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Biochemical and immunohistochemical characterisation of mucins in 8 cases of colonic disease – a pilot study

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Summary

Objectives. To characterise mucins in cancer of the colon and compare these with controls using stringent biochemical measures to avoid endogenous proteolysis.

Design. Crude mucus scrapings were collected from 12 specimens obtained by colectomy. Specimens from 3 traumatic colectomies and 1 sigmoid volvulus were used as controls, and compared with 6 specimens from colons resected for adenocarcinoma and 2 irradiated colons.

Subjects. The median age of the 4 female patients was 76 years (range 49 - 82 years), and of the 8 male patients 46.5 years (range 16 - 74 years).

Results and conclusions. The crude mucus scrapings in the 9 specimens ranged in weight from 353 mg to 7 697 mg (median 4 928 mg). The median of purified mucin in the 9 specimens was 0.72 µg/mg wet weight of scraped material. Eight samples gave non-extractable pellet material, and were treated with DTT to reduce disulphide bonds for further analysis. One of these 8 pellets was resistant to reduction and had to be digested with papain before analysis. Only 5 of these pellets had mucin. Gel filtration and SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) analysis revealed different populations of mucin based on size and extent of degradation. Western blotting and immunohistochemical analysis confirmed the presence of MUC2 in all samples, MUC5AC in 2 and MUC5B in 5 diseased specimens. Immunohistochemical analysis showed that there was no MUC1 in the normal specimens, MUC1 apoprotein (MUC1 core) in 2 cancer specimens and MUC1 in 1 cancer specimen. Histochemical analysis showed that normal tissue expressed neutral and acidic mucins and diseased specimens predominantly expressed acidic mucins. The electrophoretic behaviour of MUC2 in sigmoid volvulus was different from that in cancer of the colon.

Mucins are large glycoproteins responsible for the rheological properties of normal mucus gels. So far 5 secreted gel-forming mucins (MUC2, 5AC, 5B, 6, 19), 3 secreted non-gel-forming mucins (MUC7, 8, 9), 10 membrane-bound mucins (MUC1, 3A, 3B, 4, 11, 12, 13, 16, 17, 20) and 3 unclassified mucins (MUC14, 15, 18) have been identified.[1,2]

Mucin genes are independently regulated and their expression is organ and cell type-specific.[3] MUC2 is the dominant mucin in the human colon and is a 'large' mucin for which

Abbreviations

DTT = dithiothreitol, H&E = haematoxylin and eosin, PAS = periodic acid Schiff, HID = high iron diamine, CsCl = caesium chloride, GuHCl = guanidinium chloride, EDTA = ethylenediaminetetra-acetic acid disodium salt, NEM = *N*-ethylmaleimide, PMSF = phenylmethysulphonyl fluoride, SDS = sodium dodecyl sulphate, PAGE = polyacrylamide gel electrophoresis, NaCl = sodium chloride, HRPO = horseradish peroxidase, TBST = tris-buffered saline tween-20, TBS = tris-buffed saline tween, V_i = included volume, V_o = void volume.

cDNA has been fully sequenced.[4] The development of the majority of colorectal carcinomas is associated with a diminished expression of MUC2 in the tumour cells.[5] However, Bresalier *et al*.[6] showed that mucinous colonic carcinomas are more likely to metastasise, and that inhibition of mucin synthesis is associated with a reduction of metastatic potential.

The physiochemical and biological properties of mucus are largely conferred by mucins, large O-glycosylated, sticky, visco-elastic glycoproteins responsible for the rheological properties of normal mucus gels.[7] Mucus forms a continuous, insoluble adherent gel layer in the gastro-intestinal tract, which protects the underlying mucosa from the hostile environment of the lumen.[7-9]

A biochemical study[10] of mucin obtained from specimens of macroscopically normal human colon as well as those resected during cancer surgery, suggested that colonic mucin is an insoluble complex that cannot be solubilised by chaotropic agents. These researchers employed the most stringent biochemical procedures to extract and purify mucin, making this probably the first definitive biochemical study of human colonic mucin in disease. However, the study focused on the characterisation of colonic mucin and did not compare differences between normal and diseased colonic mucin.[10]

In the present study we isolated mucins from colectomy specimens according to the method of Hermann *et al*.;[10] coupled with immunohistochemical studies[11] we determined the changes in the mucous secretions of colonic epithelia in 6 cancer patients and 2 irradiated cases.

Materials and methods

The study was approved by the University of Cape Town Research Ethics Committee (REC REF 149/2002). Crude mucus scrapings were collected from the resected colon in 12 cases. Four of the resections (3 for trauma and 1 for sigmoid volvulus) were used as controls. Six specimens were collected from colons containing an adenocarcinoma and 2 from irradiated bowel (Table I). The 3 trauma specimens were delivered to us in formalin and were only suitable for histological and immunohistochemical analysis.

Antibodies to the different mucins were obtained from appropriate sources; the details may be obtained by contacting the authors, as may those for the processes of extrac-

TABLE I. PATIENT DATA AND HISTOLOGICAL DETAILS

Laboratory number	Age (yrs)	Gender	Specimen	Histological assessment	Histological grading
C1	**33**	**M**	**Left colon**	**Normal colonic mucosa**	**N/A**
C2	**16**	**M**	**Right colon**	**Normal colonic mucosa**	**N/A**
C3	**22**	**M**	**Left colon**	**Normal colonic mucosa**	**N/A**
C4	**77**	**F**	**Sigmoid resection**	**No tumour. Normal colonic mucosa with no evidence of inflammation or ulceration**	**N/A**
S1	**49**	**F**	**Sigmoid colon with polyp**	**Colonic adenocarcinoma extending into the muscularis propria**	**2/3**
S2	**75**	**F**	**Anus and rectum (biopsy)**	**Chronic radiation-induced change in the rectal mucosa**	**N/A**
S3	**42**	**M**	**Anal verge post radiation (biopsy)**	**Benign squamous epithelium with underlying fibrous stroma containing mildly ecstatic vessels**	**N/A**
S4	**55**	**M**	**Colonic polyps**	**Pedunculated polyp. Adenocarcinoma**	**1/3**
S5	**70**	**M**	**Rectum**	**Colonic adenocarcinoma that infiltrates the muscularis propria and extends into the subserosa**	**1/3**
S6	**51**	**M**	**Sigmoid colon and rectum**	**Rectal mucosa shows an invasive colonic adenocarcinoma that arises from the surface, infiltrates through the muscularis mucosa into the submucosa**	**Not graded**
S7	**82**	**F**	**Left colon**	**Midway descending colon. Adenocarcinoma**	**1/3**
S8	**74**	**M**	**Left colon**	**Midway descending colon. Adenocarcinoma**	**1/3**
Median	**53**	**-**	**-**	**-**	**-**

N/A = not available.

tion and purification, gel filtration, enzymatic digestion, polyacrylamide gel electrophoresis, agarose gel electrophoresis, Western blotting, and histochemical, and immunohistochemical analysis.

Results

Table I lists the patients, the type of specimen obtained, and a histological assessment of each specimen. The crude wet weight of mucus harvested had a median of 4 928 mg (range 353 - 7 697 mg). A median of 0.72 µg/mg wet weight purified mucin was obtained from the soluble portion of the crude scrapings. The non-extractable pellet material obtained after homogenisation and centrifugation had to be further treated and yielded mucin in 5 of 8 cases. The pure mucin fractions, free of contaminant protein and nucleic acid,[12] were pooled, dialysed exhaustively against distilled water and freeze-dried (Fig. 1).

Fig. 2a shows a representative gel filtration profile of the soluble mucus from a patient with carcinoma. The soluble material contained a mixture of mucin (that eluted in the void volume (V_o), suggesting that there was a mixture of large and polymeric (gel-forming) mucin)[13-15] and degraded mucins and non-mucin protein material that eluted in the included volume of the column (V_i) (Fig. 2a). Purified mucin also had a mixture of gel-forming polymeric (V_o) and degraded subunit mucin (V_i).[14] The profile (Fig. 2b) revealed different size populations of mucins in the V_i suggesting a variable degradation of mucin in disease. Most of the insoluble pellet material eluted in the included volume of the column as expected (Fig. 2c).[15]

Mucus glycoprotein was analysed by SDS/PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). An intense PAS stained band was observed at the origin on top of the gel. This was interpreted as the presence of mucus glycoproteins that had not entered the gel because of their high-molecular-weight (Fig. 3). The faint background of material in each column again suggests the presence of some degradation of colonic mucin in each of the patients.

All specimens (Fig. 4, lanes 3 - 6 and 8 - 11), except S4 lane 7, were positive for MUC2 in varying degrees, with less visible material for patient S5 (Fig. 4, lane 6) and patients S1 - 3 after treatment to reduce the disulphide bonds of the mucin. There was no visible material for patient S4 (Fig. 4, lane 7) who had a cancerous pedunculated polyp. The glycoform of MUC2 in the patient with a sigmoid volvulus (Fig. 4, lane 3) was different from that in the cancer patients.

MUC5AC, on the other hand, is a gastric mucin type and as such it is not anticipated in the colon. In this study, the patient with the adenocarcinomatous pedunculated polyp was positive for MUC5AC with Western blotting (not shown). Patient S1 (colonic adenocarcinoma) showed some reactivity for MUC5B, while patient C4R (Fig. 4, lane 9) (sigmoid volvulus) was more strongly positive for MUC5B (not shown).

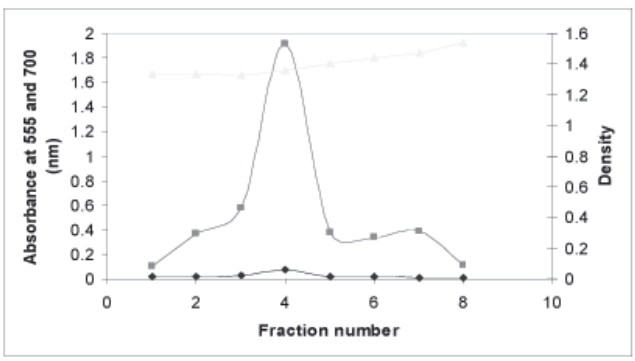

Fig. 1. Second CsCl density gradient centrifugation of human colonic mucus. CsCl was added to semi-purified mucins obtained from the first CsCl density centrifugation spin to give a starting density of 1.39 - 1.40 g/ml. After centrifugation (40 000 rpm for 48 h) the tubes were fractionated into 8 equal fractions and the density of each fraction was measured (▲). The fractions were then assayed for protein absorbance at 280 nm (◆) and analysed for carbohydrate with PAS at 555 nm (■).

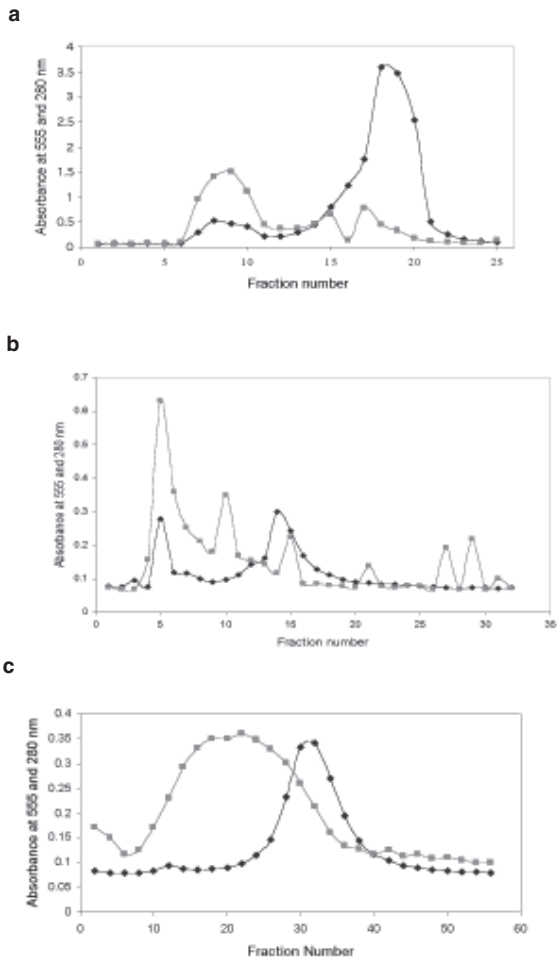

Fig. 2. Gel filtration analysis of soluble mucus and purified mucin on a Sepharose CL-2B column. Mucus scrapings were extracted in 6 M guanidinium chloride (GuHCl) containing 10 mM EDTA, 5 mM NEM and 1 mM PMSF, homogenised, and spun at 6 000 g for 1 h to remove insoluble debris. An aliquot (0.7 ml) of the soluble mucus in the supernatant was loaded on the column (a). After solubilisation mucins were isolated and purified by density gradient ultra-centrifugation, twice for 48 h at 40 000 rpm (b). Insoluble material resistant to extraction and reduction with DTT was digested with papain (see 'Materials and methods' section) and the mucin was purified (c). The column was eluted with 0.2 M NaCl: 0.02% sodium at a flow rate of 2.0 ml/min at room temperature. Fractions were analysed for carbohydrate with PAS at 555 nm (■) and protein absorbance at 280 nm (●).

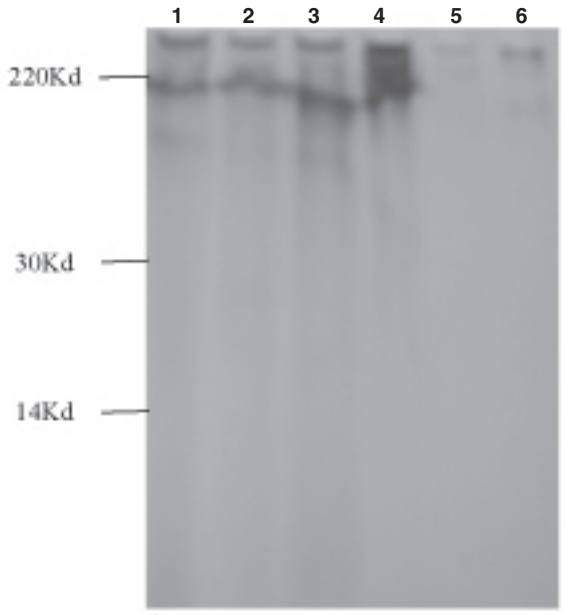

Fig. 3. A 10% SDS-PAGE gel of purified colonic mucins. Mucins were purified as described in the materials and methods section. Lane 1, sigmoid volvulus (C4); lane 2, colonic adenocarcinoma (S1); lane 3, radiation-induced damage in rectum (S2); lane 4, adenocarcinoma (S4); lane 5, colonic adenocarcinoma (S5); and lane 6 colonic adenocarcinoma (S8). The gel was stained for glycoprotein with PAS. See Table II for more details of patients' histological diagnoses.

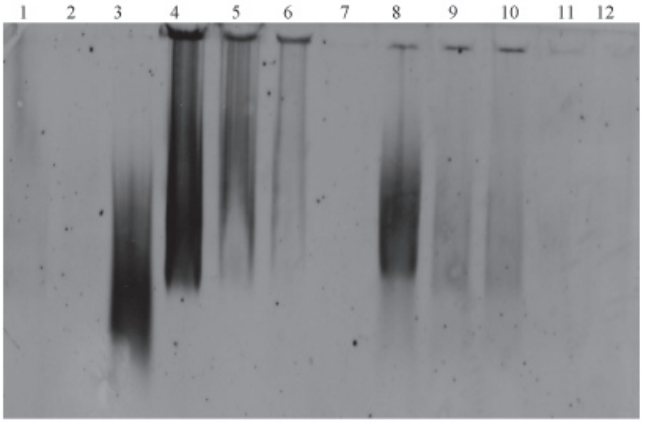

Fig. 4. Western blotting of purified colonic mucin using rabbit anti-MUC2 polyclonal antibody. Samples were separated on a 10% agarose gel and transferred to nitrocellulose membrane by vacuum blotting. The membrane was incubated for 1 h in 5% (w/v) low-fat milk powder in TBS, 0.05% Tween-20 (TBST) at 4ºC to block for nonspecific binding. The membrane was then incubated with rabbit anti-MUC2 polyclonal antibody for 2 h and then diluted in 5% (m/v) low-fat milk powder in TBST at 1:100. The membrane was then washed 3 times for 5 min with TBST and incubated for 1 h with HRPO-linked secondary antibody (goat anti-rabbit) diluted in 5% (m/v) low-fat milk powder in TBST at a dilution of 1:2 000. After another TBST wash (3 times for 5 min), bands were visualised with an ECL detection kit.

Lanes: 1, cervical mucin (+ve control); 2, normal sputum (-ve control); 3, sigmoid volvulus with no evidence of tumour or ulceration (C4); 4, colonic adenocarcinoma (S1); 5, benign squamous epithelium and underlying fibrous stroma (S3); 6, colonic adenocarcinoma (S5); 7, adenocarcinoma (S4); 8, sigmoid volvulus (C4); 9, colonic adenocarcinoma (S1); 10, chronic radiation-induced damage in the anus and rectum (S2).

Table II summarises the histological results. All controls had a mixture of neutral and acidic mucins (Fig. 5A, Table III), the latter being largely of the sulphated type (Fig. 5C, Table III). The sigmoid volvulus had less neutral and more acidic mucin of both the sulphated and non-sulphated type showing staining with both HID and alcian blue respectively. Acidic mucin was less in the cancer specimens than in controls. However acidic mucin in cancer specimens was predominantly positive for Alcian blue while the sulphated HID-positive mucin was largely absent in disease (Table III). There was less neutral mucin in the diseased tissue than in controls.

Immunohistochemistry

A summary of these results is given in Table III. Patient S4 was positive for MUC1 (Fig. 5E). Normal specimens showed no MUC1 expression, while 2 patients (S4 and S7) showed reactivity for MUC1c (mucin apoprotein) (Fig. 5F) and patient S9 was positive for MUC5AC (Table III). MUC2 was positive in controls and diseased specimens (Fig. 5G).

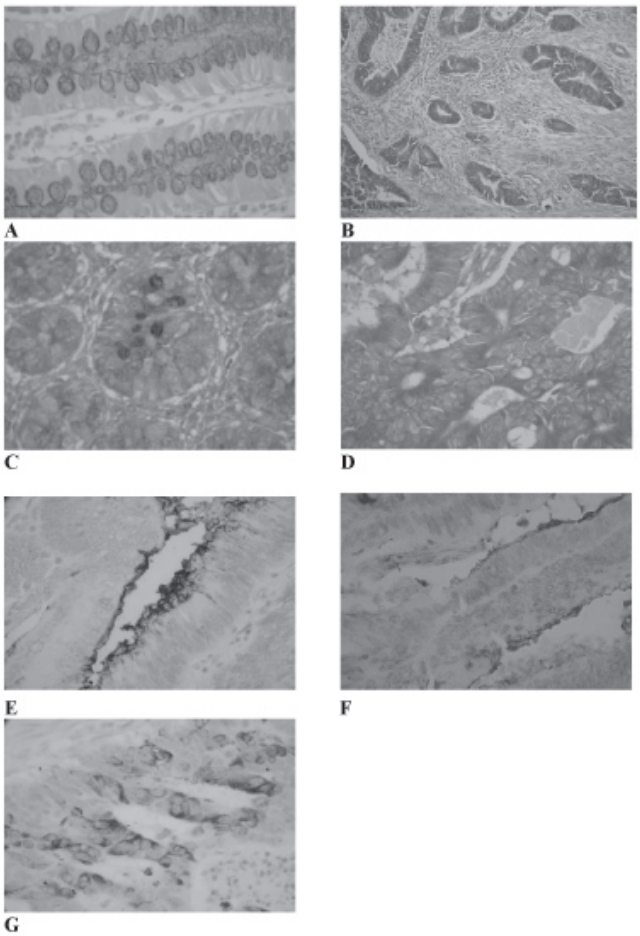

Fig. 5. Histochemical results (A - D) showing A, normal colon stained with PAS/Alcian blue (40X), B, adenocarcinoma stained with H & E (10X), C, normal colon stained with HID (10X) and D, adenocarcinoma stained with HID (10X).

Immunohistochemistry (E - G) of mucin expression in a moderately differentiated adenocarcinoma of the colon with MUC1 expression (40X) is shown in E, MUC1 CORE expression (40X) in F, and MUC2 expression (40X) in G.

TABLE II. SUMMARY OF MUCIN HISTOLOGY RESULTS

Laboratory number	Histochemistry	H and E stain	PAS/Alcian blue	HID/Alcian blue
C1	✓	**Normal No tumour**	**Mixture of acidic and neutral mucins (2+)**	**Sulphated mucin (2+)**
C2	✓	**Normal No tumour**	**Mixture of acidic and neutral mucins (2+)**	**Sulphated mucin (2+)**
C3	✓	**Normal No tumour**	**Mixture of acidic and neutral mucins (2+)**	**Sulphated mucin (2+)**
C4	✓	**Normal No tumour Sigmoid volvulus**	**Predominantly acidic mucin (2+) with focal neutral mucin (1+)**	**Predominantly non-sulphated mucin (2+) Sulphated mucin (1+)**
S1	✓	**Moderately differentiated colon adenocarcinoma**	**Predominantly extracellular neutral mucin (2+) Focal acidic mucin (1+)**	**Sulphated mucin (2+)**
S2	×	**No tissue available**	**No tissue available**	**No tissue available**
S3	×	**No tissue available**	**No tissue available**	**No tissue available**
S4	✓	**Moderately differe-ntiated colon adenocarcinoma**	**Intracytoplasmic neutral mucin (1+)**	**Non-sulphated mucin (1+)**
S5	✓	**Moderately differentiated colon adenocarcinoma**	**Apical neutral mucin (1+) Intracytoplasmic acidic mucin (1+)**	**No sulphated mucin Carboxylated mucins**
S6	✓	**Moderately differentiated colon adenocarcinoma**	**Intracytoplasmic acidic mucin (1+) Apical neutral mucin (1+)**	**Non-sulphated mucin (+1)**
S7	✓	**Moderately differentiated colon adenocarcinoma**	**Acidic mucin (1+)**	**Non-sulphated mucin (1+)**
S8	✓	**Moderately differentiated colon adenocarcinoma**	**Acidic mucin (1+)**	**Non-sulphated mucin (1+)**

TABLE III. SUMMARY OF MUCIN IMMUNOHISTOCHEMICAL RESULTS

Laboratory number	Immunohistochemistry	MUC1	MUC1 core	MUC2	MUC5AC	MUC5B	MUC6
C1	✓	**-ve**	**-ve**	**+ve (3+)**	**-ve**	**+ve (4+)**	**-ve**
C2	✓	**-ve**	**-ve**	**+ve (3+)**	**-ve**	**+ve (4+)**	**-ve**
C3	✓	**-ve**	**-ve**	**+ve (3+)**	**-ve**	**+ve (2+)**	**-ve**
C4?	✓	**-ve**	**-ve**	**+ve (2+)**	**-ve**	**+ve (1+)**	**-ve**
S1	✓	**-ve**	**-ve**	**+ve (4+)**	**-ve**	**+ve (1+)**	**-ve**
S4	✓	**+ve (2+)**	**+ve (2+)**	**+ve (1+)**	**-ve**	**-ve**	**-ve**
S5	✓	**-ve**	**-ve (< 5%)**	**+ve (1+)**	**-ve**	**-ve**	**-ve**
S6	✓	**-ve**	**-ve**	**-ve (< 5%)**	**-ve**	**-ve**	**-ve**
S7	✓	**-ve**	**+ve (1+)**	**+ve (1+)**	**-ve**	**+ve (1+)**	**-ve**
S8	✓	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**

Discussion

In early studies on the biochemical characterisation of colonic mucin, mucins were extracted in the absence of denaturing media that could have allowed for endogenous proteolysis.[16] The first definitive biochemical study on human colonic mucus was done by Herrmann *et al*.[10] However their study focused on the biochemical characterisation of colonic mucins and was not a comparison between normal and cancerous tissue. The biochemical aspect of our study was undertaken in carefully controlled conditions in which extraction and solubilisation of mucins were done in denaturing conditions, to prevent endogenous proteolysis according to the method of Carlstedt *et al*.[16] Besides comparing mucins in controls and diseased states, we also used immunohistochemical analysis to determine the type of mucin expressed in the different states.

In this study 8 samples gave non-extractable pellet material; on further analysis only 5 of these pellets were shown to have mucin. The median wet weight of the crude scraped material was 4 928 mg, which is higher than in previous studies. However, despite the higher yield of crude mucus in patients in this study (none of whom had received bowel-prep), the presence of varying amounts of faecal material in the resected specimens made the scraping of the mucosal surface and the extraction and purification of mucin very difficult.

Attempts at using postmortem samples of colonic mucus as controls[17] have been unsuccessful because cadavers were only available 48 - 72 hours after death, for medico-legal reasons. Also, tissue samples taken from the colons of these cadavers showed a variable degree of autolysis. The advantage of obtaining tissue from resections for trauma was that it was fresh. Unfortunately, in this study the tissue was supplied to us in formalin, making it unsuitable for biochemical studies and therefore used only for histological and immunohistochemical analysis. A resection for sigmoid volvulus (that might perhaps be considered 'normal') was also used as a control sample.

MUC2 is the major colonic mucin, and crude colonic mucus is found both as a soluble phase and an insoluble glycoprotein complex.[10] However in our study only 5 of the 8 non-extractable pellets had mucin. The reason for this is unknown. Reduction of the disulphide bonds has in some cases generated oligomers apparently joined by a novel reduction-insensitive bond that required treatment with trypsin for solubilisation.[10] The presence of a 'tough' mucus gel on the colonic surface is peculiar to the colon and is not found in other tracts in the body. The physiological relevance of this is not known but it could be that the intestinal surface needs both a highly resistant gel and one that allows for substances to be able to pass through from the intestinal lumen into the mucosa.

Our study also confirms the increased expression of acidic mucins in cancer of the colon compared with normals.[18] MUC2 was present in all specimens, both normal and diseased. The sigmoid volvulus specimen gave a MUC2 of different charge from the rest of the samples, and we do not understand the significance of this. MUC5AC (a gastric and respiratory mucin) has reportedly been found in 86% of mucinous carcinomas.[19] We found expression of MUC5AC in 2 and MUC5B in 5 diseased specimens. However the numbers are too small to be able to interpret these findings.

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