Antigenic and structural features of goblet-cell mucin of human small intestine

Michèle MANTLE, Gordon G. FORSTNER and Janet F. FORSTNER

Research Institute, Hospital for Sick Children, and Department of Biochemistry, University of Toronto,
Toronto, Ont. M5G 1X8, Canada

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With the use of a newly developed solid-phase radioimmunoassay method, the major antigenic determinants of human small-intestinal goblet-cell mucin were investigated and related to the overall tertiary structure of the mucin. Preliminary hapten inhibition studies with various oligosaccharides of known sequence and structure suggested that the determinants did not reside in carbohydrate. Exhaustive thiol reduction, however, almost abolished antigenicity, caused breakdown of the mucin into small heterogeneous glycopeptides, and liberated a 'link' peptide of M_r 118 000. Western 'blots' of reduced mucin from polyacrylamide gels on to nitrocellulose sheets showed that a small amount of residual antigenicity remained in large- M_r glycopeptides (M_r > 200 000). The 'link' peptide was not antigenic. Timed Pronase digestion of native mucin resulted in a progressive loss of antigenic determinants. Gel electrophoresis revealed that after 8 h of digestion the 118 000- M_r peptide had disappeared, whereas antigenicity, which was confined to large- M_r glycopeptides, was destroyed much more slowly with time (70% by 24 h, 100% by 72 h). Despite the loss of antigenicity, 72 h-Pronase-digested glycopeptides retained all of the carbohydrate of the native mucin. Therefore the antibody to human small-intestinal mucin appears to recognize a 'naked' (non-glycosylated and Pronase-susceptible) peptide region(s) of mucin glycopeptides. For full antigenicity, however, disulphide bonds are required to stabilize a specific three-dimensional configuration of the 'naked' region.

Human small-intestinal mucin is synthesized in and secreted by goblet cells of the small intestine as a large- M_r glycoprotein complex consisting of oligosaccharide side chains (> 70% by weight) attached to a peptide core. The carbohydrate is distinguished by its lack of mannose and the presence of *O*-glycosidic linkages between *N*-acetylgalactosamine on the oligosaccharide chains and threonine (or serine) residues in the peptide. The peptide is distinguished by the prominence of serine, threonine and proline residues (Jabbal *et al.*, 1976; Wesley *et al.*, 1983).

There is a growing recognition that mucins are relevant to the pathophysiology of intestinal diseases such as chronic inflammatory bowel disease (Clamp *et al.*, 1981; Ehsanullah *et al.*, 1982a,b), secretory diarrhoeas of bacterial and parasitic origin (Sherr *et al.*, 1979; Miller & Yawa, 1979; Forstner *et al.*, 1981), gastric and duodenal ulceration (Younan *et al.*, 1982), cholelithiasis (Lee, 1981; Lee *et al.*, 1981), colonic adenocarcino-

ma (Gold & Miller, 1978; Filipe & Fenger, 1979; Boland *et al.*, 1982a,b) and mucus obstruction in cystic fibrosis (di Sant'Agnese *et al.*, 1981; Wesley *et al.*, 1983).

In previous studies we have shown that an antibody developed in rabbits against purified goblet-cell mucin of human small intestine can be localized by immunofluorescence specifically to goblet cells. By using a double-antibody radioimmunoassay method we further demonstrated that the antibody was highly specific to human small-intestinal mucin, giving weak or no cross-reactivity with mucins of other organs and species, and no reaction with brush-border membranes, connective tissues or human serum (Qureshi *et al.*, 1979).

The specificity of the antibody indicated that human mucin contains unique antigenic determinants not shared by other mucins of similar general composition and size, such as rat (Fahim *et al.*, 1983) or pig (Mantle & Allen, 1981) small-intestinal mucins. With the use of a competitive solid-phase radioimmunoassay method, we under-

Abbreviation used: SDS, sodium dodecyl sulphate.

took the present study to determine whether these unique determinants reside in the three-dimensional configuration of the mucin molecule or its carbohydrate or peptide moieties. Our findings suggest that antigenicity resides in 'naked' peptide segments within the core of large mucin glycopeptide components.

Methods

Preparation of human mucin

Human small intestine was obtained within 8 h *post mortem* from patients with cystic fibrosis or others having no known intestinal disease. Goblet-cell mucin was isolated and purified by the method of Wesley *et al.* (1983) or Mantle & Allen (1981). In both cases the final mucin samples were highly purified, as judged by SDS/polyacrylamide-gel electrophoresis and the absence of mannose and uronic acid. Protein constituted approx. 10–16% by weight, as shown earlier by Jabbal *et al.* (1976).

Radioimmunoassay of mucin

The antibody to mucin was a 40%-(w/v)- $(\text{NH}_4)_2\text{SO}_4$ fraction of immune rabbit sera described and used earlier in a double-antibody radioimmunoassay (Qureshi *et al.*, 1979).

A more convenient solid-phase radioimmunoassay method was used in the present study, and the optimum procedure is outlined in Table 1. At each stage of the procedure, conditions were optimized by investigating variables of time (0–24 h), temperature (4–37°C) and relative concentrations of antigen and antibody. All solutions were made in phosphate-buffered saline (0.1 M-NaCl/0.1 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4) containing 0.02% NaN_3 . Radioactivities in wells were counted in a Beckman 8000 γ -radiation counter. Standard curves were constructed by plotting ^{125}I radioactivity (c.p.m.) bound to the wells against the amount of mucin antigen (protein) added to the preincubation mixture. The curves were characterized by their B_{50} values (the amount of mucin antigen required to produce half-maximal binding of ^{125}I -labelled protein A) 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, in order to compare the affinity of the mucin antibody for different mucin antigens or haptens. Control incubations (without antibody) were included in all experiments to ensure that the non-specific binding of ^{125}I -labelled protein A to mucin antigen or bovine serum albumin in the wells was less than 5% of the B_0 or maximum value [i.e. the radioactivity (c.p.m.) value obtained without antigen added to preincubation mixtures].

Hapten inhibition studies

These were carried out with amino acids (Sigma Chemical Co., St. Louis, MO, U.S.A.), monosaccharides (Pfanstiehl Laboratories, Waukegan, IL, U.S.A.), conjugated monosaccharides, disaccharides and trisaccharides (synthesized by Dr. K. Matta, Buffalo, NY, U.S.A.), and four oligosaccharides from rat colonic mucin (purified by Dr. A. Slomiany and Dr. B. Slomiany, New York, NY, U.S.A.).

Thiol reduction of mucin

Mucin samples were reduced by treatment with 0.2 M-2-mercaptoethanol in 0.18 M-NaCl/0.02 M-sodium barbitone buffer, pH 8.6, for 5 min at 100°C (with or without 1% SDS). Samples were dialysed exhaustively against phosphate-buffered saline, and where necessary SDS was extracted by the method of Henderson *et al.* (1979). Appropriate controls were carried out to determine the effects of boiling and/or SDS extraction on mucin antigenicity.

Pronase digestion

Intestinal mucin (300 μg of protein/ml in phosphate-buffered saline) was digested at 37°C at pH 7.0 for periods up to 72 h with Pronase (type XIV from *Streptomyces griseus*; Sigma Chemical Co.). Enzyme was added at the start of incubations (enzyme/mucin protein weight ratio 1:1), after 24 h (ratio 1:0.5) and at 48 h (final ratio 1:0.3). Control mucin samples were incubated similarly but without enzyme.

Separate controls containing Pronase but no mucin confirmed that the enzyme did not generate false positive or negative results during the radioimmunoassay procedure.

Column chromatography

Mucin (containing 0.45 mg of protein) was applied before and after reduction or Pronase treatment to Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) columns (180 cm $\times$ 1.5 cm) and eluted by upward flow with 0.2 M-NaCl containing 0.02% NaN_3 . Eluate fractions were analysed by the periodate/Schiff assay method of Mantle & Allen (1978).

In some experiments reduced mucin samples (containing 0.2–0.5 mg of protein) were applied to Sepharose 4B columns (60 cm $\times$ 2.5 cm), eluted with 0.1% (w/v) SDS, and the glycopeptides were eluted in the excluded volume. In other experiments Pronase-digested mucin samples were chromatographed on Sephadex G-150 columns (60 cm $\times$ 2.5 cm), eluted with 0.2 M-NaCl containing 0.02% NaN_3 , and the glycopeptides were harvested from the void volume. Recovery of mucin from the various columns ranged from 85 to 95%, as judged by the periodate/Schiff assay.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of mucin samples (4–10 μ g of protein) was carried out by the method of Laemmli (1970) on a vertical slab gel system (16cm $\times$ 20cm gels; BRL, Gaithersburg, MD, U.S.A.). Bands were detected by the silver staining procedure of Merril *et al.* (1981), and M_r assignments made by comparison with standard proteins (M_r 14000 to 200000) obtained from BioRad Laboratories (Richmond, CA, U.S.A.). Electrophoretic transfer of bands to nitrocellulose sheets (Western 'blot') was performed by using the method of Towbin *et al.* (1979), and antigenic bands were detected by autoradiography after sequential treatment with 20ml each of 3% (w/v) bovine serum albumin, mucin antibody [1:400 dilution in 3% (w/v) bovine serum albumin] and 2 μ Ci of 125 I-labelled protein A (specific radioactivity 30mCi/mg; The Radiochemical Centre, Amersham, Bucks., U.K.) in 3% (w/v) bovine serum albumin.

Chemical analyses

Carbohydrate was analysed by g.l.c. of trifluoroacetate derivatives of mucin hydrolysates as described earlier (Wesley *et al.*, 1983). Protein was analysed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and by amino acid analysis of samples hydrolysed for 24h *in vacuo* at 110°C in 5.5M-HCl with the use of a Durrum D-500 automatic amino acid analyser.

Preparative gel electrophoresis

Mucin samples containing 1 mg of protein were reduced at 100°C for 5min in 0.2M-2-mercaptoethanol, in the presence of 1% SDS, in 0.1M-Tris/borate buffer, pH8.6. Electrophoresis was carried out in 7.5%-polyacrylamide slab gels for 17h at 7mA/gel in 0.1% SDS, with 0.2M-Tris/borate, pH8.6, as the electrode buffer. The resulting 118000- M_r band was removed by electro-elution and subjected to dialysis and ion-pair extraction (Henderson *et al.*, 1979) to remove mercaptoethanol, buffer salts and detergent.

Results

Radioimmunoassay of human mucin

The optimum conditions of immunoassay are provided in Table 1. Since the assay involves preincubation of standard or test mucin with the antibody, the radioactivity in the micro-titre wells is inversely proportional to the concentration of mucin (or effective hapten) in the preincubation mixture. The required dilution of antibody (step 3, Table 1) was determined from an antibody titre

Table 1. Procedure for the solid-phase radioimmunoassay of human intestinal mucin

1. Antigen coating of wells:
Add 50ng of mucin protein in 50 μ l of phosphate-buffered saline to wells
Incubate for 24h at 4°C
Aspirate and wash three times with phosphate-buffered saline
2. Saturation of binding sites:
Add 200 μ l of bovine serum albumin (20mg/ml in phosphate-buffered saline) to wells
Incubate for 2h at 22°C
Aspirate and wash three times with phosphate-buffered saline
3. Preincubation mixture:
Mix 75 μ l of antibody (1:6000 final dilution) with 75 μ l of antigen solution containing 3–200ng of mucin protein
Incubate for 24h at 37°C
4. Antibody binding to wells:
Add 50 μ l of the preincubation mixture to antigen-coated wells
Incubate for 24h at 22°C
Aspirate and wash three times with phosphate-buffered saline
5. 125 I-labelled protein A binding to wells:
Add 50 μ l of 125 I-labelled protein A (approx, 0.01 μ Ci)
Incubate for 4h at 22°C
Aspirate and wash three times with phosphate-buffered saline, drain dry
6. Cut wells from plate, count radioactivity in each well in a γ -radiation counter

Fig. 1. Antibody titre curve for human small-intestinal mucin

Microtitre wells were coated with 50ng of mucin (protein), and serial dilutions of mucin antibody (50 μ l in phosphate-buffered saline) were added in duplicate. The mixtures were incubated for 24h at 22°C, washed, and bound antibody was detected by 125 I-labelled protein A binding as described in Table 1. The antibody titre was 1:14000, and an antibody dilution of 1:6000 (arrow) was chosen for subsequent routine radioimmunoassays.

Fig. 2. Radioimmunoassay of human small-intestinal mucin: standard curves

The final antibody dilutions in preincubation mixtures with mucin were: Δ , 1:2000; $\circ$, 1:4000; $\bullet$, 1:6000; $\square$, 1:12000; $\blacktriangle$, 1:24000. Bars indicate the S.E.M. for ten independent assays.

curve (Fig. 1). Subsequent standard curves (Fig. 2) led us to choose an antibody dilution of 1:6000 (arrow) for routine use. Under these conditions, the B_0 value was 75% of maximal binding, the B_{50} was 32 ng of mucin (protein), the range of antigen detected was 6–100 ng of mucin (protein), and the inter-assay variation was less than 10% for each point.

Carbohydrate cross-reactivity

The following carbohydrates were used as haptens in the radioimmunoassay and gave no reaction with the mucin antibody: (a) monosaccharides (3 nmol–25 μ mol/assay): fucose, *N*-acetylneuraminic acid, galactose, glucose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, all sugars together; (b) conjugated monosaccharides (50–400 nmol/assay): β -*p*-NO₂Ph-Gal, β -*p*-NO₂Ph-GlcNAc, α -*p*-NO₂Ph-GlcNAc, β -*p*-NO₂Ph-GalNAc, α -Ph-GalNAc; (c) disaccharides (25–200 nmol/assay): Gal- β 1-3-GalNAc, Gal- β 1-3-(α -*o*-NO₂Ph)-GalNAc, Gal- β 1-3-(β -*p*-NO₂Ph)-GalNAc, Gal- β 1-3-GlnNAc, Gal- β 1-3-(α -Me)-GlcNAc, Gal- β 1-3-(β -*p*-NO₂Ph)-GlcNAc, GlcNAc- β 1-6-(α -*o*-NO₂Ph)-GalNAc, Fuc- β 1-3-(α -PhCH₂)-GalNAc; (d) trisaccharides (25–200 nmol): Gal- β 1-3-(GlcNAc- β 1-6)(α -PhCH₂)-GalNAc, Fuc- α 1-2-Gal- β 1-3-GalNAc-ol, Fuc- α 1-2-Gal- β 1-3-(α -Ph)-GalNAc; (e) oligosaccharides from rat colonic mucin (2.5–10 μ g/assay): NeuAc- α 2-6-GalNAc-ol, Gal- β 1-4-GlcNAc- β 1-3-GalNAc-ol, GlcNAc-

β 1-3-(NeuAc- α 2-6)-GalNAc-ol, Gal- β 1-3-GlcNAc- β 1-3-(NeuAc- α 2-6)-GalNAc-ol.

Since all of the sugar sequences and linkages present in the above structures would be expected to occur in human intestinal mucin, their lack of reactivity, even at high concentrations, suggests that they do not contribute to antigenic determinants in the mucin.

Amino acid cross-reactivity

Hapten inhibition studies similar to those used to assess carbohydrate cross-reactivity were also performed with individual amino acids. They were pooled into groups as follows, and gave no reaction: (a) serine, threonine, proline (0.45–7.2 μ mol/assay); (b) aspartate, glutamate (0.12–1.9 μ mol/assay); (c) alanine, glycine, valine, leucine, isoleucine (0.36–5.8 μ mol/assay); (d) tyrosine, phenylalanine, histidine, lysine, arginine (0.22–3.6 μ mol/assay); (e) methionine, cysteine, cystine (0.15–2.3 μ mol/assay).

Effect of thiol reduction

Experiments were undertaken to determine if a relationship existed between the three-dimensional structure of human mucin and its antigenic reactivity. In early experiments human mucin showed only a moderate degree of dissociation when treated with 10 mM-dithiothreitol in 6M-

Fig. 3. Sepharose 2B chromatography of human small-intestinal mucin

Mucin samples (3 mg dry wt.) were chromatographed before (a) and after (b) reduction at pH 8.6 in 0.2M-2-mercaptoethanol for 5 min at 100°C. The eluting solution was 0.2M-NaCl in 0.02% NaN₃. Glycoprotein was measured by the periodate/Schiff assay. V_0 and V_i are the excluded volume and the totally included volume respectively.

Table 2. *Effect of thiol reduction on the radioimmunoassay of human small-intestinal mucin*

After each treatment the mucin samples were dialysed exhaustively against phosphate-buffered saline at 4°C. SDS was removed by ion-pair extraction. Five dilutions of each sample in duplicate were subjected to radioimmunoassay to generate the slope and B_{50} data.

Mucin treatment	B_{50} (ng of protein)	Slope (relative to standard = 1.0)
Mucin 1 (patient GM)		
No treatment	45	1.00
Boiled (5 min, 100°C, pH 8.6) (control)	400	0.92
Reduced and boiled (5 min, 100°C, pH 8.6, with 0.2M-2-mercaptoethanol)	> 10 ⁶	0.3
Mucin 2 (patient CAM)		
No treatment	55	1.0
SDS (pH 8.6, 1% SDS, room temp., 30 min)	78	1.2
Boiled (pH 8.6, 100°C, 5 min)	135	1.0
SDS and boiled (pH 8.6, 1% SDS, 100°C, 5 min)	400	0.94
SDS, boiled and reduced (pH 8.6, 1% SDS, in 0.2M-2-mercaptoethanol)	> 10 ⁶	0.14

guanidinium chloride, pH 8.5, for 4 h at 22°C (Forstner *et al.*, 1979). To achieve more complete reduction in the present study, mucin samples were reduced at pH 8.6 in 0.2M-2-mercaptoethanol with or without 1% SDS for 5 min at 100°C. (Reduction for 48 or 72 h at 4°C gave similar results.) Three mucin samples all showed a significant decrease in size after reduction (Fig. 3), with about 80% of the periodate/Schiff-reactive material forming a broad peak in the partially included volume, suggesting a heterogeneous distribution of glycoproteins of various sizes. There was no significant change in the overall carbohydrate profile of reduced mucin (results not shown), but a major decrease occurred in the slope and B_{50} of the radioimmunoassay (Table 2). Some loss in the number of antigenic determinants (as reflected by increased B_{50} values) was due to boiling (or boiling with SDS), but the major loss of antigen-antibody affinity (i.e. decreased slopes) was clearly a result of reduction. Thus intra- or inter-molecular disulphide bonds in the peptide are very important for the antigenicity of human mucin.

Mucin samples were examined by silver staining in 7.5%-polyacrylamide gels containing 0.1% SDS (Fig. 4a). As expected, untreated or native mucin barely penetrated the gel, and no other bands were detected. After reduction, however, a strong band of higher mobility appeared and, by comparison with protein standards, was assigned an M_r value of 118000. This finding was similar to that observed for rat intestinal mucin (Fahim *et al.*, 1983), in which a non-glycosylated peptide of apparent M_r 118000 appeared on gels after reduction. It is tentatively assumed therefore that the band from human mucin is a similar 'link' peptide. The gels were subjected to electrophoretic transfer (Western 'blot') on to nitrocellulose sheets and treated sequentially with mucin antibody

(1:400 dilution) and ¹²⁵I-labelled protein A. Autoradiography (Fig. 4b) indicated that the 'link' peptide was not significantly antigenic and that, after reduction, the large- M_r components carrying residual antigenicity penetrated the gel to a greater extent than in the case of unreduced mucin. This finding is in keeping with the Sepharose 2B column data (Fig. 3) showing similar and more heterogeneous mucin components after reduction.

In separate experiments, the 118000- M_r band was electro-eluted from preparative 7.5%-polyacrylamide slab gels, and gave no reactivity in the radioimmunoassay (slope < 0.15), thus confirming the relative inactivity of this peptide as an antigen.

Pronase digestion of reduced mucin

To discover the nature of the residual antigen seen after a Western 'blot' of reduced mucin (Fig. 4b), a sample of native mucin was first reduced in the presence of 1% (w/v) SDS, and the glycoprotein fraction was isolated in the excluded volume of Sepharose 4B columns. A portion of this peak was kept as a control, and the remainder was digested with Pronase for 72 h at 37°C. A subsequent Western 'blot' step (Fig. 4c) showed that the radioactivity present in the control reduced mucin sample disappeared after Pronase treatment. Thus the residual antigenicity must have been contained within (or was strongly dependent upon) non-glycosylated ('naked') regions of the peptide backbone of the large- M_r glycopeptides of the mucin.

Pronase digestion of native mucin

In an effort to discover whether the major antigenic determinants of native mucin (i.e. before reduction) could also be attributed to peptide, timed digestions with Pronase were carried out and the products tested for alterations in structure and antigenicity.

Fig. 4. SDS/polyacrylamide-gel electrophoresis and Western 'blot' of human small-intestinal mucin before and after thiol reduction and Pronase digestion

(a) Mucin (4.9 μ g of protein in 1.0% SDS) was applied to 7.5%-polyacrylamide gels before (–) and after (+) reduction for 5 min at 100°C in 0.2M-2-mercaptoethanol. The stacking gel was 5.7%. The M_r -marker positions are shown on the left. Bands were detected by silver staining. (b) The same gels were subjected to electrophoretic transfer (Western 'blot') on to nitrocellulose, incubated with mucin antibody (1:400 dilution), and antigenic bands were detected by using 125 I-labelled protein A and autoradiography (exposure time 18 h). (c) Human mucin was reduced for 5 min at 100°C in 0.2M-2-mercaptoethanol and 1% (w/v) SDS at pH 8.6. The glycopeptides were harvested in the void volume of Sepharose 4B columns, and digested with Pronase (final enzyme/mucin protein ratio 1:0.3) for 72 h at 37°C. Samples (8 μ g of protein) taken before (–) and after (+) Pronase treatment were subjected to Western 'blot'. Note the loss of antigenicity after the Pronase treatment.

It was clear from Sepharose 2B chromatography (Fig. 5) that major degradative changes occurred in human mucin during Pronase treatment. By 24 h

Fig. 5. Sepharose 2B chromatography of Pronase-digested human small-intestinal mucin

(a) Control mucin (2mg dry wt.) ($\square$) and 24h-Pronase-digested mucin (3mg dry wt.) ($\blacksquare$) were chromatographed on Sepharose 2B (150cm $\times$ 1.5cm), eluted with 0.2M-NaCl containing 0.02% NaN_3 . (b) 72h-Pronase-digested mucin (4mg) was chromatographed similarly on a longer Sepharose 2B column (180cm $\times$ 1.5cm) to show multiple degradation species. Glycoprotein was measured in all cases by the periodate/Schiff assay as described in the Methods section. V_i and V_0 are the included volume and the totally excluded volume respectively.

Table 3. Effect of Pronase digestion on the antigenicity of human mucin

Mucin samples containing 300 μ g of protein were incubated with Pronase at 37°C at pH 7.0 for increasing time periods, and the mucin radioimmunoassay was performed on five dilutions of each incubation mixture in order to calculate slope and B_{50} (ng of protein) values at each time period. Pronase enzyme alone did not react in the radioimmunoassay. The percentage of antigenic determinants remaining at each time period was calculated from the B_{50} value. Abbreviation: N.D. Not determined.

Digestion time	... 0h		8h		24h		48h		72h	
Mucin sample	Determinants remaining		Determinants remaining		Determinants remaining		Determinants remaining		Determinants remaining	
	Slope	(%)	Slope	(%)	Slope	(%)	Slope	(%)	Slope	(%)
1 (KL)	1.0	100	1.0	40	1.1	30	N.D.	N.D.	1.0	0
2 (GM)	1.0	100	1.0	69	N.D.	N.D.	N.D.	N.D.	0.9	0
3 (RB)	1.0	100	1.2	75	0.8	30	0.8	30	0.7	0
4 (CAM)	1.0	100	1.1	36	1.1	20	1.1	8	1.2	0

over 80% of the glycoprotein was distributed in a broad included peak, and by 72h several included peaks could be discerned. The carbohydrate profile of 72h digests was preserved (results not shown), implying no gross alteration in the composition of oligosaccharide side chains. By weight, the loss of mucin protein after 72h varied from 23 to 30% (three independent digestion experiments).

Table 3 shows a gradual loss of antigenic determinants with Pronase digestion in four different mucin preparations. In most cases over 50% of the total 72h loss was experienced within the first 24h of digestion. It is particularly noteworthy that slope values remained normal (approx. 1.0) throughout the digestion, suggesting that the action of Pronase was to decrease the number of antigenic determinants without compromising the affinity of the remaining determinants for the mucin antibody. This was true despite a major change in size and, presumably, polymerization of the mucin. It was unlike the effect of reduction presented above, where the slope values fell precipitously (Table 2), indicating a major loss of antigen-antibody affinity after disruption of disulphide bonds.

Polyacrylamide-gel-electrophoretic patterns with corresponding Western 'blots' are shown in Fig. 6. After 8h of Pronase digestion no 118000- M_r 'link' peptide remained. Indeed, no silver-stained bands (with the exception of Pronase enzyme) could be detected in any of the digests beyond 8h, either before or after thiol reduction. Therefore Pronase must have degraded the 'link' and probably other 'naked' peptide in the mucin to fragments having M_r values less than 15000 (the lower limit of detection on our polyacrylamide gels). Western 'blots' (Fig. 6b) both before and after reduction of Pronase-digested mucin (preparation from patient CAM) showed that antigenic activity was in the top region of the gel (including the stacking gel), was never seen in an area corresponding to M_r 118000 or in smaller- M_r bands, and was decreased dramatically after 8h of digestion. In the case of mucin from patient RB (results not shown), the same dramatic decrease required 24h, but in all samples virtually no antigen remained after 72h. These findings support the interpretation that within the native mucin most of the antigenicity arises from 'naked' peptide segments in the large glycopeptide components. These segments are apparently somewhat

Fig. 6. Polyacrylamide-gel electrophoresis (a) and Western 'blot' (b) of Pronase-digested human small-intestinal mucin (patient CAM) was digested with Pronase for 72h at 37°C. Portions taken at various times and containing 7.0 µg of mucin protein were reduced and subjected to SDS/polyacrylamide-gel electrophoresis (a) followed by a Western 'blot' (b). 1, 2 and 7 represent 0h, 8h and 72h control incubations (no Pronase) respectively, and 3, 4, 5 and 6 represent 8h-, 24h-, 48h- and 72h-Pronase-digested mucin samples respectively. Key: O, origin (separating the stacking gel and the running gel); 116 (position of marker of M_r 116000); A (mercaptoethanol artifact); P (Pronase enzyme band).

resistant to Pronase, since prolonged digestion was required to destroy antigenicity completely.

Discussion

In the present investigation we have explored, by means of a newly developed solid-phase radioimmunoassay method, the requirements for antigenicity of goblet-cell mucin from human small intestine. The present assay method represents an improvement over the double-antibody radioimmunoassay method previously reported (Qureshi *et al.*, 1979), since (a) it does not involve immunoprecipitation, and therefore requires much less mucin antibody (1:6000 instead of 1:1000 dilution) and no second antibody, (b) it has a stable low background of about 5%, (c) it produces a much greater range of radioactivity between maximum and minimum binding, thereby giving more reliable results, (d) it allows many more samples to be analysed simultaneously (300 compared with 60 per day), and (e) it does not require radioactive labelling of either the mucin antigen or antibody.

Despite the high content of carbohydrates in the mucin, we were unable to find evidence to suggest a primary antigenic role for them. Previous work had demonstrated that the antibody was indifferent to the ABH and Lewis blood-group status of human mucins (Qureshi *et al.*, 1979), and the present experiments failed to elicit a hapten response to mono-, di-, tri- and oligo-saccharides typical of mucin structures.

After exhaustive reduction of disulphide bonds, several structural changes took place in the mucin, some of which were no doubt responsible for the marked decrease in the affinity of the mucin for its antibody. The major change was a decrease in size, indicating that the mucin macromolecule(s) had depolymerized (or disaggregated) into a broad spectrum of heterogeneous glycoprotein (or glycopeptide) components. SDS/polyacrylamide-gel electrophoresis demonstrated the release of a 118000- M_r peptide, assumed by analogy with rat (Fahim *et al.*, 1983) and pig (Mantle *et al.*, 1981) mucins to represent a disulphide-bonded 'link' peptide in the native mucin. When examined by Western 'blot' or radioimmunoassay, this peptide was not significantly antigenic. The large glycopeptides, however, retained some antigenicity, and the latter was abolished by digestion with Pronase. Thus non-glycosylated or 'naked' peptide regions in the peptide core of the large glycopeptides appear to play an important part in creating antigenic sites on the mucin.

Greater insight into the importance of peptide was provided by timed Pronase digestion of native (or intact) mucin preparations. By 8 h of digestion considerable degradation of the mucin occurred,

Fig. 7. Schematic models of intestinal mucin to show the location of antigenic determinants

The determinants (■) are within a non-glycosylated ('naked') peptide region at the ends (a) or in the middle (b) of heavily glycosylated large glycopeptide components. Tertiary configuration of the determinant region is stabilized by intramolecular S-S bands. Thiol reduction destroys antibody affinity, and Pronase digestion removes antigenic determinants, as well as the 'link' peptide of M_r 118000 (ZZZ).

and the 'link' peptide could no longer be detected before or after reduction. At the same time, however, most of the mucin preparations retained considerable numbers of antigenic determinants (40–75% by radioimmunoassay) and a normal affinity of those determinants for the antibody. Only with prolonged digestion and extensive mucin degradation were the antigenic sites abolished. Yet only 23–30% of the original mucin peptide (by weight) was lost after 72 h. We conclude therefore that the major antigen is peptide in nature, and, because it is relatively resistant to Pronase, it is probably located in a region close to the attachment of oligosaccharide chains (Fig. 7). In addition, disulphide bonds (intramolecular) probably stabilize a specific tertiary configuration of the antigenic 'naked' peptide at the ends (Fig. 7a) or in the middle (Fig. 7b) of glycopeptide components.

In summary, the antibody to human mucin seems to recognize a specific segment of peptide that is needed for attachment of glycopeptide(s) to 'link' peptide, and which is stabilized by disulphide bonds. Future applications of interest may thus include immunological evaluation of proteolytically degraded or altered mucin produced in various states of health and disease.

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