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

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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 $M_r$ 118000. 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 ($M_r > 200000$). 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 8h of digestion the 118000-$M_r$ peptide had disappeared, whereas antigenicity, which was confined to large-$M_r$ glycopeptides, was destroyed much more slowly with time (70% by 24h, 100% by 72h). Despite the loss of antigenicity, 72h-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-acetylglalactosamine 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 (Jabbar 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 adenocarcinoma (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-
took the present study to determine whether these unique determinants reside in the three-dimen-
sional 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 glyco-
peptide 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)–
(NH₄)₂SO₄ fraction of immune rabbit sera de-
scribed and used earlier in a double-antibody
radioimmunoassay (Qureshi et al., 1979).

A more convenient solid-phase radioimmuno-
assay 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 concen-
trations of antigen and antibody. All solutions
were made in phosphate-buffered saline (0.1M-NaCl/
0.1M-Na₂HPO₄/NaH₂PO₄ buffer, pH 7.4) con-
taining 0.02% NaN₃. Radioactivities in wells were
counted in a Beckman 8000 γ-radiation counter.
Standard curves were constructed by plotting ¹²⁵I
radioactivity (c.p.m.) bound to the wells against
the amount of mucin antigen (protein) added to the
preincubation mixture. The curves were character-
ized by their B₅₀ values (the amount of mucin
antigen required to produce half-maximal binding
of ¹²⁵I-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 ¹²⁵I-
labelled protein A to mucin antigen or bovine
serum albumin in the wells was less than 5% of the
B₀ 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.), monosac-
charides (Pfanstiehl Laboratories, Waukegan, IL,
U.S.A.), conjugated monosaccharides, disacchar-
ides and trisaccharides (synthesized by Dr. K.
Matta, Buffalo, NY, U.S.A.), and four oligosac-
charides 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
0.2m-2-mercaptoethanol in 0.18m-NaCl/0.02m-
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 antigenic-
ity.

Pronase digestion

Intestinal mucin (300µg of protein/ml in phos-
phate-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 0.45µg of protein) was ap-
plied before and after reduction or Pronase
treatment to Sepharose 2B (Pharmacia Fine
Chemicals, Uppsala, Sweden) columns
(180 cm × 1.5 cm) and eluted by upward flow with
0.2m-NaCl containing 0.02% NaN₃. Eluate frac-
tions were analysed by the periodate/Schiff assay

In some experiments reduced mucin samples
(containing 0.2–0.5 mg of protein) were applied to
Sepharose 4B columns (60 cm × 2.5 cm), eluted
with 0.1% (w/v) SDS, and the glycopeptides were
eluted in the excluded volume. In other experi-
ments Pronase-digested mucin samples were chro-
matographed on Sephadex G-150 columns
(60 cm × 2.5 cm), eluted with 0.2m-NaCl contain-
ing 0.02% NaN₃, 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.

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Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of mucin samples (4-10 μg of protein) was carried out by the method of Laemmli (1970) on a vertical slab gel system (16 cm × 20 cm gels; BRL, Gaithersburg, MD, U.S.A.). Bands were detected by the silver staining procedure of Merril et al. (1981), and Mr assignments made by comparison with standard proteins (Mr 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 20 ml each of 3% (w/v) bovine serum albumin, mucin (or inversely proportional) with standard, and by amino acid analysis of samples hydrolysed for 24 h in vacuo at 110°C in 5.5M-HCl with the use of a Durrum D-500 automatic amino acid analyser.

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 24 h in vacuo at 110°C in 5.5M-HCI 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 5 min in 0.2M-2-mercaptoethanol, in the presence of 1% SDS, in 0.1M-Tris/borate buffer, pH 8.6. Electrophoresis was carried out in 7.5% polyacrylamide slab gels for 17 h at 7mA/gel in 0.1% SDS, with 0.2M-Tris/borate, pH 8.6, as the electrode buffer. The resulting 118000-Mr 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>
<thead>
<tr>
<th>Table 1. Procedure for the solid-phase radioimmunoassay of human intestinal mucin</th>
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<tbody>
<tr>
<td>1. Antigen coating of wells:</td>
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<tr>
<td>Add 50 ng of mucin protein in 50 μl of phosphate-buffered saline to wells</td>
</tr>
<tr>
<td>Incubate for 24 h at 4°C</td>
</tr>
<tr>
<td>Aspirate and wash three times with phosphate-buffered saline</td>
</tr>
<tr>
<td>2. Saturation of binding sites:</td>
</tr>
<tr>
<td>Add 200 μl of bovine serum albumin (20 mg/ml in phosphate-buffered saline) to wells</td>
</tr>
<tr>
<td>Incubate for 2 h at 22°C</td>
</tr>
<tr>
<td>Aspirate and wash three times with phosphate-buffered saline</td>
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<tr>
<td>3. Preincubation mixture:</td>
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<tr>
<td>Mix 75 μl of antibody (1:6000 final dilution) with 75 μl of antigen solution containing 3-200 ng of mucin protein</td>
</tr>
<tr>
<td>Incubate for 24 h at 37°C</td>
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<tr>
<td>4. Antibody binding to wells:</td>
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<tr>
<td>Add 50 μl of the preincubation mixture to antigen-coated wells</td>
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<tr>
<td>Incubate for 24 h at 22°C</td>
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<tr>
<td>Aspirate and wash three times with phosphate-buffered saline</td>
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<tr>
<td>5. 125I-labelled protein A binding to wells:</td>
</tr>
<tr>
<td>Add 50 μl of 125I-labelled protein A (approx, 0.01 μCi)</td>
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<tr>
<td>Incubate for 4 h at 22°C</td>
</tr>
<tr>
<td>Aspirate and wash three times with phosphate-buffered saline, drain dry</td>
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<tr>
<td>6. Cut wells from plate, count radioactivity in each well in a γ-radiation counter</td>
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</table>

![Fig. 1. Antibody titre curve for human small-intestinal mucin](image)

Microtitre wells were coated with 50 ng of mucin (protein), and serial dilutions of mucin antibody (50 μl in phosphate-buffered saline) were added in duplicate. The mixtures were incubated for 24 h at 22°C, washed, and bound antibody was detected by 125I-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.
\[ \text{Gal-fil-3-}(\alpha\text{-pNO}_{2}\text{Ph}-)\text{GalNAc}, \ \text{Gal-1-3l-4-GlcNAc-p1-3-GalNAc-ol}, \ \text{11-3-GalNAc}, \ \text{Gal-fil-3-}(\alpha\text{-pNO}_{2}\text{Ph}-)\text{GalNAc}, \ \text{Fuc-al-2-}(\text{GlcNAc-fil-6})(\alpha\text{-Ph})\text{-GalNAc}, \ \text{Gal-1-3-}(\text{NeuAc-\text{\(a2-6\)}})\text{GalNAc-ol}, \ \text{Gal-\(\beta1-3\)}-\text{GlcNAc-ol}, \ \text{Gal-\(\beta1-3\)}-\text{GlcNAc-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 \(\mu\text{mol/assay}\)); (b) aspartate, glutamate (0.12–1.9 \(\mu\text{mol/assay}\)); (c) alanine, glycine, valine, leucine, isoleucine (0.36–5.8 \(\mu\text{mol/assay}\)); (d) tyrosine, phenylalanine, histidine, lysine, arginine (0.22–3.6 \(\mu\text{mol/assay}\)); (e) methionine, cysteine, cystine (0.15–2.3 \(\mu\text{mol/assay}\)).

**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\(\text{mM}\)-dithiothreitol in 6M-

**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 \(\mu\text{mol/assay}\)): fucose, \(\text{N}\)-acetylneuraminic acid, galactose, glucose, mannose, \(\text{N}\)-acetylglucosamine, \(\text{N}\)-acetylgalactosamine, all sugars together; (b) conjugated monosaccharides (50–400 nmol/assay): \(\beta\text{-pNO}_{2}\text{Ph-Gal, } \beta\text{-pNO}_{2}\text{Ph-GlcNAc, } \alpha\text{-pNO}_{2}\text{PhGlcNAc, } \beta\text{-pNO}_{2}\text{Ph-GalNAc, } \alpha\text{-Ph-GalNAc}; \) (c) disaccharides (25–200 nmol/assay): \(\text{Gal-\(\beta1-3\)}-\text{GalNAc, } \text{Gal-\(\beta1-3\)}-(\alpha\text{-NO}_{2}\text{Ph})\text{GalNAc, } \text{Gal-\(\beta1-3\)}-(\beta\text{-pNO}_{2}\text{Ph})\text{GalNAc, } \text{Gal-\(\beta1-3\)}-\text{GlnNAc, } \text{Gal-\(\beta1-3\)}-(\alpha\text{-Me})\text{GlcNAc, } \text{Gal-\(\beta1-3\)}-(\beta\text{-pNO}_{2}\text{Ph})\text{-GlcNAc, GlcNAc- } \beta1-6-(\alpha\text{-NO}_{2}\text{Ph})\text{-GlcNAc, Fuc-\(\beta1-3\)}-(\alpha\text{-PhCH}_{2})\text{GlcNAc; } \) (d) trisaccharides (25–200 nmol): \(\text{Gal-\(\beta1-3\)}-\text{(GlcNAc-}\beta1-6)(\alpha\text{-PhCH}_{2})\text{GlcNAc, Fuc-\(\alpha1-2\)}-\text{Gal-\(\beta1-3\)}-\text{GalNAc-ol, Fuc-\(\alpha1-2\)}-\text{Gal-\(\beta1-3\)}-(\alpha\text{-Ph})\text{-GalNAc; } \) (e) oligosaccharides from rat colonic mucin (2.5–10 \(\mu\text{g/assay}\)): NeuAc-\(\alpha2-6\)-GalNAc-ol, \(\text{Gal-\(\beta1-4\)}\text{-GlcNAc-}\beta1-3\text{-GalNAc-ol, GlcNAc-} \]

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

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

- 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-mercaptopethanol for 5 min at 100°C. The eluting solution was 0.2M-NaCl in 0.02% NaN\(_3\). Glycoprotein was measured by the periodate/Schiff assay. \(V_0\) and \(V_e\) are the excluded volume and the totally included volume respectively.
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 ^{125}I-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.
It was clear from Sepharose 2B chromatography (Fig. 5) that major degradative changes occurred in human mucin during Pronase treatment. By 24 h

![Figure 4: SDS/polyacrylamide-gel electrophoresis and Western 'blot' of human small-intestinal mucin before and after thiol reduction and Pronase digestion.](image)

![Figure 5: Sepharose 2B chromatography of Pronase-digested human small-intestinal mucin.](image)

**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 μg 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 Mr-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 125I-labelled protein A and autoradiography (exposure time 18h). (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 72h at 37°C. Samples (8 μg of protein) taken before (−) and after (+) Pronase treatment were subjected to Western 'blot'. Note the loss of antigenicity after the Pronase treatment.

![Graph showing Glycoprotein mg vs Elution volume ml.](image)

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

(a) Control mucin (2mg dry wt.) (□) and 24h-Pronase-digested mucin (3mg dry wt.) (■) were chromatographed on Sepharose 2B (150cm x 1.5cm), eluted with 0.2M-NaCl containing 0.02% NaN₃. (b) 72h-Pronase-digested mucin (4mg) was chromatographed similarly on a longer Sepharose 2B column (180cm x 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_r and V_o are the included volume and the totally excluded volume respectively.

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

<table>
<thead>
<tr>
<th>Mucin sample</th>
<th>Digestion time</th>
<th>Determinants remaining (%)</th>
<th>Determinants remaining (%)</th>
<th>Determinants remaining (%)</th>
<th>Determinants remaining (%)</th>
<th>Determinants remaining (%)</th>
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<tbody>
<tr>
<td></td>
<td>0h</td>
<td>8h</td>
<td>24h</td>
<td>48h</td>
<td>72h</td>
<td></td>
</tr>
<tr>
<td>1 (KL)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>2 (GM)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>3 (RB)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>4 (CAM)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Mucin samples containing 300 μg 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.
over 80% of the glycoprotein was distributed in a broad included peak, and by 72 h several included peaks could be discerned. The carbohydrate profile of 72 h 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 72 h 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 72 h loss was experienced within the first 24 h 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 8 h of Pronase digestion no 118 000- Mr 'link' peptide remained. Indeed, no silver-stained bands (with the exception of Pronase enzyme) could be detected in any of the digests beyond 8 h, either before or after thiol reduction. Therefore Pronase must have degraded the 'link' and probably other 'naked' peptide in the mucin to fragments having Mr values less than 15 000 (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 Mr 118000 or in smaller-Mr bands, and was decreased dramatically after 8 h of digestion. In the case of mucin from patient RB (results not shown), the same dramatic decrease required 24 h, but in all samples virtually no antigen remained after 72 h. 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

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**Fig. 6.** Polyacrylamide-gel electrophoresis (a) and Western 'blot' (b) of Pronase-digested human small-intestinal mucin

Mucin (patient CAM) was digested with Pronase for 72 h 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 0 h, 8 h and 72 h control incubations (no Pronase) respectively, and 3, 4, 5 and 6 represent 8 h, 24 h, 48 h and 72 h Pronase-digested mucin samples respectively. Key: O, origin (separating the stacking gel and the running gel); 116 (position of marker of Mr, 116 000); A (mercaptoethanol artefact); 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, 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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References

Boland, R. C., Montgomery, C. K. & Kim, Y. S. (1982a) Gastroenterology 82, 664–672