromatography and are also characterized by different reactivities with various histochemical procedures. Density-gradient centrifugation of intact mucins indicates the presence of different components, the glycopeptides of which are clearly resolved as separate species by ion-exchange h.p.l.c. Together these data suggest that each mucin population is composed of only a single type of oligosaccharide cluster.

Biochemical and histochemical studies on CF bronchial mucins have indicated an increase in their sulphate content (Boat *et al.*, 1976; Lamblin *et al.*, 1977). Chace *et al.* (1983) isolated a CF respiratory mucin, rich in sulphate and relatively poorly reactive with PAS, which was seen to increase in amount with the severity of the disease. We have observed a similar component in the purified mucins from the sputum of four out of five CF individuals. The other sample does not have this highly acidic HID-rich component. Instead, a less acidic HID-positive species was found. Thus it seems likely that the increase in sulphate observed in CF mucins is due to an increase in the proportion of the most acidic mucin species.

In this study we have shown that there may be distinct populations of CF respiratory mucins, the proportions of which vary between individuals. We do not know if the pattern of mucins is specific for the individual, is related to CF, is correlated with the severity of the disease or is due to bacterial infection. The cellular origin of the mucins is not known, but it is tempting to speculate that they might arise from different cell types, such as the mucous cells of the submucosal glands and the goblet cells. To answer these questions it will be necessary to study a larger number of individuals and to develop probes for the different mucins.

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