Intestinal mucins from normal subjects and patients with cystic fibrosis

Variable contents of the disulphide-bound 118 kDa glycoprotein and different reactivities with an anti-(118 kDa glycoprotein) antibody

Michèle MANTLE* and Gordon STEWART

Gastro-intestinal Research Group, Departments of Medical Biochemistry and Paediatrics, University of Calgary, Calgary, Alberta T2N 4N1, Canada

1. A specific antibody was developed against the disulphide-bound 118 kDa glycoprotein of human intestinal mucin and used to establish an e.l.i.s.a. Fourteen purified mucins [eight normal (N) and six cystic fibrosis (CF)] had the same affinity for the antibody in the e.l.i.s.a., but their relative immunoreactivities varied widely (approx. 100000-fold). In general, CF mucins were more antigenic than N mucins. 2. Variations (approx. 10-fold) were detected in the 118 kDa glycoprotein content of both N and CF mucins (assessed from Coomassie Blue-stained polyacrylamide gels), but these did not appear to be responsible for the differences in mucin immunoreactivity. 3. Variations (approx. 6-fold) were also observed in the size of the 118 kDa peak produced by N and CF mucins on Western blots. These were mostly due to differences in the 118 kDa glycoprotein content of mucins, although a small proportion resulted from changes in the number of antigenic determinants within individual 118 kDa glycoproteins. 4. After concanavalin A affinity chromatography of four reduced mucins (two N and two CF), purified 118 kDa glycoprotein was recovered in the bound fractions from the column, specifically eluted by methyl α -mannoside. 5. The amounts of 118 kDa glycoprotein isolated from the four mucins varied as predicted from the size of their 118 kDa bands on Coomassie Blue-stained gels. 6. Three 118 kDa glycoproteins (one N and two CF) showed almost identical reactivity in the e.l.i.s.a.; the fourth had fewer antigenic determinants. 7. Since differences in 118 kDa glycoprotein content and in the number of antigenic determinants within the 118 kDa glycoprotein did not account for variations in the reactivity of native mucins in the e.l.i.s.a., it appeared that accessibility of the 118 kDa glycoprotein to antibody binding may be critical in determining mucin immunoreactivity. This suggests that the three-dimensional conformation of CF mucins may differ from that of N mucins, leading to increased antigenicity.

INTRODUCTION

Many purified mucins, including pig and human gastric (Pearson *et al.*, 1981), pig, human, rat and rabbit intestinal (Fahim *et al.*, 1983, 1987; Mantle & Thakore, 1988; Mantle *et al.*, 1981, 1984a,b) and human and dog tracheobronchial (Ringler *et al.*, 1987) mucins, have similar polymeric structures comprised of large heterogeneous glycoprotein monomers and a smaller discrete (glyco)protein component (65–120 kDa) held together by disulphide bonds. Compared with the mucin glycoprotein monomers, the 118 kDa glycoproteins of human and rat intestinal mucins have a higher proportion of acidic and aliphatic amino acids in their peptide cores and a lower carbohydrate content (approx. 50% by wt.) with large amounts of mannose (approx. 9 mol% of the total carbohydrate), suggesting the presence of *N*-linked oligosaccharide chains (Fahim *et al.*, 1983, 1987; Mantle *et al.*, 1984b). The 70 kDa and 65 kDa disulphide-bound components of pig gastric and dog tracheobronchial mucins respectively have amino acid profiles remarkably

similar to those of the 118 kDa glycoproteins from intestinal mucins, but are not glycosylated (Pearson *et al.*, 1981; Ringler *et al.*, 1987). The function of the disulphide-bound (glyco)proteins has not yet been determined. Examination of the effects of thiol reduction and proteolysis on the macromolecular structure of human and rat intestinal mucins has provided indirect evidence to suggest that the 118 kDa glycoproteins may serve as 'link' components by forming disulphide bridges with cysteine residues in non-glycosylated regions of the peptide core of the mucin glycoprotein monomers (Fahim *et al.*, 1983, 1987; Mantle *et al.*, 1984a,b).

The objective of the present study was to establish an immunoassay for the 118 kDa glycoprotein of human intestinal mucin and to use the assay to compare mucins from normal (N) subjects and patients with cystic fibrosis (CF). Considerable variability was observed in the relative immunoreactivities of individual mucin preparations, with CF mucins being in general more antigenic than N mucins. In exploring the reasons for the differences in immunoreactivity, we found variations in

Abbreviations used: CF, cystic fibrosis; N, normal; PAGE, polyacrylamide-gel electrophoresis; e.d.g.c., equilibrium density-gradient centrifugation; Con A, concanavalin A; PBS, phosphate-buffered saline (0.1 M-NaCl/0.1 M-Na₂HPO₄/NaH₂PO₄ buffer, pH 7.4).

* To whom correspondence should be addressed, at: Department of Medical Biochemistry, Health Sciences Centre, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada.

the 118 kDa glycoprotein content of both N and CF mucins and also in the number of antigenic determinants within individual 118 kDa glycoproteins. However, these factors together did not account for the immunological differences among mucins, suggesting that reactivity of the native mucin polymer with the antibody may depend to a large extent on the accessibility of the 118 kDa glycoprotein, and this may be greater in CF mucins.

METHODS

Isolation and purification of mucin

Human small intestine was obtained within 12 h *post mortem* from N subjects with no history of gastrointestinal disease and from patients with CF. Mucin was purified from mucosal homogenates in the presence of proteinase inhibitors (5 mM- Na_2EDTA , 1 mM-phenylmethanesulphonyl fluoride and 10 mM-*N*-ethylmaleimide) by e.d.g.c. in CsCl (twice) followed by gel filtration on Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden), as described previously (Mantle *et al.*, 1984b). High-molecular-mass polymeric mucin was harvested from the void-volume fractions of the column. The absence of non-mucin protein bands on SDS/PAGE and the failure to detect DNA (Hinegardner, 1971), mannose, glucose or uronic acid (by g.l.c.) established the purity of all mucin preparations. Three mucin samples (GM, JK and JR, kindly supplied by Dr. J. F. Forstner and Dr. G. G. Forstner, Hospital for Sick Children, Toronto, Ont., Canada) were purified by gel filtration of mucosal homogenates on Sepharose 4B followed by chromatography of the void-volume fractions on Sepharose 2B and recovery of the excluded and partially included mucin peaks (Wesley *et al.*, 1983). Two mucins (H13 and CF4) were extracted with chloroform/methanol (2:1, v/v) (as described by Slomiany *et al.*, 1983) to ensure complete removal of non-covalently bound lipid.

Isolation of the 118 kDa glycoprotein

Procedure 1: Preparative gel electrophoresis. After reduction of mucin with 0.2 M-2-mercaptoethanol [5 min at 100 °C in 1% (w/v) SDS], the 118 kDa glycoprotein was purified by two-step preparative gel electrophoresis and electro-elution in the presence of 10 mM-2-mercaptoethanol (Mantle *et al.*, 1984b). After an overnight dialysis against 10 mM-2-mercaptoethanol, SDS was removed from the 118 kDa glycoprotein by ion-pair extraction (Henderson *et al.*, 1979).

Procedure 2: Density-gradient centrifugation. Reduced mucin (approx. 20 mg, treated with 0.2 M-2-mercaptoethanol for 5 min at 100 °C) was subjected to e.d.g.c. in CsCl at a starting density of 1.35 g/ml. After fractionation of the gradient, each fraction was dialysed against 10 mM-2-mercaptoethanol for 3–4 h before SDS/PAGE. In some cases the reduced mucin was alkylated with iodoacetamide (0.5 M for 16 h at 4 °C and pH 8.6 in the dark) before the addition of CsCl and centrifugation. In other cases density-gradient centrifugation of reduced or reduced and alkylated mucin was carried out in the presence of 4 M-guanidinium chloride.

Procedure 3: Gel filtration. Reduced mucin [approx. 20 mg, treated with 0.2 M-2-mercaptoethanol in 5%

(w/v) SDS for 5 min at 100 °C] was applied to a Sepharose 2B column (1 cm $\times$ 120 cm) eluted with 10 mM-mercaptoethanol in 5% (w/v) SDS, and 1 ml fractions were collected. Slot-blot assays (see below) were used to identify fractions enriched in the 118 kDa glycoprotein. Those fractions were pooled, dialysed briefly against 10 mM-mercaptoethanol, concentrated by freeze-drying and applied to a TSK G5000PW column (LKB-Produkter AB, Bromma, Sweden) eluted with 0.25% (w/v) SDS and 10 mM-mercaptoethanol in 0.2 M- NaCl . Fractions (1 ml) were collected and analysed.

Procedure 4: Con A affinity chromatography. Affi-Gel-Con A (containing 9 ± 1 mg of Con A/ml of gel; Bio-Rad Laboratories, Richmond, CA, U.S.A.) was thoroughly washed according to the method of Zanetta & Gombos (1976). This ensured complete removal of non-covalently bound lectin from the gel matrix and prevented contamination of mucin components in subsequent experiments by Con A leaching from the column. Purified mucin (10 mg) was reduced with 0.2 M-2-mercaptoethanol in 0.1% (w/v) SDS (5 min at 100 °C) and alkylated by addition of iodoacetamide. After dialysis against Tris buffer [10 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M- NaCl and 0.01% (w/v) SDS], reduced and alkylated mucin samples were applied at room temperature to Affi-Gel-Con A columns (5 ml bed volume) equilibrated in the same Tris buffer. After 30 min incubation, the column was eluted with Tris buffer. Non-bound fractions (eluted in the first column volume) and retarded fractions (eluted in the next two column volumes) were collected (Narasimhan *et al.*, 1979). Addition of 0.5 M-methyl α -mannoside to the elution buffer and thorough washing (2 column volumes) removed bound material from the column. (A high concentration of methyl α -mannoside was chosen to elute the column to ensure complete removal of bound material; the effects of lower concentrations were not assessed.) Non-bound, retarded and bound fractions were analysed by SDS/PAGE and Western blots after dialysis for 3 h against 0.01% SDS. For amino acid and monosaccharide determinations, samples were thoroughly dialysed first and SDS was removed by ion-pair extraction (Henderson *et al.*, 1979).

Preparation of antibody

A polyclonal antibody was developed in New Zealand White rabbits (two) against purified 118 kDa glycoprotein isolated by preparative gel electrophoresis from an N human intestinal mucin. Immunization was carried out as described by Qureshi *et al.* (1979). After 8 weeks, animals were exsanguinated under halothane anaesthesia and euthanized with Euthanyl (MTC Pharmaceuticals, Cambridge, Ont., Canada). Serum from both animals was tested for the presence of antibody by performing a titre curve (see below). Both serum samples reacted positively. By using the serum with the highest antibody titre, an immunoglobulin-rich fraction was prepared by precipitation with 40%-saturated $(\text{NH}_4)_2\text{SO}_4$. After dialysis against PBS, the final antibody preparation was divided into 100 μl portions and stored at -80 °C.

A polyclonal antibody against purified N human intestinal mucin was also developed in New Zealand White rabbits by the same protocol.

Development of an e.l.i.s.a. for the 118 kDa glycoprotein

Wells of a micro-titre plate (Dynatech Laboratories, Alexandria, VA, U.S.A.) were coated overnight at 4 °C with 500 ng of purified standard mucin in 50 μ l of PBS. At the same time, various amounts of standard mucin (0–80 ng) or test material (in 75 μ l) were pre-incubated at 37 °C with the anti-(118 kDa glycoprotein) antibody (75 μ l of a 1:200 dilution). On the following day, the micro-titre plate was washed three times with PBS containing 0.1 % (v/v) Tween (PBS/Tween), and the remaining binding sites on the wells were blocked by addition of bovine serum albumin (200 μ l of 20 mg/ml solution in PBS) for 1 h at room temperature. After the micro-titre plate had been washed again (three times with PBS/Tween), 50 μ l of the antigen/antibody pre-incubation mix was added to the antigen-coated wells and incubated for a further 1 h at room temperature. Unbound material was removed from the plate by washing with PBS/Tween (three times). Horseradish-peroxidase-conjugated Protein A (50 μ l of a 1:1000 dilution in PBS; Zymed, San Francisco, CA, U.S.A.) was added to the wells and allowed to bind for 1 h at room temperature. After thorough removal of the unbound Protein A conjugate (four washes in PBS/Tween), 150 μ l of horseradish-peroxidase substrate [0.6 mg of *o*-phenylenediamine (BDH Chemicals, Poole, Dorset, U.K.) and 1 μ l of H₂O₂ per ml of sodium citrate buffer, pH 5.5] was added to each well and the colour was allowed to develop for 30 min. The reaction was stopped by addition of 50 μ l of 2.5 M-H₂SO₄. Absorbance in the wells was measured on an e.l.i.s.a. plate reader at 492 nm against a reagent blank to which pre-incubated PBS had been added in place of the antigen-antibody mix. All assays were performed in duplicate. At each stage of the procedure conditions were optimized by investigating variables of time (1–24 h), temperature (23 °C and 37 °C) and relative concentrations of antigen, antibody and Protein A conjugate. Standard curves were constructed by plotting A_{492} in the well (ordinate, linear scale) against the amount of standard antigen in the pre-incubation mix (abscissa, logarithmic scale). The curves were characterized by their B_{50} values (the amount of antigen required to produce half-maximal Protein A binding) and their slope. The slope of the standard curve was normalized to 1.0 and the slopes of test samples were expressed relative to the standard. The 118 kDa-glycoprotein e.l.i.s.a. is an indirect assay in which the measured absorbance in the wells is proportional to the amount of 'free' antibody remaining after the pre-incubation step.

To determine the appropriate concentration of antibody for the e.l.i.s.a., a titre curve was first performed. Serial dilutions of the antibody (50 μ l in PBS, in duplicate) were added in place of the pre-incubation mix to the antigen-coated wells and allowed to bind for 1 h at room temperature. In all other respects the titre curve was carried out as described for the standard assay above. The concentration of antibody required to produce 60–70 % of maximal binding (1:200) was selected for use in the standard assay procedure.

PAGE

SDS/PAGE was carried out by the method of Laemmli (1970). Bands were detected by the Bio-Rad silver stain (Bulletin 1089; Bio-Rad Laboratories) and molecular-

mass assignments were made by comparison with standard proteins (14–200 kDa). In some experiments gels were stained with Coomassie Blue and scanned on a model 620 video densitometer (Bio-Rad Laboratories). Electrophoretic transfer on to nitrocellulose sheets (Western-blot analysis) was performed by the method of Towbin *et al.* (1979), and antigenic bands were detected by the Immun-blot assay with the rabbit antibody preparations [1:800 dilution of the anti-(118 kDa glycoprotein) antibody or 1:1000 dilution of the anti-mucin antibody] followed by goat anti-(rabbit IgG) antibody conjugated to horseradish peroxidase (1:2000 dilution) and peroxidase substrate (as described by Bio-Rad Laboratories). Western blots were also scanned on the densitometer.

Chemical analyses

The protein and glycoprotein contents of fractions from e.d.g.c. or gel-filtration columns were determined by using slot-blot assays. Standards (0–5 μ g of bovine serum albumin or 0–20 μ g of purified mucin in PBS) and test samples were applied to nitrocellulose sheets and stained with either Fast Green (to detect protein) or periodic acid/Schiff reagent (to detect glycoprotein) (Tseng, 1985). Blots were scanned on a densitometer, and standard curves were constructed by plotting the areas of the standard peaks against the amount of protein or glycoprotein applied to the nitrocellulose. The protein or glycoprotein content of unknown samples was determined by reading their peak areas against the standard curve. Slot blots (unstained) were also probed with anti-mucin or anti-(118 kDa glycoprotein) antibodies (as described for Western-blot analyses) in order to detect their respective antigens in gradient or column fractions. Test samples containing detergent, reducing agent or high salt concentrations were analysed by slot-blot assays without prior dialysis. Reducing agents and salts were removed by thorough washing of the nitrocellulose sheets with water, and detergents were removed by rinsing the nitrocellulose briefly in 20 % (v/v) methanol after application of test samples.

Amino acids were quantified on a Beckman 6300 amino acid analyser (Beckman Instruments, Palo Alto, CA, U.S.A.) after hydrolysis for 24 h at 110 °C in 6 M-HCl *in vacuo*. Carbohydrates were quantified by g.l.c. (Chaplin, 1982).

RESULTS

As in earlier studies (Mantle *et al.*, 1948b), the 118 kDa glycoprotein from human intestinal mucin was purified by two-step preparative gel electrophoresis (Fig. 1). Since the chemical composition of the isolated 118 kDa glycoprotein was consistent with that determined previously, an antibody was developed against it in rabbits. On Western blots, the anti-(118 kDa glycoprotein) antibody reacted with the intact native mucin, with the 118 kDa band released from the mucin after reduction and with the purified 118 kDa glycoprotein preparation (Fig. 1, tracks 5–7). No reaction was detected with the mucin glycoprotein monomers (note absence of stain in the upper regions of the gel in Fig. 1, track 6). The antibody produced against the intact native mucin reacted with both the reduced glycoprotein monomers (staining the stacking gel and the upper 2 cm of the running gel) and the 118 kDa glycoprotein (Fig. 1, tracks

Fig. 1. SDS/PAGE and Western blots of purified human intestinal mucin and 118 kDa glycoprotein

Purified mucin and 118 kDa glycoprotein (isolated by preparative gel electrophoresis) were subjected to SDS/PAGE on 7.5% separating gels with 5.7% stacking gels. After electrophoresis, gels were silver-stained. Western blots were treated with either anti-(118 kDa glycoprotein) antibody (1:800 dilution) or anti-mucin antibody (1:1000 dilution), and antigenic bands were detected by the Immun-blot assay. Track 1, molecular-mass marker proteins; tracks 2 and 3, purified human intestinal mucin before and after reduction with 0.2 M-2-mercaptoethanol respectively; track 4, purified 118 kDa glycoprotein; tracks 5-7, Western blots of tracks 2-4 respectively treated with the anti-(118 kDa glycoprotein) antibody; tracks 8-10, Western blots of tracks 2-4 respectively treated with the anti-mucin antibody. Over-development of either gels or blots leads to the appearance of 2-mercaptoethanol artifacts (▲).

8 and 9). However, Western blots of the isolated 118 kDa glycoprotein probed with the anti-mucin antibody only resulted in positive staining at the molecular-mass position of 118 kDa (Fig. 1, track 10).

Mucin immunoreactivity in the e.l.i.s.a.

With the use of the anti-(118 kDa glycoprotein) antibody, an e.l.i.s.a. was established. The assay was highly reproducible, with less than 10% inter-assay variability (Fig. 2). Fourteen purified intestinal mucins produced parallel slopes in the e.l.i.s.a., indicating the presence of identical antigenic determinants with the same affinity for the anti-(118 kDa glycoprotein) antibody (Table 1). However, wide variations were seen in the B_{50} values of the mucins (expressed either as mucin dry weight or as mucin protein), indicating considerable differences in their relative immunoreactivities. In general, it appeared that N mucins were less immunoreactive than CF mucins (i.e. had higher B_{50} values). No relationship was detected by linear-regression analysis between the B_{50} value of a mucin (whether N or CF) and either its protein content or the time *post mortem* that the intestinal tissue was obtained ($P > 0.1$). Delipidation of two mucins with widely different B_{50} values (H13 and

Fig. 2. E.l.i.s.a. standard curve

The e.l.i.s.a. standard curve was constructed with the use of H24 mucin (0-80 ng) and a 1:200 dilution of the anti-(118 kDa glycoprotein) antibody. B_{50} indicates the amount of mucin required to produce half-maximal binding of horseradish-peroxidase conjugated Protein A and, hence, antibody. Bars indicate the S.E.M. for eight independent analyses.

CF4) changed neither their slope nor their B_{50} value in the e.l.i.s.a., suggesting that differences in their immunoreactivity were not due to the presence of lipid.

Assessment of the 118 kDa glycoprotein content of mucins

Densitometric analysis of Coomassie Blue-stained SDS/polyacrylamide gels indicated considerable variability in the size of the 118 kDa band produced by different mucin preparations, even though the same amount of material was applied to the gel (Table 1). Assuming that all the 118 kDa glycoproteins stained similarly with Coomassie Blue, the results suggest that different mucins contained different amounts of 118 kDa glycoprotein. However, there was no statistical difference between N and CF mucins in terms of their 118 kDa glycoprotein content ($P > 0.1$). There was no apparent correlation between the 118 kDa glycoprotein content of a mucin and either its protein content or the time *post mortem* that the tissue was acquired ($P > 0.1$ for both N and CF mucins). There was also no significant relationship between the B_{50} value of a mucin in the e.l.i.s.a. and its 118 kDa glycoprotein content.

Assessment of the number of antigenic determinants in 118 kDa glycoproteins

Western blots treated with the anti-(118 kDa glycoprotein) antibody also showed considerable variability in the size of the 118 kDa band from different mucin preparations (Table 1). The area of 118 kDa peak on the blot was not related to the B_{50} value of the mucin ($P > 0.1$ for N and CF mucins), but did correlate significantly with the size of the 118 kDa peak on the Coomassie Blue-stained gel. The linear-regression equation for N mucins was 'Western-blot area = $1.4 \times (\text{Coomassie Blue area}) + 0.7$ ' ($r^2 = 0.87$, $P < 0.01$) and that for CF mucins was very similar ['Western-blot area = $1.2 \times (\text{Coomassie Blue area}) + 0.3$ ' ($r^2 = 0.9$, $P < 0.01$)]. As judged from these data, most of the variability in the 118 kDa peak areas on Western blots was attributable to variations in the amount of 118 kDa

Table 1. Intestinal mucins: immunoreactivity and assessment of their 118 kDa glycoprotein content

Small intestine was obtained from N subjects and patients with CF. Time *post mortem* refers to the time *post mortem* that the intestinal tissue was acquired. Mucins were purified from mucosal homogenates by e.d.g.c. and gel filtration on Sepharose 2B. Protein content refers to their protein contents determined from amino acid analyses. Each purified mucin was analysed in the e.l.i.s.a. twice, in duplicate: slope refers to their slopes, expressed relative to the slope of the standard mucin (H24) used in every assay, and B_{50} refers to the amount of mucin required to produce half-maximal antibody binding. After reduction, mucins (500 μ g) were subjected to SDS/PAGE and gels were stained with Coomassie Blue and scanned on a laser densitometer: values refer to the area under the 118 kDa peak. Western blots, treated with the anti-(118 kDa glycoprotein) antibody, were also scanned on the densitometer: values refer to the area under the 118 kDa peak. Abbreviation: N.A., not analysed.

	Time <i>post mortem</i> (h)	Protein content (%)	E.l.i.s.a.		Size of 118 kDa band (<i>A</i> ·mm ²)	
			Slope	<i>B</i> ₅₀ (ng of mucin)	On polyacrylamide gels	On Western blots
N mucins:						
H24	5	14.6	1.0	5	5.4	10.9
H35	4.5	10.0	0.9	5	5.8	7.6
H11	12	15.6	1.2	580	6.3	12.2
H21	5.5	12.0	1.2	1400	10.3	14.3
H20	7	19.5	1.0	10000	12.6	16.5
H14	4	12.4	1.0	90000	N.A.	N.A.
H13	4	7.0	1.0	120000	4.3	3.9
GM	3	18.0	1.0	120000	4.9	8.1
CF mucins:						
CF2	7	20.0	0.7	0.2	3.4	4.0
JK	2	15.0	1.0	0.5	3.0	2.5
CF1	11	18.4	0.8	0.7	3.4	4.9
CF3	5.5	13.5	0.8	0.7	8.4	9.0
CF4	7	7.0	0.9	9.0	1.3	2.9
JR	12	18.0	1.0	800	7.3	10.7

glycoprotein present in N and CF mucins. However, a proportion also appeared to be due to differences in the number of antigenic determinants present within individual 118 kDa glycoproteins.

Large-scale isolation of the 118 kDa glycoprotein

In view of the results obtained from Coomassie Blue-stained gels and Western blots, it was desirable to recover purified 118 kDa glycoproteins quantitatively from N and CF mucins and compare their immunoreactivities. Quantitative recovery cannot be accomplished by two-step preparative gel electrophoresis because of the number of manipulations involved in the procedure. However, 118 kDa glycoproteins isolated from two mucins (H35 and CF3) by this technique were analysed in the e.l.i.s.a. The slopes of both preparations were parallel to the standard mucin, and B_{50} values of 3 and 1.2 ng of amino acid protein were obtained for the H35 and CF3 118 kDa glycoproteins respectively. We then assessed other potential isolation procedures for the 118 kDa glycoprotein.

E.d.g.c. in CsCl. In previous studies (Mantle *et al.* 1984b), e.d.g.c. in CsCl (starting density 1.4 g/ml) was used to isolate the 118 kDa glycoprotein. However, owing largely to problems encountered with the solubility of the 118 kDa glycoprotein on removal of the salt, it was not possible to determine whether separation from the glycoprotein monomers was complete or what proportion of the 118 kDa glycoprotein was recovered. We attempted to improve on those earlier experiments by seeking conditions that would (a) ensure complete

separation of the component parts of the mucin macromolecule and (b) maintain the solubility of the 118 kDa glycoprotein. Centrifugation was therefore carried out with the use of a lower starting density (1.35 g/ml) to create a shallower gradient, and fractions were dialysed against 10 mM-2-mercaptoethanol to preserve reducing conditions. Despite these modifications, analysis of gradient fractions on SDS/PAGE and Western blots (Fig. 3, tracks 2–9) showed that most of the 118 kDa glycoprotein banded in the same position (fractions 5–8) as the mucin glycoprotein monomers (ρ 1.35–1.45 g/ml). Only very small amounts of 118 kDa glycoprotein were detected in the lower-density fractions of the gradient (ρ 1.24–1.32 g/ml). If gradient fractions were dialysed briefly against distilled water before electrophoresis, the 118 kDa glycoprotein barely penetrated the stacking gel (Fig. 3, compare tracks 8 and 9 with tracks 11 and 12). Incorporation of 4 M-guanidinium chloride into the CsCl gradient resulted in recovery of both the 118 kDa glycoprotein and the mucin glycoprotein monomers in fractions of density 1.3–1.37 g/ml (Fig. 4). Alkylation of the reduced mucin before e.d.g.c. (with or without guanidinium chloride) had no effect on the distribution of the 118 kDa glycoproteins and the mucin glycoprotein monomers on the gradient (results not shown).

Since dialysis of gradient fractions against water resulted in loss of the 118 kDa band on SDS/PAGE and Western blots, we wondered whether aggregation of the 118 kDa glycoprotein (or re-association with the mucin glycoprotein monomers) occurred by thiol-dependent and/or -independent mechanisms. Therefore a sample of

Fig. 3. SDS/PAGE and Western blots of fractions from CsCl density gradients of reduced intestinal mucin

Purified mucin (H14) was reduced with 0.2 M-2-mercaptoethanol for 5 min at 100 °C and subjected to e.d.g.c. in CsCl (starting density 1.35 g/ml) in the presence of 10 mM-2-mercaptoethanol. Fractions from the gradient were dialysed against either 10 mM-2-mercaptoethanol or distilled water for approx. 3 h before SDS/PAGE on 7.5% separating gels with 5.7% stacking gels. After electrophoresis, gels were silver-stained. Western blots of the gels were treated with anti-mucin antibody (1:1000 dilution) and antigenic bands were detected by the Immun-blot assay. Track 1, molecular-mass marker proteins; tracks 2-9, fractions 1 (low density) to 8 (high density) respectively from the CsCl gradient after dialysis against 10 mM-2-mercaptoethanol; track 10, Western blot of fraction 6 from the density gradient after dialysis against 10 mM-2-mercaptoethanol; tracks 11 and 12, fractions 6 and 7 respectively from the density gradient after dialysis against distilled water; tracks 13 and 14, Western blots of tracks 11 and 12 respectively.

mucin was reduced with 0.2 M-2-mercaptoethanol and divided into three portions. One portion was alkylated, dialysed for 3 h against distilled water and analysed on SDS/PAGE and Western blots. The other two portions were dialysed against either 10 mM-2-mercaptoethanol or distilled water for the same length of time before analysis. Both the alkylated and non-alkylated mucin samples that were dialysed against distilled water showed essentially no 'free' 118 kDa glycoprotein on the gels; only the mucin that was maintained in reducing conditions contained 'free' 118 kDa glycoprotein (Fig. 5). If the water-dialysed samples were boiled in SDS (or reduced again in the case of non-alkylated mucin) immediately before PAGE, 'free' 118 kDa glycoprotein could often (but not always) be seen on gels. It appeared to become much more difficult to release the 118 kDa glycoprotein from previously reduced mucins after extended periods of dialysis against water (approx. 24 h).

Gel-filtration chromatography. Although previous attempts to purify the 118 kDa glycoprotein of human intestinal mucin by chromatography on Sepharose 4B

Fig. 4. SDS/PAGE and Western blots of reduced intestinal mucin after e.d.g.c. in CsCl containing 4 M-guanidinium chloride

Purified mucin (H14) was reduced with 0.2 M-2-mercaptoethanol for 5 min at 100 °C and subjected to e.d.g.c. in CsCl (starting density 1.35 g/ml) containing 4 M-guanidinium chloride and 10 mM-2-mercaptoethanol. Fractions from the gradient were dialysed against 10 mM-2-mercaptoethanol for approx. 3 h before SDS/PAGE on 7.5% separating gels with 5.7% stacking gels. Gels were silver-stained. Western blots of the gels were treated with anti-mucin antibody (1:1000 dilution), and antigenic bands were detected by the Immun-blot assay. Track 1, molecular-mass marker proteins; tracks 2-9, fractions 1 (low density) to 8 (high density) respectively from the gradient; tracks 10 and 11, Western blots of fractions 1 and 2 respectively from the density gradient.

were unsuccessful (Mantle *et al.*, 1984b), rat intestinal mucin has been fractionated into its component parts on Sepharose 2B (Fahim *et al.*, 1983). Therefore reduced human intestinal mucin was applied to a Sepharose 2B column eluted with 10 mM-2-mercaptoethanol in 5% SDS. By use of slot-blot assays reaction with the anti-mucin antibody was observed in eluate fractions 45-100, whereas reaction with the anti-(118 kDa glycoprotein) antibody occurred in fractions 85-100 (excluded and included volumes of the column were fractions 30-40 and 110-120 respectively). Although fractions 85-100 were enriched in the 118 kDa glycoprotein, some mucin glycoprotein monomers were also present (Fig. 6, tracks 2 and 3). Re-chromatography of fractions 85-100 on a TSK G5000PW column only slightly improved the separation of the 118 kDa glycoprotein and the remaining mucin glycoprotein monomers (Fig. 6). Other gel-filtration matrices (such as Sephadex G150, Sepharose 4B or Sepharose 2B), eluted with low or high concentrations of detergent in the presence or in the absence of reducing agent, were also incapable of resolving the 118 kDa glycoprotein from the smaller mucin monomers.

Con A affinity chromatography. Since the 118 kDa glycoprotein is known to contain mannose, suggesting

Fig. 5. SDS/PAGE of reduced intestinal mucin after dialysis against 2-mercaptoethanol or water

Purified mucin was reduced with 0.2 M-2-mercaptoethanol for 5 min at 100 °C. Portions were dialysed against 10 mM-2-mercaptoethanol or distilled water for 3 h. The remainder of the reduced mucin was alkylated by addition of 0.5 M-iodoacetamide (16 h at 4 °C in the dark) and dialysed against water for a comparable length of time. Mucin samples were then subjected to SDS/PAGE on 7.5% separating gels with 5.7% stacking gels. After electrophoresis, gels were silver-stained. Track 1, molecular-mass marker proteins; track 2, reduced mucin after dialysis against 10 mM-2-mercaptoethanol; track 3, reduced mucin dialysed against water and re-reduced with 0.2 M-2-mercaptoethanol (5 min at 100 °C) immediately before electrophoresis; track 4, reduced mucin after dialysis against water (non-reduced on the gel); track 5, reduced and alkylated mucin after dialysis against water (non-reduced on the gel). Samples in tracks 2, 4 and 5 were applied to gels without prior heating to 100 °C. ▲, 2-Mercaptoethanol artifacts.

the presence of *N*-linked oligosaccharide chains (Mantle *et al.*, 1984b), we speculated that it may be capable of interacting with Con A. Therefore reduced and alkylated mucins (two N and two CF) were subjected to Con A affinity chromatography in the presence of SDS. From Western-blot analyses it appeared that all the glycoprotein monomers from the four mucins were recovered in the non-bound fractions of the column, but the 118 kDa glycoprotein was distributed among the non-bound, retarded and bound fractions (Fig. 7). Densitometric analysis of blots treated with the anti-(118 kDa glycoprotein) antibody indicated that over 70% of the 118 kDa glycoprotein present in the original mucins was recovered from the Con A column, with (on average) 36%, 24% and 40% occurring in the non-bound, retarded and bound fractions respectively. After re-chromatography of the non-bound fractions on Affi-Gel-Con A, only a further 10% of the 118 kDa glycoprotein separated into the retarded and bound fractions from the column.

The chemical compositions of the Con A-non-bound and -bound fractions from two mucins are shown in Table 2. Compared with native mucins, the Con A-bound fractions had a higher content of acidic and aliphatic amino acids and mannose but a lower content of *N*-acetylgalactosamine. Similar compositional differences between native mucins and their 118 kDa glycoproteins purified by gel electrophoresis have been noted previously (Mantle *et al.*, 1984b). The non-bound fractions from the Con A column had an increased serine/threonine/proline content and a decreased content of all other amino acids compared with the native mucins. The carbohydrate profiles of the non-bound fractions differed only slightly (notably in the *N*-acetylgalactosamine content) from those of the native mucins. The Con A-retarded fractions (containing approx. 7% of the protein applied to the column for all four mucins) had an overall chemical composition very similar to that of the non-bound fractions (results not shown).

The amount of protein recovered in the Con A-bound fractions from all four mucins (Table 3) reflected their 118 kDa glycoprotein content assessed from Coomassie Blue-stained polyacrylamide gels and Western blots treated with the anti-(118 kDa glycoprotein) antibody (Table 1). Of the four mucins analysed by Con A chromatography, the ones that yielded the largest and smallest amounts of 118 kDa glycoprotein in the bound fractions also gave respectively the strongest-staining (CF3) and the weakest-staining (CF4) 118 kDa band on gels and blots. Since the Con A-bound fractions from the four mucins contained between 1.5% and 7% of the protein applied to the column and also approx. 40% of the 118 kDa glycoprotein present in the original mucins, then the 118 kDa glycoprotein apparently constituted 4–17% of the total mucin protein (equivalent to 1.1–4.6% by wt. of the total mucin, taking into account the carbohydrate moieties).

The relative immunoreactivities of the isolated 118 kDa glycoproteins in the e.l.i.s.a. partially reflected the immunoreactivities of their respective native mucins (Table 3). Three preparations derived from highly immunoreactive mucins reacted strongly in the e.l.i.s.a., whereas one obtained from a very poorly immunoreactive mucin (H13) showed less reactivity in the immunoassay. However, in the latter case the immunoreactivity of the purified 118 kDa glycoprotein was proportionately much greater than the immunoreactivity of the native mucin, suggesting that its lower content of antigenic determinants was not entirely responsible for the very weak antigenicity of the mucin.

DISCUSSION

In the present study, 14 highly purified human intestinal mucins (eight N and six CF) were compared in an e.l.i.s.a. for their reactivity with a specific antibody developed against the disulphide-bound 118 kDa glycoprotein component. Although all mucins had the same affinity for the antibody, indicating the presence of identical antigenic determinants, considerable variability was observed in their relative immunoreactivities. In general, CF mucins were more antigenic than N mucins, although two N mucins showed comparably high levels of immunoreactivity. The variable antigenicity of mucins was surprising, but did not appear to be due to degradation *in vitro* and loss of mucin protein, since (a)

Fig. 6. SDS/PAGE and Western blots of reduced mucin after Sepharose 2B chromatography and h.p.l.c.

Purified mucin was reduced with 0.2 M-2-mercaptoethanol (5 min at 100 °C in 5 % SDS) and applied to a Sepharose 2B column eluted with 10 mM-2-mercaptoethanol and 5 % SDS. Fractions 45–84 and 85–100 were pooled and analysed on Western blots reacted with the anti-mucin antibody (1:1000 dilution). Antigenic bands were detected by the Immun-blot assay. Fractions 85–100 were then applied to a TSK G5000PW column eluted with 10 mM-2-mercaptoethanol in 0.25 % SDS. Eluate fractions were subjected to SDS/PAGE on 7.5 % separating gels with 5.7 % stacking gels. Gels were silver-stained. Western blots of eluate fractions were performed as above. Track 1, molecular-mass marker proteins; track 2, Western blot of fractions 45–84 from the Sepharose 2B column; track 3, Western blot of fractions 85–100 from the Sepharose 2B column; tracks 4–10, fractions 14–20 respectively from the TSK G5000PW column; tracks 11–17, Western blots of tracks 4–10 respectively. ▲, 2-Mercaptoethanol artifacts.

Table 2. Affinity chromatography of intestinal mucin on Con A: composition of fractions

Reduced and alkylated mucins (10 mg) were applied to an Affi-Gel-Con A column. Non-bound fractions were eluted with 10 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl and 0.01 % (w/v) SDS. Bound fractions were recovered by addition of 0.5 M-methyl α -mannoside to the elution buffer. After dialysis and removal of SDS, fractions were analysed for amino acids and monosaccharides. Abbreviations: N.D., not detected.

	Composition (mol/100 mol)					
	H35 Native	Fractions from Con A column		CF3 Native	Fractions from Con A column	
		Non-bound	Bound		Non-bound	Bound
Ser/Thr/Pro	58.5	72.7	33.0	55.0	66.3	32.6
Asp/Glu	10.5	7.8	20.2	12.1	9.6	19.9
Gly/Ala/Val	13.6	9.8	21.0	14.1	11.0	19.8
Leu/Ile	8.8	5.8	9.4	8.5	6.7	10.1
Rest	8.6	3.9	16.4	10.3	6.3	17.6
Fuc	12.1	10.6	8.0	12.6	10.1	10.9
Gal	19.1	16.9	15.8	16.4	15.5	12.6
Man	N.D.	N.D.	9.4	N.D.	N.D.	9.9
GlcNAc	15.4	15.3	19.9	16.4	16.6	18.7
GalNAc	32.5	36.9	26.5	34.6	36.9	25.6
NeuAc	20.9	20.3	20.4	20.0	20.9	22.3

purification was carried out in the presence of proteinase inhibitors, (b) mucins were not exposed to reducing agents during purification and (c) mucins were apparently in their native polymeric state. Whether changes in the mucins *post mortem* contribute to some of the variability is not clear, but no direct relationship was detected between the time *post mortem* that intestinal tissue was

obtained and the immunoreactivity of the isolated mucin, its protein content or its 118 kDa glycoprotein content. Although it has been reported that CF mucins contain more associated lipid than do N mucins and that lipid may modulate the physical properties of mucins (Slomiany *et al.*, 1983, Takagi *et al.*, 1986), we could not attribute the immunological differences of N and CF

Fig. 7. Western blots of fractions from the Affi-Gel-Con A affinity column

Reduced and alkylated mucin (H13; 10 mg in 2 ml) was applied to an Affi-Gel-Con A column. Non-bound fractions (eluted in the first column volume) and retarded fractions (eluted in the next two column volumes) were collected. Bound material was removed from the column by addition of 0.5 M-methyl α -mannoside to the elution buffer. Non-bound, retarded and bound fractions were each concentrated down to 2 ml, and 40 μ l portions were subjected to SDS/PAGE on 10% separating gels with 5.7% stacking gels. Western blots of gels were treated with anti-mucin antibody (1:1000 dilution; tracks 1–4) or anti-(118 kDa glycoprotein) antibody (1:800 dilution; tracks 5–8), and antigenic bands were detected by the Immunoblot assay. Tracks 1 and 5, reduced and alkylated mucin (10 μ l portion) before Con A chromatography; tracks 2 and 6, non-bound fractions from the Con A column; tracks 3 and 7, retarded fractions from the Con A column; tracks 4 and 8, bound fractions from the Con A column. ▲, 2-Mercaptoethanol artifacts.

mucins to the presence of lipid. To examine the reasons for the differences in mucin antigenicity, we investigated the amount of 118 kDa glycoprotein present in individual mucins and the number of antigenic determinants within the 118 kDa glycoprotein itself.

The 118 kDa glycoprotein content of mucins (both N and CF) varied considerably when assessed from Coomassie Blue-stained SDS/polyacrylamide gels. However, mucins that reacted strongly in the e.l.i.s.a. did not always contain more 118 kDa glycoprotein than weakly reacting mucins. Since the 118 kDa glycoprotein content of mucins varied over a 10-fold range whereas mucin immunoreactivity in the e.l.i.s.a. varied approx. 100000-fold, it seemed unlikely that variations in

Table 3. Recovery and immunoreactivity of 118 kDa glycoprotein in bound fractions from the Affi-Gel-Con A column

Reduced and alkylated mucins (10 mg) were applied to an Affi-Gel-Con A column and bound material (118 kDa glycoprotein) was recovered as described in Table 2. After dialysis and extraction of SDS, the amount of protein (μ g) in the bound fraction was determined from amino acid analyses. Dialysed and extracted material was also analysed in the e.l.i.s.a.: slopes are expressed relative to the standard mucin (H24) used in the assay, and B_{50} values are given in ng of protein (from amino acid analysis).

	H13	H35	CF3	CF4
Protein (μ g)	21.53	44.8	91.45	10.3
Slope	0.9	1.0	1.0	0.8
B_{50} (ng of protein)	2000.0	4.0	2.0	5.9

118 kDa glycoprotein content were, on their own, responsible for the differences in immunoreactivity.

On Western blots treated with the anti-(118 kDa glycoprotein) antibody, variations (approx. 6-fold) were observed in the size of the 118 kDa band produced by N and CF mucins. This largely resulted from differences in the amount of 118 kDa glycoprotein present in the mucins, although some of the variability also appeared to arise from differences in the number of antigenic determinants within individual 118 kDa glycoproteins. Since, under the reducing and denaturing conditions of a blot, the reactivity of 'free' 118 kDa glycoproteins with the antibody did not correlate with (or vary to the same extent as) the reactivity of native N and CF mucins in the e.l.i.s.a., it seemed likely that changes in the accessibility of the 118 kDa glycoprotein for antibody binding may be responsible for the differences in N mucin and CF mucin antigenicity in the immunoassay.

To investigate further the 118 kDa glycoprotein content of mucins and its antigenic properties, we wanted to isolate it quantitatively from the mucin glycoprotein monomers. Since this is not possible by two-step preparative electrophoresis owing to the complexity of the procedure, we attempted to use techniques of gel filtration and e.d.g.c. Considerable problems were encountered during these experiments as a result of (1) strong non-covalent interactions between the component parts of the mucin, (2) the broad size heterogeneity of the mucin glycoprotein monomers, (3) the high buoyant density of the 118 kDa glycoprotein, and (4) the ability of the 118 kDa glycoprotein to aggregate or re-associate with the mucin glycoprotein monomers via the formation of disulphide bonds. Thus gel filtration of reduced mucin on Sepharose 2B eluted with buffer containing 2-mercaptoethanol but no SDS resulted in poor separation of the mucin glycoprotein monomers and the 118 kDa glycoprotein (M. Mantle & G. Stewart, unpublished work). Although elution with detergent as well as 2-mercaptoethanol improved separation, a small proportion of the mucin monomers was still co-eluted with the 118 kDa glycoprotein, and these could not be resolved by re-chromatography on Sepharose 2B or other gel-filtration matrices, regardless of the concentration of detergent or reducing agent present.

In earlier experiments (Mantle *et al.*, 1984b) it appeared

that the 118 kDa glycoprotein had separated from the reduced mucin glycoprotein monomers on e.d.g.c. because none was detected by SDS/PAGE in the higher-density (monomer-containing) fractions. However, gradient fractions were analysed after prolonged dialysis against water, which was clearly shown in the present studies to cause self-aggregation of the 118 kDa glycoprotein and/or re-association with the mucin glycoprotein monomers. Since aggregation/re-association was largely reversed by reduction and was not prevented by alkylation, then it is likely that the phenomenon can occur by either thiol-dependent (covalent) or thiol-independent (non-covalent) mechanisms. In order to avoid aggregation of the 118 kDa glycoprotein (particularly in the absence of detergent or denaturant), it was necessary to maintain reducing conditions. When the solubility of the 118 kDa glycoprotein was preserved after e.d.g.c., most of it was found in the same fractions as the mucin monomers. Since mucin was boiled in 0.2 M-2-mercaptoethanol for 5 min before e.d.g.c. and reducing agent was present throughout the 72 h centrifugation step, it is unlikely that the lack of separation on the gradient was due to incomplete reduction. Evidently therefore, the carbohydrate content of the 118 kDa glycoprotein (approx. 50% by wt.; Mantle *et al.*, 1984b) is sufficiently high to give the molecule a buoyant density comparable with that of the mucin glycoprotein monomers. As in earlier studies, a small amount of purified 118 kDa glycoprotein could be isolated by e.d.g.c. (from fractions immediately above the mucin glycoprotein monomers), but recovery was unacceptably low for the purposes of our present experiments.

Although an improvement over e.d.g.c. and gel filtration, separation of the component parts of mucin by affinity chromatography on Con A was still only partially successful, since the 118 kDa glycoprotein distributed in the non-bound fractions (with the majority of the mucin glycoprotein monomers) and in the retarded and bound fractions from the column. Our results were not due to incomplete reduction or to the occurrence of non-covalent interactions with the mucin monomers (SDS was present throughout the procedure) or to saturation of binding sites on the column (since only a further 10% of the 118 kDa glycoprotein was bound on re-chromatography of the non-bound fractions). Therefore it seems that there may be microheterogeneity within the 118 kDa glycoprotein, with some molecules containing either no *N*-linked sugar chains or, more probably, *N*-linked chains with different structures.

The mucin glycoprotein monomers (non-bound by the Con A column) showed changes in their amino acid profile (compared with the native mucin) consistent with the removal of some 118 kDa glycoprotein, namely an increased content of serine, threonine and proline and a decreased content of all other amino acids. Surprisingly, in view of the appearance of Western blots showing only a strongly stained 118 kDa band, the retarded fractions also largely comprised mucin glycoprotein monomers. Since the amount of material recovered in the retarded fractions was only small, it seems probable that glycoprotein monomers were eluted in this position as a result of trailing of the non-bound peak and that our failure to detect their presence on Western blots reflected inadequate loading.

The 118 kDa glycoprotein recovered in the bound

fractions from the Con A column had a chemical composition similar to that of 118 kDa glycoprotein purified by preparative electrophoresis. The amount of 118 kDa glycoprotein isolated from four mucins was variable but proportional to the size of their 118 kDa bands on Coomassie Blue-stained polyacrylamide gels and Western blots, thus confirming that individual mucins contained different amounts of 118 kDa glycoprotein. It appeared from our calculations that, in the four mucins subjected to Con A affinity chromatography, the 118 kDa glycoprotein represented between 4 and 17% of the mucin protein (or approx. 1.1–4.6% by wt. of the total mucin). Since other mucins contained more 118 kDa glycoprotein (as judged from SDS/PAGE), then it is likely that the 118 kDa glycoprotein can constitute up to approx. 26% of the mucin protein. This value is in the same range as that estimated previously (approx. 30%) from differences in the carbohydrate/protein ratios of two mucins and their reduced glycoprotein monomers isolated from a density gradient or a Sepharose 4B column (Mantle *et al.*, 1984b).

Three 118 kDa glycoproteins isolated by Con A chromatography from highly immunoreactive mucins (H35, CF3 and CF4) had almost identical immunoreactivity in the e.l.i.s.a., with the same number of antigenic determinants. In two cases (CF3 and H35) the 118 kDa glycoproteins also had very similar antigenic properties to the same 118 kDa glycoproteins isolated by preparative gel electrophoresis, suggesting that antigenicity was unaffected by the purification procedure. By comparison, the 118 kDa glycoprotein isolated from a poorly immunoreactive mucin (H13) was not very reactive in the e.l.i.s.a., having fewer antigenic determinants. Interestingly, this particular mucin also produced a smaller 118 kDa band on a Western blot than would be expected from the size of the 118 kDa band on a Coomassie Blue-stained gel, a finding also consistent with the presence of fewer antigenic determinants in the 118 kDa glycoprotein. Despite the decreased numbers of antigenic determinants, the immunoreactivity of H13 mucin in the e.l.i.s.a. was still relatively lower than would be expected from the immunoreactivity of its isolated 118 kDa glycoprotein, suggesting that other factors contributed to the diminished antigenicity of the native mucin.

Taking into consideration differences in the 118 kDa glycoprotein content of individual mucins (assessed either from the amount recovered after Con A chromatography or from SDS/PAGE of mucins), together with differences in the number of antigenic determinants in the 118 kDa glycoprotein (assessed from Western blots or the immunoreactivity of isolated 118 kDa glycoproteins), we could not account for the large variations in mucin immunoreactivity in the e.l.i.s.a. Therefore it seems that the immunoreactivity of native mucins may depend on the accessibility of the 118 kDa glycoprotein (and its antigenic determinants) to the antibody. This suggests that the three-dimensional conformation of mucins can vary and that CF mucins in general may differ in macromolecular structure from N mucins, possibly being more 'extended' to allow greater antibody binding. Such a conformational change may contribute to the abnormal visco-elastic properties often noted in CF mucus secretions.

It is not yet clear how or why the immunological and

apparent conformational difference arose between N and CF mucins, but a number of possible causes have been considered, including changes in mucin biosynthesis, proteolysis *in vivo* and proteolysis *post mortem*. (1) The presence of longer carbohydrate chains (Wesley *et al.*, 1983) may alter the conformation of CF mucin at the level of biosynthesis. (2) Diminished production of pancreatic digestive enzymes in CF patients may decrease proteolysis *in vivo* and lead to the isolation of a more intact mucin. Increased sulphation (Morrissey & Tymvios, 1978) may also decrease proteolytic digestion of CF mucins *in vivo* (Slomiany *et al.*, 1985). Since, however, all our mucins were purified from tissue homogenates (not from secretions), one would not perhaps expect them to be affected by luminal enzyme activities. Alternatively, greater release of cellular proteinases from a less healthy CF mucosa may increase proteolysis of intestinal mucin *in vivo* and thereby induce an alteration in the conformation of the molecule. This also seems unlikely, as there is no evidence for histological change or damage in the intestinal mucosa in CF that may increase release of cellular proteinases (Morrissey & Tymvios, 1978; Neutra & Trier, 1978). (3) Since there is no reason to believe that the facts controlling mucin degradation *post mortem* differ in N and CF tissue, and since the immunoreactivity, protein content and 118 kDa glycoprotein content of a mucin did not correlate with the time *post mortem* that the intestinal tissue was acquired, it seems unlikely that proteolysis occurring after death but before mucin isolation caused a conformational change in CF mucins. If one also takes into consideration that the protein and 118 kDa glycoprotein contents of CF and N mucins were not statistically different, that all mucins apparently maintained their polymeric structure, and that low-molecular-mass digestion products (peptides other than the 118 kDa glycoprotein) were not detected on silver-stained polyacrylamide gels after reduction of the purified mucins, it becomes very difficult to attribute the immunological and conformational differences between N and CF mucins to proteolysis either *in vivo* or *post mortem*. Although some of the variability in the protein and 118 kDa glycoprotein contents of mucins may well have been introduced by proteolysis *in vivo* or *post mortem*, the cause of the altered conformation of CF mucins must await future studies.

Since the function of the 118 kDa glycoprotein and the three-dimensional conformation of intestinal mucin are not yet known, it is difficult to explain fully variation in 118 kDa glycoprotein content in terms of the overall structure of mucins. If the 118 kDa glycoprotein functions as a centrally located 'link' component holding together a fixed number of mucin glycoprotein monomers, then less variability in the amount present in different native mucin preparations (whether N or CF) would be expected. Possibly, therefore, the number of glycoprotein monomers attached to the 118 kDa glycoprotein may vary among mucins. Alternatively, the number of glycoprotein monomers per mucin polymer may be constant but the proportion of polymers containing a 118 kDa glycoprotein may vary. In this case, mucin polymerization would have to occur by disulphide bonding of the glycoprotein monomers with

the 118 kDa glycoprotein attached to only some molecules, perhaps helping to stabilize the polymer. Further studies are now required to elucidate the role of the 118 kDa glycoprotein and to determine whether and how it affects the physical properties of mucin. Such studies may provide insight into the macromolecular structure of mucins, and may be particularly important in view of the suggestion from the data reported here that mucin conformation may be different in CF.

We thank Dr. J. F. Forstner and Dr. G. G. Forstner (Hospital for Sick Children, Toronto, Ont., Canada) for supplying mucins JK, JR and GM used in this study. Financial support was provided by the Canadian Cystic Fibrosis Foundation and the Alberta Heritage Foundation for Medical Research.

REFERENCES

- Chaplin, M. R. (1982) *Anal. Biochem.* **123**, 336–341
- Fahim, R. E. F., Forstner, G. G. & Forstner, J. F. (1983) *Biochem. J.* **209**, 117–124
- Fahim, R. E. F., Specian, R. D., Forstner, G. G. & Forstner, J. F. (1987) *Biochem. J.* **243**, 631–640
- Henderson, L. E., Oroszlan, S. & Konigsberg, W. (1979) *Anal. Biochem.* **93**, 153–157
- Hinegardner, R. T. (1971) *Anal. Biochem.* **39**, 197–201
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Mantle, M. & Thakore, E. (1988) *Biochem. Cell Biol.* **66**, 1045–1054
- Mantle, M., Mantle, D. & Allen, A. (1981) *Biochem. J.* **195**, 277–285
- Mantle, M., Forstner, G. G. & Forstner, J. F. (1984a) *Biochem. J.* **217**, 159–167
- Mantle, M., Forstner, G. G. & Forstner, J. F. (1984b) *Biochem. J.* **224**, 345–354
- Morrissey, S. M. & Tymvios, M. C. (1978) *J. Pathol.* **126**, 197–208
- Narasimhan, S., Wilson, J. R., Martin, E. & Schachter, H. (1979) *Can. J. Biochem.* **57**, 83–96
- Neutra, M. R. & Trier, J. S. (1978) *Gastroenterology* **75**, 701–710
- Pearson, J. P., Allen, A. & Parry, S. (1981) *Biochem. J.* **197**, 155–162
- Qureshi, R., Forstner, G. G. & Forstner, J. F. (1979) *J. Clin. Invest.* **64**, 1149–1156
- Ringler, N. J., Selvakumar, R., Woodward, H. D., Simet, I. M., Bhavanandan, V. P. & Davidson, E. A. (1987) *Biochemistry* **26**, 5322–5328
- Slomiany, A., Slomiany, B. L., Witas, H., Aono, M. & Newman, L. J. (1983) *Biochem. Biophys. Res. Commun.* **113**, 286–293
- Slomiany, B. L., Laszewicz, W. & Slomiany, A. (1985) *Scand. J. Gastroenterol.* **20**, 857–860
- Takagi, A., Slomiany, A. & Slomiany, B. L. (1986) *Ann. N. Y. Acad. Sci.* **463**, 154–156
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Tseng, S. C. G. (1985) *Ophthalmology* **92**, 728–733
- Wesley, A., Forstner, J. F., Qureshi, R., Mantle, M. & Forstner, G. G. (1983) *Pediatr. Res.* **17**, 65–69
- Zanetta, J.-P. & Gombos, G. (1976) in *Concanavalin A as a Tool* (Bittiger, H. & Schnebli, H. P., eds.), pp. 389–398, John Wiley and Sons, London and New York