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Colonic mucins in ulcerative colitis: evidence for loss of sulfation

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Colonic tissue obtained at surgery from control individuals and patients with ulcerative colitis was used to isolate mucins and to prepare mucin glycopolypeptides by pronase digestion. These were compared with mucins labelled with [^{35}S] sulfate and [^{3}H]-glucosamine after organ culture tissue samples from the same patients. A significant loss of mucin sulfation was detected in the colitis patients by both metabolic labelling and chemical analysis of the glycopolypeptides. A change in the size distribution of purified mucin oligosaccharides fractionated on BioGel P6 after release by β-elimination was seen in both radiolabelled and non-labelled colitis mucins compared with controls. Amino acid analysis of the glycopolypeptides showed a close similarity to the expected ratio of serine:threonine:proline for MUC2 and did not vary between control and colitis groups. Analysis of the mucins confirmed >90% purity in the labelling experiments, characteristic behaviour on density gradient centrifugation and agarose gel electrophoresis in control and ulcerative colitis groups and differences in sulfation and turnover at various sites in the normal colon.

Keywords: Mucin, mucus, ulcerative colitis, MUC2, sulfation, colon, metabolic labelling

Abbreviations: WGA, wheat germ agglutinin; UC, ulcerative colitis; HRP, horseradish peroxidase

Introduction

The gastrointestinal tract is coated with an adherent, viscoelastic mucus defensive barrier secreted by the mucosal cells [1]. The properties of the adherent mucus gel are governed largely by the physicochemical properties of the mucins, the major secretory product of the mucous and goblet cells in the mucosal layer [2, 3]. Although the mucins synthesized throughout the gastrointestinal tract form viscoelastic gels, they have a site specific structure and can be differentiated on the basis of their chemical composition [2]. Furthermore, pathological modifications in the structure of the mucosal barrier mucins affect their normal defensive properties and their rate of degradation and therefore directly influence the viability of the defensive barrier [1, 4].

Both the secreted mucins, forming the extracellular gels [2], and cell membrane associated (glycocalyx) mucins, typically MUC1 type [2, 3] involved in cell surface interactions are implicated in the cell surface and adherent gel layer changes occurring during gastrointestinal cancer and inflammatory bowel diseases such as ulcerative colitis (UC) [4, 5]. Histological study has identified goblet cell depletion [6] and changes in goblet cell vesicle staining for sulfated and sialylated mucin [3, 4] associated with UC. The loss of a mucin subform, separated by ion-exchange chromatography has been described [7], although this has been challenged as an artefact of the fractionation procedure [8].

Normal human colonic secreted mucins are rich in

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sulfate and O-acetylated sialic acids [9, 10]. These terminal moieties impart negative charge and govern the rate of mucin breakdown by enteric bacterial enzymes [11]. Further characteristic properties of the oligosaccharides include 65–75% having 4–12 sugars per chain, 30–40% as neutral structures and a predominance of Core 3 (GlcNAcβ1-3GalNAc-Ser/Thr) based oligosaccharides [12]. Backbone structures are based on Gal-GlcNAc repeats with branching at galactose residues and the sialic acids are mostly linked α2-6 to galactose and GalNAc. The substitution of sulfate in oligosaccharides has not been rigorously examined, although enzymic analyses suggest the presence of 3-O-sulfation of galactose [13].

In recent years a number of mucin (*MUC*) genes have been identified coding for mucin polypeptides [14]. These have typically two domains, firstly threonine, serine, proline rich regions present as tandem repeats and constituting the major part of the O-glycosyl-linkage sites for the oligosaccharides. Secondly, there are cysteine rich, poorly glycosylated peptide sequences, susceptible to proteolytic attack and involved in the end to end disulphide bond linkage between individual mucin subunits. The sequence of the tandem repeat units is novel for each *MUC* gene and constitutes part of the individual properties of these mucins. In the human colon MUC2 has been reported to be the major *MUC* gene product [15, 16].

Attempts to assess the nature of alterations in mucin production, secretion and structure during normal development and disease have adopted various approaches. Direct measurement of the thickness of mucus gel layer in colonic biopsies has recently been reported [17], showing a reduction in thickness associated with UC. Chemical characterization of isolated mucins has been reported where sufficient tissue could be obtained [7] and metabolic labelling methods including dual labelling with [^{35}S]-sulfate and [^{3}H]-glucosamine have provided a valuable assessment of mucin structure and turnover [5, 18–20]. The use of the dual labelling method has shown conflicting results with regard to mucin sulfation in UC [5, 18–21].

The present study is designed to resolve the discrepancies observed in the metabolic labelling experiments and to correlate the sulfation and glycosylation patterns of colonic mucins with the MUC gene product in control and UC cases. The results show that UC is associated with a modification of mucin glycosylation, a depletion of sulfate, but with retention of a predominantly MUC2 based polypeptide.

Patients

Tissue samples were all obtained at surgery from a total of 10 control patients, undergoing colectomy for prolapse ($n = 6$) or chronic constipation ($n = 4$). Samples were taken from rectal tissue alone in five cases and at multiple sites in the remaining five cases. These patients were found to have macroscopically and histologically normal rectal mucosa [21]. Rectal tissue was also taken from 10 patients with severe ulcerative colitis undergoing restorative proctocolectomy and two obtained at colonoscopy as rectal biopsies from patients with established inactive colitis. Diagnostic classification was based on biopsies taken from the same site for routine histology and mucin histochemistry [21].

Materials and methods

MATERIALS

Radioactive materials [^{3}H]-glucosamine (740 GBq mmol^{-1}), sodium [^{35}S]-sulfate (2.04 GBq mmol^{-1}) and [^{3}H]-thymidine (740 GBq mmol^{-1}) and [^{3}H]-Hyperfilm were obtained from Amersham International plc, Amersham, UK. Cell culture products were purchased from Gibco Ltd, Edinburgh, Scotland. Sepharose CL 4B and CL 2B were products of Pharmacia/LKB, Milton Keynes, UK. Immobilon P (PVDF) was from Millipore, Watford, UK. Biogel P6, was from BioRad, Hemel Hempstead, UK. Seakem LE agarose was from Flowgen Instruments, Sittingbourne, UK. Chondroitinase ABC, heparinase, heparitinase and hyaluronidase were from Boehringer UK, Lewes, UK. Hoechst dye 33258 was obtained from Hoeffer, Newcastle Under Lyme, UK. Wheatgerm agglutinin-horseradish peroxidase conjugate was a product of Vector Labs, Peterborough, UK. Antibody PR 3A5 was obtained from the Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin was from Dako, High Wycombe, UK. Pronase, guanidine hydrochloride, caesium chloride, 3,3′-diaminobenzidine and other analytical grade biochemicals were from the Sigma Chemical Co Ltd, Poole, UK.

HISTOCHEMISTRY

Tissue samples were fixed in 10% buffered formalin, processed routinely and sectioned at 2 μm. Sections were stained with Haematoxylin-eosin and high iron diamine/Alcian blue (HID/AB) with a fresh 60% solution of ferric chloride and controlled with a standard composite block of ileum, gastric body and right colon [21].

COLONIC TISSUE

Tissue obtained at surgery was kept at ambient temperature after resection. Mucosal samples for organ culture were dissected free of the muscularis propria. Maximum duration between removal of tissue and initiation of culture was 60 min, most samples were processed within 30 min. Tissue samples used to prepare mucin subunits and glycopolypeptide were irrigated with PBS-protease inhibitor buffer [24] and scraped as described before [22].

The extracted mucus was separated by chromatography on Sepharose CL 2B, the excluded V_0 fraction being collected and pooled [22]. Samples of this fraction were submitted to density gradient centrifugation to prepare mucin subunits, or exhaustively digested with pronase and chromatographed on Sephadex G150 to prepare mucin glycopolypeptides [23].

ORGAN CULTURE

Organ culture was carried out as described before [24] in minimal Eagles medium with 10% fetal calf serum. This medium contains 0.8 mM magnesium sulfate and 5.6 mM glucose. Each incubation contained 925 kBq sodium [^{35}S]-sulfate (2.04 GBq mmol^{-1}) and 370 kBq [^{3}H]-D-glucosamine (740 GBq mmol^{-1}). After incubation the medium was collected and the tissue homogenized and aliquots taken for DNA analysis [24]. Incubations with [^{3}H]-thymidine were under the same culture conditions and contained 370 kBq [^{3}H]-thymidine (740 GBq mmol^{-1}). After various times the medium was removed and the tissue collected and homogenized in 500 μl PBS-inhibitor buffer. A sample (20 μl) was taken for DNA measurement and a portion (200 μl) precipitated with 2 ml of 10% trichloroacetic acid at 4 °C for 30 min and centrifuged at 12 000 × **g** and 4 °C for 10 min. The pellet was washed three times with 95% ethanol before determining the radioactivity.

GEL FILTRATION

Organ culture samples were fractionated on 30 × 1 cm columns of Sepharose CL 4B as before [24]. Non-radiolabelled colonic scrapings were separated on Sepharose CL 2B the V_0 peak was pooled, submitted to exhaustive pronase digestion and the V_0 fraction collected from Sephadex G150 chromatography [22, 23].

DENSITY GRADIENT CENTRIFUGATION

Density gradient centrifugation in PBS/4 M guanidine hydrochloride was carried out as described before [21].

AGAROSE GEL ELECTROPHORESIS AND VACUUM BLOTTING

Agarose gel electrophoresis was carried out on 15 cm × 15 cm, 1.0% agarose gels as described by Thornton *et al.* [25]. Native or reduced and alkylated [24] samples were loaded into 1.5 mm slots. After electrophoresis the gels were vacuum blotted onto PDVF membranes [25]. The blots were stained with wheat germ agglutinin-horseradish peroxidase conjugate or with PR3A5 antibody.

Beta-elimination

Mucin samples were subjected to alkali catalysed β-elimination and purified as described before [23]. The products were run on Sepharose CL 2B and the low molecular weight peak was recovered and run on a column of BioGel P6 (100 × 1 cm) in 0.1 M pyridine acetate pH 5.0. Samples of glycopolypeptide prepared by pronase digestion of non-radioactive mucin were analysed in the same way and the products detected by sialic acid and hexose assays. Pools from BioGel P6 chromatography were analysed for their total carbohydrate composition by gas chromatography [26].

Acid hydrolysis to remove sialic acid and sulfate

Oligosaccharides released by beta-elimination and purified by Sepharose CL 2B chromatography were dissolved in 0.1 M HCl and incubated for 1 h at 100 °C in sealed glass tubes under nitrogen. The samples were rotary evaporated to dryness at 30 °C, passed through columns of Dowex 1 × 8 formate form and washed with five column volumes of distilled water. The non-retained fraction was resubmitted to Biogel P6 chromatography. Bound material was eluted with 1 M formic acid and freeze dried.

Enzyme digests

Digestion and chondroitinase ABC, testicular hyaluronidase and heparinase/heparitinase were as before [21]. The products were analysed by Sepharose CL 2B. Non-radioactive samples were detected by *N*-acetyl hexosamine, hexose and sialic acid colorimetric assays.

Detection of mucins

The presence of glycoproteins including mucins was detected by slot blotting of aliquots (10–50 μl) onto PVDF membranes [27], and probing with the PAS stain, wheat germ agglutinin-HRP conjugate (0.7 μg ml^{-1}) or antibody PR3A5 followed by horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin at 1:3000 dilution [28]. Visualization of HRP conjugates was carried out using 3,3′-diaminobenzidine. After scanning of blots by a Hewlett Packard HP2C Scanner, their intensity was measured densitometrically using Optimas Bioscan Software. Fractions from separation techniques were probed by liquid assays for hexose, sialic acids and optical density at 280 nm.

Other methods

Protein was measured using the dye-binding method of Bradford [29]. DNA, [21], hexoses [30], *N*-acetyl hexasamines and sialic acids [31] were measured as before. Amino acids were measured after acid hydrolysis using a modified Waters Picotag system [32]. Cysteine was detected as carboxymethyl cysteine after reduction and alkylation. Sulfate was detected by the method of Silvestri *et al.* [33]. Radioactivity was measured and corrected as described [21].

Statistical analysis of the disease groups was carried out using the Mann-Whitney U test.

Results

Histological assessment of surgical specimens and organ culture validation

Histological examination of colitic colonic tissue confirmed the morphological abnormalities and sulfate depletion reported before [21] when compared with normal controls (data not shown). Tissue placed in organ culture for periods up to 48 h showed preservation of the initial morphology up to 36–48 h. No abnormalities were detected in tissue samples incubated for 24 h and this time was taken as a standard maximum for all incubations.

The incorporation of [^{3}H]-thymidine into acid precipitable biopsy DNA during the 24 h incubation showed a regular increase throughout this period indicating the viability of the cultures, no difference was observed between control and UC tissue tested (Fig. 1a). Incorporation of [^{3}H]-glucosamine and [^{35}S]-sulfate into the Sepharose CL 4B V_0 mucin fraction (Fig. 2) was found to increase regularly over 48 h with the cellular mucin (Fig. 1b) and exhibited the expected initial lag period for the secreted mucin fraction (Fig. 1c). A lower incorporation into UC mucins was found after 20 h incubation for cellular mucin and after 30 h for secreted mucin. These experiments also showed a reduction in sulfate over glucosamine incorporation in both cellular and secreted mucins (Fig. 1b,c).

CHARACTERIZATION AND COMPARISON OF CONTROL AND ULCERATIVE COLITIS MUCINS

In order to compare control and UC mucins in the metabolic labelling experiments with non-labelled and organ culture mucins from the same tissue an enriched mucin fraction was obtained by pooling the Sepharose CL 4B V_0 peak. This material was further examined by gel filtration, enzyme digestion, beta-elimination, agarose gel electrophoresis and density gradient centrifugation.

Gel filtration

Representative Sepharose CL 4B profiles for metabolically labelled secreted and cellular soluble fractions are shown in Fig. 2 for normal and colitis patients. A good separation of the high molecular weight mucin fraction at the V_0 of the column was obtained in all cases. Non-labelled mucin extracts gave a mucin enriched fraction in the V_0 under the same conditions. Rechromatography in 4 M-guanidine hydrochloride did not affect the elution profile for both normal and colitis mucin fractions tested (data not shown). The fragmentation of this high molecular weight fraction by reduction and alkylation or pronase treatment of this V_0 fraction gave a partial displacement of the V_0 peak into the included volume on Sepharose CL 2B, with both control and colitis mucins (data not shown). This pattern corresponds to the expected behaviour of native mucins forming reduced subunits and mucin glycopolypeptide [1–3, 14]. The fractionation of pronase digested non-radioactive mucin on Sephadex G150 yielded a high molecular weight mucin glycopolypeptide fraction eluting at the V_0 of the column and containing >90% of the total carbohydrate.

Metabolic labelling of mucins eluting in the Sepharose CL 4B V_0 peak showed a significant reduction in the ratio of [^{35}S]:[^{3}H] for both secreted and cellular mucin in UC patients (Table 1), reflecting the differences seen in

Table 1. Incorporation of radioactive precursors into mucus glycoproteins in organ culture. The incorporation of label into the V_0 mucin fraction isolated on Sepharose CL 4B was measured after metabolic labelling in organ culture. The ratio of [^{35}S]:[^{3}H] and the turnover relative to the total DNA in the cultured tissue is shown.

	Ratio (Mean ± SEM)	*Turnover (Mean ± SEM)*	
Sample	*[^{35}S]:[^{3}H]*	*[^{35}S] per μg DNA*	*[^{3}H] per μg DNA*
Control ($n = 10$)			
Cellular	2.23 ± 0.25	9316 ± 1814	4173 ± 733
Secreted	1.58 ± 0.24	5900 ± 1213	3680 ± 1370
p	NS	NS	NS
Ulcerative colitis ($n = 12$)			
Cellular	0.49 ± 0.10	857 ± 307	1656 ± 470
Secreted	0.52 ± 0.12	547 ± 213	1196 ± 269
p	NS	NS	NS
p control versus ulcerative colitis			
Cellular	<0.0001	<0.001	<0.01
Secreted	<0.002	<0.02	NS

The significance of the results was tested using the Mann-Whitney U test.
NS, not significant.

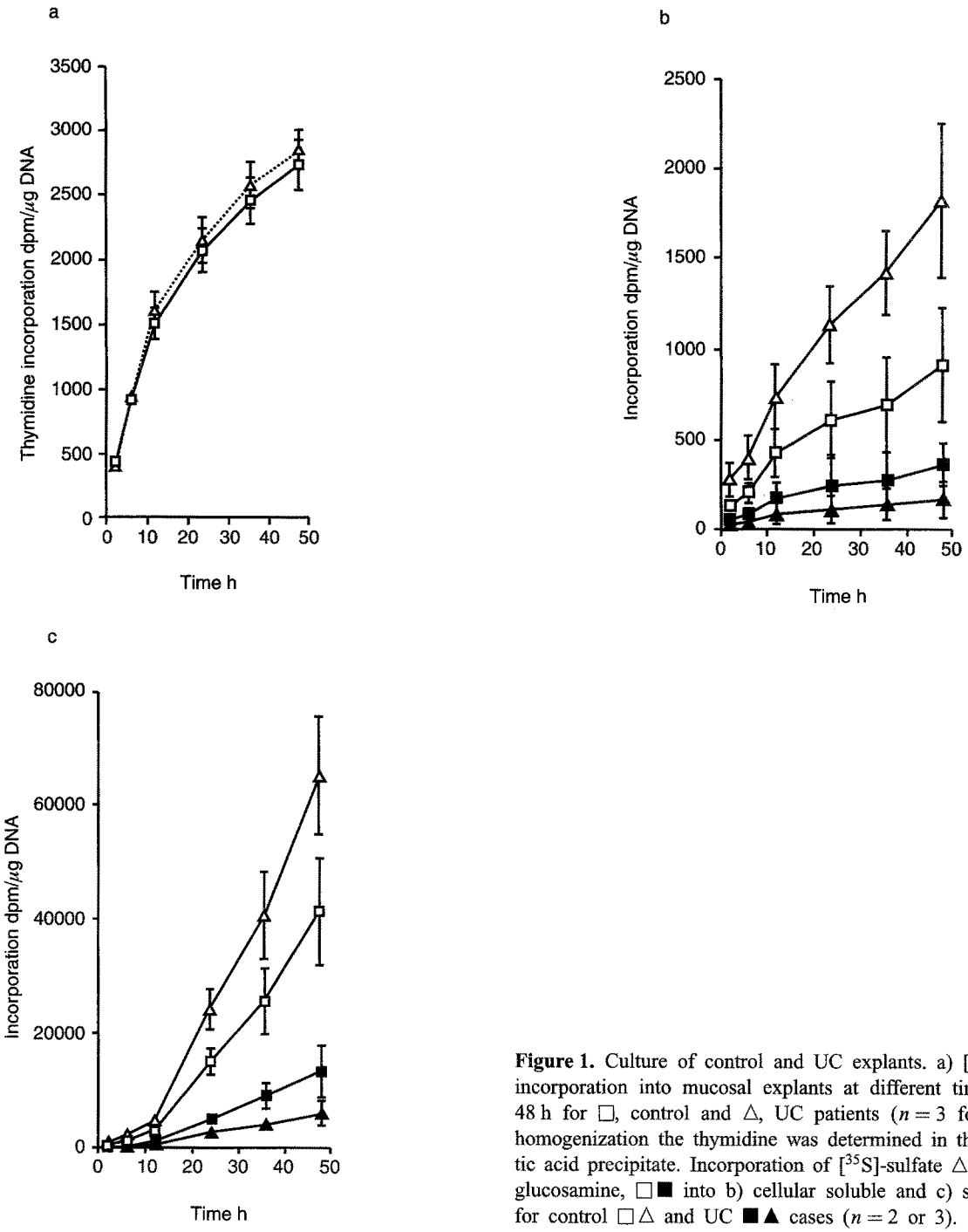

Figure 1. Culture of control and UC explants. a) [^{3}H]-Thymidine incorporation into mucosal explants at different time points over 48 h for □, control and △, UC patients ($n = 3$ for each). After homogenization the thymidine was determined in the trichloroacetic acid precipitate. Incorporation of [^{35}S]-sulfate △▲ and D-[^{3}H]-glucosamine, □■ into b) cellular soluble and c) secreted mucin, for control □△ and UC ■▲ cases ($n = 2$ or 3).

Figs 1 and 2. This loss of sulfation was also seen in the turnover values with significant reduction of the mucin [^{35}S] dpm per μg DNA. A reduction in turnover was also seen for the mucin [^{3}H] dpm per μg DNA in UC mucin indicating additional modification of UC mucin glycosylation, but this was only significant in the cellular fraction.

The chemical analysis of sulfate in the pronase digested mucin glycopolypeptide from controls and UC patients showed a significant reduction, whether related to the dry weight of the sample or the molar ratio of galactose (Table 2), supporting the result obtained with metabolic labelling. Recovery of material in all of these experiments was 96 ± 7% for [^{3}H] and [^{35}S] and 95 ± 6% for hexose in non-radioactive samples.

Enzyme digestion

Digestion of four control and six colitic, dual labelled secreted and cellular mucin fractions and five control and five colitic non-labelled Sepharose CL 2B V_0 fractions with chondroitinase ABC or a mixture of heparinase and heparitinase did not change the elution profile of these

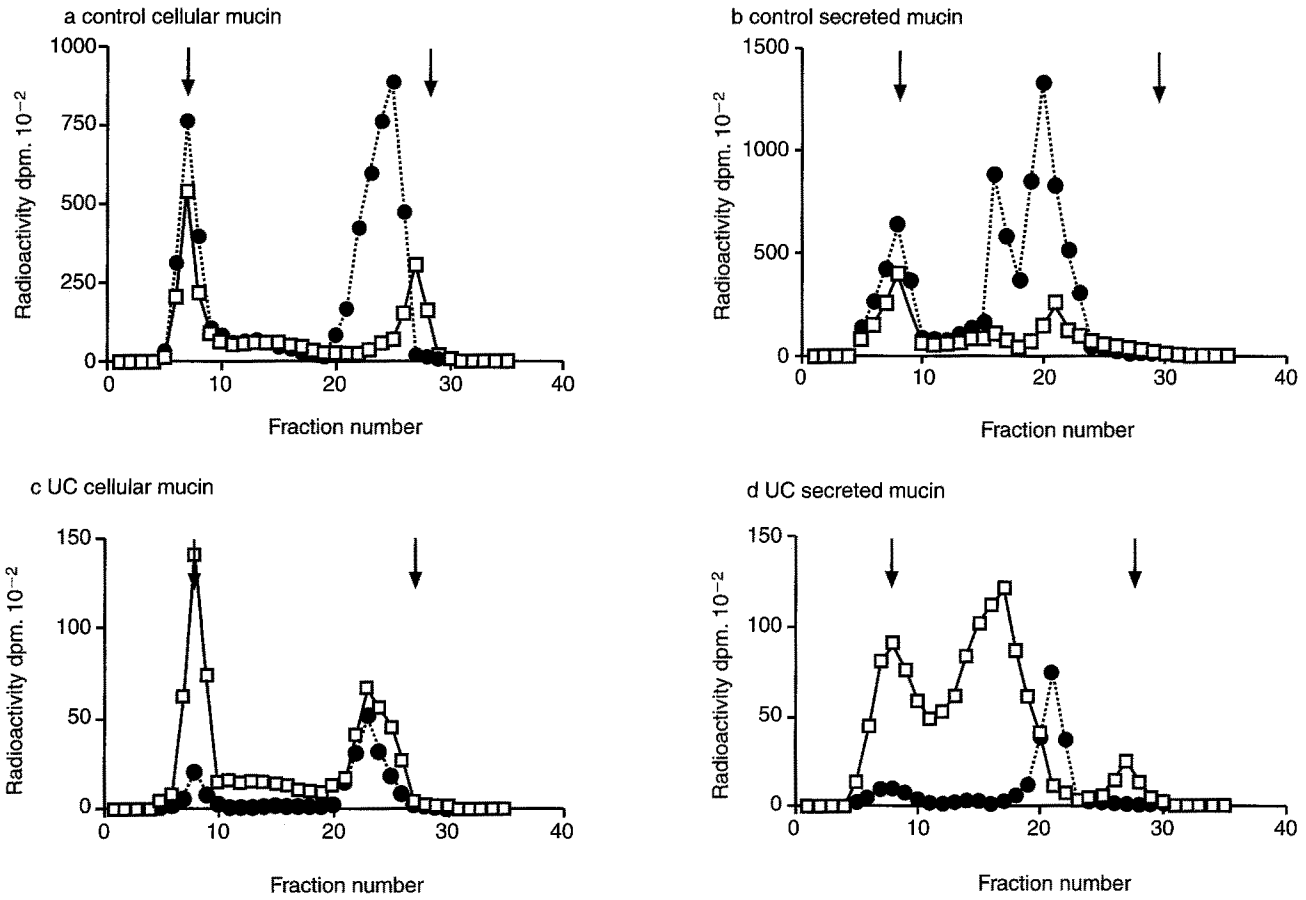

Figure 2. Sepharose CL 4B chromatography on 30 × 1 cm columns of the soluble fractions obtained from cellular (a, c) and secreted (b, d) fractions after organ culture of colonic mucosal explants. The radioactivity as □, D-[^{3}H]-glucosamine and ●, [^{35}S]-sulfate in 1 ml fractions was measured. The V_0 of the columns was determined at fractions 7–8 and the V_t at fractions 27–28 in all cases, positions are shown by arrows. Representative samples of control (a, b) and UC patients (c, d) are shown.

samples on Sepharose CL 4B chromatography (data not shown). These experiments indicate that the material eluting at the V_0 of the Sepharose CL 4B column is not glycosaminoglycan or proteoglycan. Hyaluronidase digestion of both cellular and secreted mucin resulted in a peak of [^{3}H] amounting to less than 8% of the total [^{3}H] in these incubations eluting at the V_t of the Sepharose columns, this was not detectable using the non-radioactive samples.

Table 2. Sulfate content of mucin glycopolypeptides from control and ulcerative colitis patients. The sulfate content of the mucin glycopolypeptide preparations was measured as detailed in the methods and expressed relative to the dry weight or the galactose content.

	Sulfate content	
Patient group	(*nmol mg*$^{-1}$) (*Mean* ± *SEM*)	*molar ratio*
Control ($n = 10$)	379 ± 37	3.40 ± 0.36
Ulcerative colitis ($n = 10$)	122 ± 12	0.98 ± 0.13
p	<0.0001	<0.0001

*The molar ratio was calculated relative to 6 mol of galactose as determined by gas liquid chromatography (Table 3). The statistical significance of the differences between control and colitis cases was tested by the Mann-Whitney U test.

Density gradient centrifugation

Centrifugation of non-reduced mucins in 4 M guanidine hydrochloride showed a major peak of radiolabelled material having a density of approximately 1.40 g ml^{-1} in the case of five out of seven control samples analysed (representative example shown in Fig. 3a). The remaining two control samples revealed more material appearing at a lower density of approximately 1.35 g ml^{-1} (Fig. 3b). Colitis samples showed 2/6 with a similar density distribution to 3a and 4/6 with type 3b, both with lower sulfation. Not all samples could be analysed by this technique due to the lack of material. Recovery of radioactivity was 73 ± 9% for all samples examined. The profiles of non-labelled mucins detected by wheat

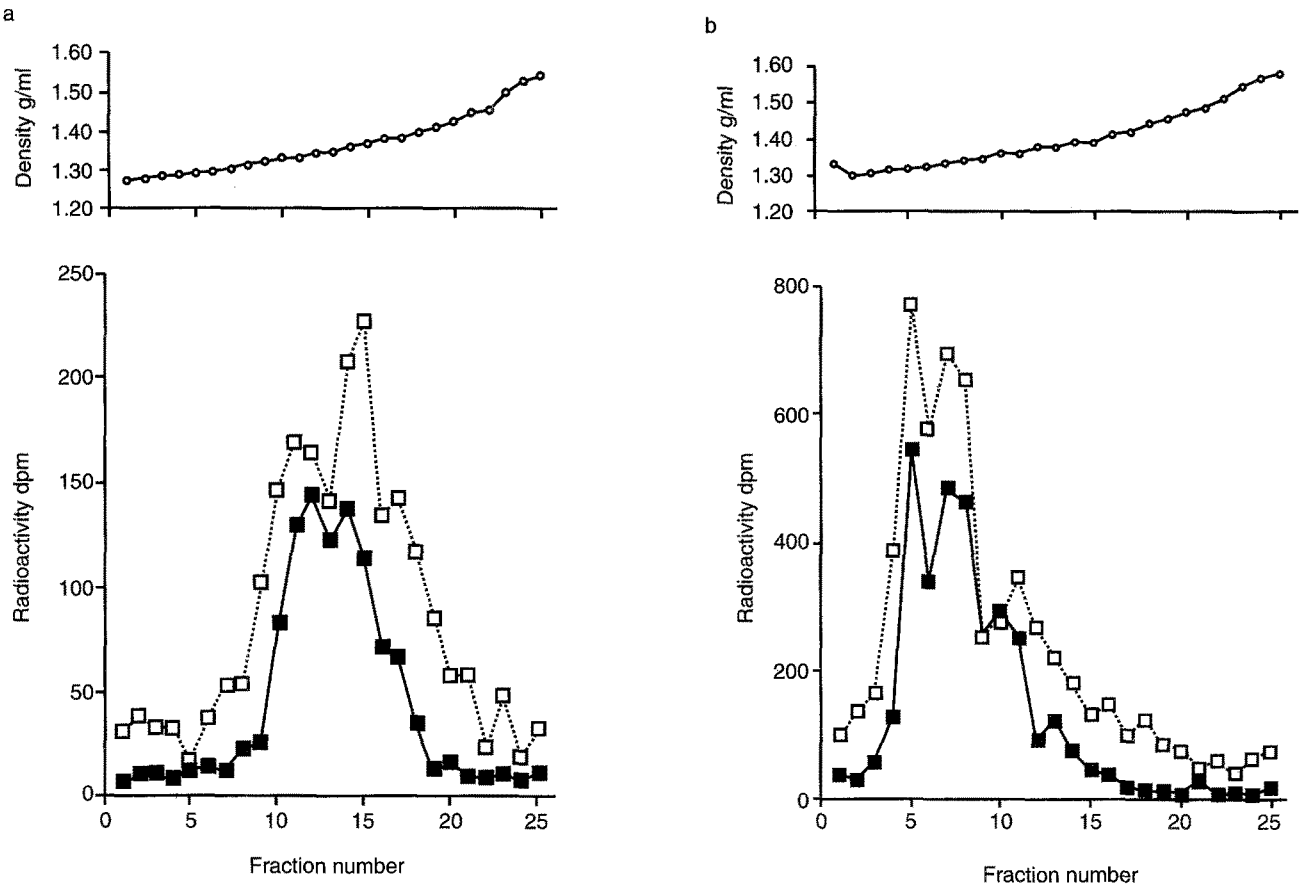

Figure 3. Density gradient centrifugation in CsCl of the Sepharose CL 4B, V_0 mucin fractions for secreted mucin from two control individuals a and b. The total V_0 fraction was centrifuged in 13 ml and 0.5 ml fractions were collected from the top of the tube after the gradient was formed. The fractions were weighed to determine the density ○, and the radioactivity as ■, D-[^{3}H]-glucosamine and □, [^{35}S]-sulfate measured. The profiles show examples of the two main patterns of radiolabel distribution found in both control and UC patients. Further details are given in the Methods.

germ agglutinin showed a single peak of material similar to the [^{3}H]-glucosamine trace in Fig. 3a in 6/9 control and 4/10 UC cases. The remainder exhibited a single peak at approx 1.35 g ml^{-1}. Reduced and alkylated samples were not examined. Recovery of 89 ± 6% of the radioactivity or hexose was found in the major mucin peaks detected for all samples tested.

Beta-elimination

Alkaline borohydride treatment resulted in a shift in the elution profile of >95% of the radioactive label to a position near to the lower included limit, V_t of a Sepharose CL 2B column for secreted and cellular mucins for five control and five UC cases. This material was collected and fractionated on BioGel P6, yielding a major peak close to the excluded volume with coincident [^{35}S] and [^{3}H] profiles for the control samples (representative sample shown in Fig. 4a). The UC samples showed a greater distribution of material in the lower molecular weight range (example given Fig. 4b). Acid hydrolysis of the total oligosaccharide pool collected from Sepharose CL 2B control and UC under conditions removing sulfate and sialic acid resulted in the complete loss of the [^{35}S]- and 20–27% of the [^{3}H]-glucosamine label. The [^{3}H]-glucosamine labelled material was retained on Dowex 1 × 8 formate column and after elution with 1 M-formic acid ran as a single component on cellulose thin-layer chromatography with an Rf identical to that of authentic *N*-acetylneuraminic acid (data not shown). The remaining neutral [^{3}H]-labelled material from control samples migrated as a major included peak on BioGel P6 as shown in Fig. 4c, while UC samples showed most material eluting at the same position but with a significant proportion in the lower molecular weight range (Fig. 4d). The recovery of all radioactivity from ion-exchange bound and unbound fractions was 87 ± 9% for samples analysed.

Beta-elimination of pronase digested mucin glycopolypeptide samples from control and UC mucins resulted in similar profiles on BioGel P6 (representative examples

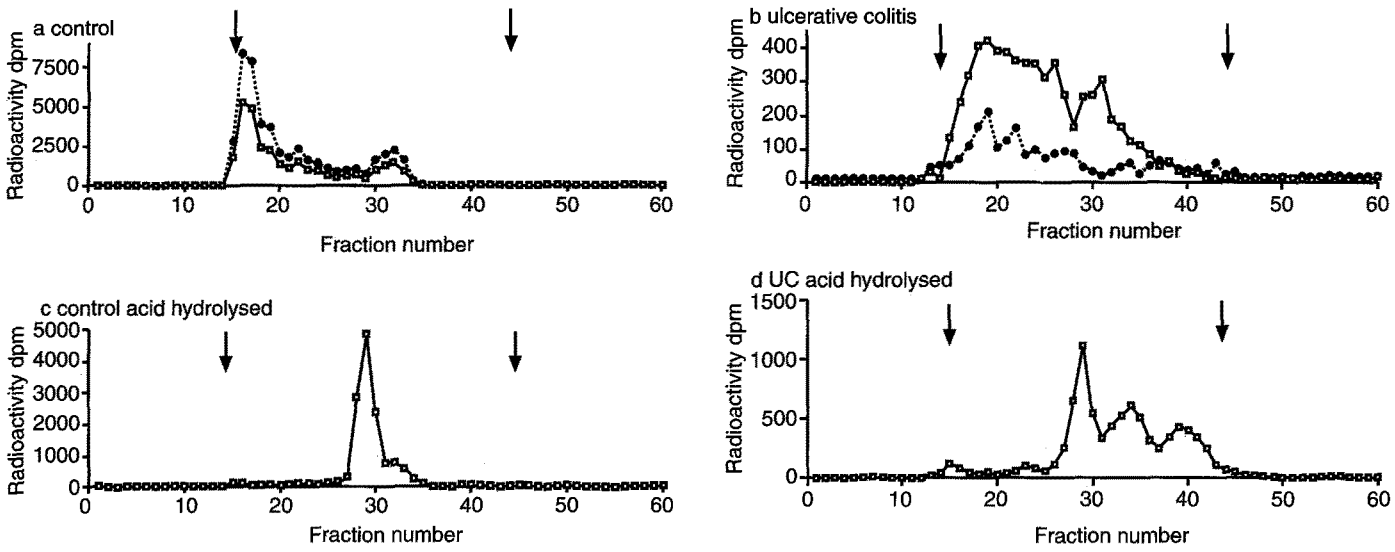

Figure 4. Fractionation of the products of beta-elimination of Sepharose CL 4B V_0 purified secreted mucin on BioGel P6 (100 × 1 cm) eluted with 0.1 M pyridine acetate pH 5.0. The products of beta-elimination were previously fractionated on Sepharose CL 2B and the single included peak collected, concentrated and applied to the P6 column. Fractions were assayed for □, D-[^{3}H]-glucosamine and ●, [^{35}S]-sulfate in representative control (a) and UC (b) patients. The total Sepharose CL 2B oligosaccharide pool for control (c) and UC (d) cases was acid hydrolysed to remove sialic acid and sulfate and chromatographed on BioGel P6 measuring the [^{3}H] profile, □. The V_0 of the BioGel P6 column was determined at fractions 14–16 and the V_t at fractions 43–45 as shown by arrows.

shown in Fig. 5a,b). An increase in the proportion of lower molecular weight material was also seen in the UC samples (Fig. 5b), but this was not as marked as that seen in the metabolically labelled mucin oligosaccharides (Fig. 4b). The proportion and monosaccharide composition of 6 oligosaccharide pools of decreasing molecular size across the profiles is shown in Table 3. A significant reduction in the proportion of the major high molecular weight peak (P6 1) was found in the UC patients with increases in the lower molecular weight pools. An increase in GlcNAc was found in the total glycopolypeptide fraction (G150) in UC samples and was also seen in the pools P6 1, 2, 3 and 5. An increase in sialic acid was found in these same fractions. GalNAc-ol was below detection limits in control pool P6 1, and detection in the same pool for UC samples suggests a shortening of chain

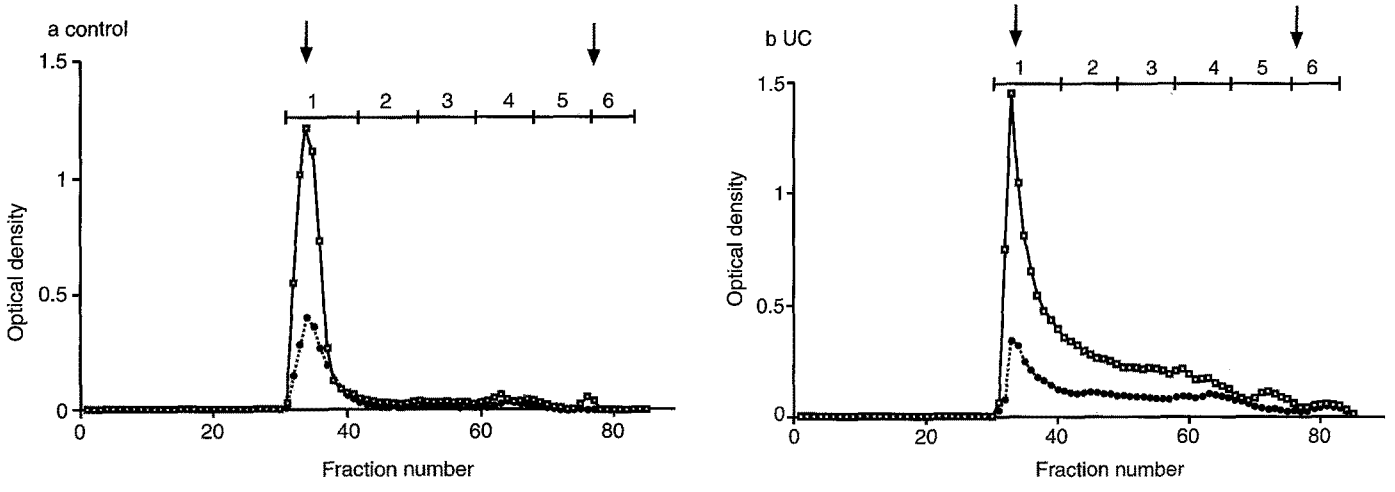

Figure 5. Fractionation of the products of beta-elimination of the mucin glycopolypeptides from control (a) and UC (b) cases, on BioGel P6 (100 × 1 cm) eluted with 0.1 M pyridine acetate pH 5.0. Fractions were assayed for hexose □, and sialic acid ●, and collected as six pools as indicated. The V_0 determined at fractions 34–35 and the V_t at fractions 76–77 are shown by arrows. Further details are given in the Methods.

Table 3. Carbohydrate composition of the glycopolypeptide fraction from Sephadex G150 and oligosaccharide pools separated by BioGel P6 chromatography after β-elimination. Results are presented as a percentage of the dry weight of the total oligosaccharides recovered after β-elimination. The glycopolypeptide G150 fraction is considered to represent 100% of the carbohydrate, the relative dry weight recoveries of total oligosaccharides after β-elimination were 75 ± 6% for control and 78 ± 4% for UC samples.

		Molar ratios (Gal = 6.0) Mean ± SEM						
Sample	*wt%*	*Fuc*	*Man*	*Gal*	*GlcNAc*	*GalNAc*	*Neu5Ac*	*GalNAc-ol*
Control ($n = 7$)								
G150	100	2.2 ± 0.3	0.4 ± 0.05	6.0	6.5 ± 0.3	7.1 ± 0.3	7.1 ± 0.4	–
P6 1	65 ± 12	1.7 ± 0.2	0.4 ± 0.04	6.0	5.9 ± 0.2	5.8 ± 0.4	5.7 ± 0.5	ND
P6 2	8 ± 1	1.5 ± 0.2	ND	6.0	5.5 ± 0.4	3.2 ± 0.3	4.4 ± 0.6	0.4 ± 0.05
P6 3	7 ± 1	1.7 ± 0.2	ND	6.0	5.1 ± 0.4	2.5 ± 0.3	4.8 ± 0.4	1.5 ± 0.4
P6 4	6 ± 1	1.8 ± 0.3	ND	6.0	5.7 ± 0.3	2.6 ± 0.3	6.3 ± 0.8	2.0 ± 0.3
P6 5	8 ± 1	2.4 ± 0.4	ND	6.0	5.0 ± 0.3	2.1 ± 0.4	6.8 ± 0.6	2.7 ± 0.1
P6 6	6 ± 3	2.2 ± 0.3	ND	6.0	3.9 ± 0.6	1.0 ± 0.4	4.7 ± 0.7	3.0 ± 0.5
Ulcerative colitis ($n = 9$)								
G150	100	1.9 ± 0.2	ND***	6.0	7.4 ± 0.5**	7.6 ± 0.6	7.3 ± 0.6	–
P6 1	50 ± 10**	2.0 ± 0.2	ND***	6.0	6.6 ± 0.2**	5.7 ± 0.5	6.8 ± 0.9**	0.1 ± 0.05**
P6 2	11 ± 1*	2.0 ± 0.2	ND	6.0	6.4 ± 0.4**	3.3 ± 0.4	5.5 ± 0.9*	0.4 ± 0.1
P6 3	10 ± 2*	1.9 ± 0.2	ND	6.0	6.3 ± 0.2**	2.6 ± 0.3	5.8 ± 1.0*	1.2 ± 0.1
P6 4	9 ± 2**	1.9 ± 0.2	ND	6.0	5.8 ± 0.4	2.5 ± 0.3	6.0 ± 1.0	1.8 ± 0.1
P6 5	11 ± 2*	2.2 ± 0.2	ND	6.0	6.2 ± 0.4**	2.5 ± 0.2	9.3 ± 1.4**	3.4 ± 0.3*
P6 6	9 ± 2*	2.6 ± 0.3	ND	6.0	4.6 ± 0.2	1.3 ± 0.3	4.0 ± 0.8	3.4 ± 0.3

The molar ratios of each monosaccharide are expressed as mean ± SEM relative to Gal = 6.0. Statistically significant differences between control and UC groups were assessed by Mann Whitney U test, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. ND, not detectable.

length in this fraction. Increased GalNAc-ol was also detected in UC pool P6 5. The origin of the GalNAc may be from blood group A antigen, 2/7 control and 3/9 UC samples were from Blood group A individuals.

Mannose was detected in the total G150 fraction of control samples and was recovered in the P6 1 fraction suggesting the presence of some glycopolypeptide residue in this fraction. The UC samples were devoid of mannose (Table 3). Insufficient material was available to carry out sulfate analysis on all of these non-radioactive samples.

Indication of MUC *gene identity by amino acid analysis*

The amino acid content of the pronase digested mucin glycopolypeptide purified from non-labelled mucosal scrapings showed significant differences between control and UC samples (Table 4). Notably, reductions in proline, threonine and cysteine and a large increase in alanine were found in UC glycopolypeptide. No change in serine was found and the proportions of Glx and Asx changed in opposite directions, but with an overall decrease for these two amino acids in UC. Comparison of the ratio of serine:threonine:proline showed no variation between control and UC glycopolypeptides (Table 5). The ratios were also compared with those reported for the different *MUC* gene tandem repeat sequences. Both control and UC ratios have good agreement with the predicted value for MUC2 characterized by the low content of serine and no correlation with other *MUC* gene tandem repeat structures.

Agarose gel electrophoresis

Agarose gel electrophoresis was used in place of SDS-PAGE because of poor resolution of very high molecular weight components and uncertainties about the proportion of each loaded sample migrating into the gels. The presence of at least four bands was detected using WGA (representative examples given in Fig. 6a,b) and this was detected in 5 control cases (Fig. 6a). The rapidly migrating bands appeared to be diminished with a change in intensity of the other main bands (Fig. 6b) in all 4 UC samples analysed. Staining of control mucins separated by density gradient centrifugation with the anti colonic mucin antibody PR3A5 revealed three bands (Fig. 6c). The fast band detected with WGA was only weakly stained.

COMPARISON OF DUAL LABELLED MUCINS IN DIFFERENT SITES IN THE NORMAL COLON

The ratio of [^{35}S] to [^{3}H]-label incorporated into the Sepharose CL 4B V_0 cellular soluble mucin fraction did not vary significantly with the site in the normal colon. However, a significant difference to the rectum was found for the secreted samples at sigmoid, transverse and caecum ($p > 0.01$) and for ascending and descending where $p > 0.001$ (Fig. 7a). The turnover of both [^{35}S]- and [^{3}H]-mucin label was higher in the normal rectum

Table 4. Amino acid analysis of glycopolypeptide prepared from control and ulcerative colitis patients: Glycopolypeptide from 10 control and 10 colitis patients were analysed. All samples were reduced and alkylated to ensure measurement of cysteine[a] as carboxymethyl cysteine.

	Mean ± SEM (nmol per mg dry weight)		
Amino acid	*Controls* (n = *10*)	*Ulcerative colitis* (n = *10*)	
Ala	9.55 ± 1.37	20.19 ± 3.05	<0.006
Arg	1.49 ± 0.60	5.00 ± 0.65	<0.0006
Asx	4.94 ± 0.46	7.80 ± 1.10	<0.05
Cys[a]	5.14 ± 1.34	0.93 ± 0.29	<0.004
Glx	22.46 ± 2.34	15.23 ± 2.29	<0.03
Gly	31.45 ± 3.08	29.33 ± 3.11	NS
His	0.54 ± 0.18	2.42 ± 0.50	<0.003
Ile	21.06 ± 1.82	16.89 ± 2.10	NS
Leu	11.04 ± 1.11	9.50 ± 1.36	NS
Lys	3.17 ± 0.33	2.96 ± 0.56	NS
Met	0.63 ± 0.43	0.47 ± 0.18	NS
Phe	1.05 ± 0.22	2.71 ± 0.35	<0.001
Pro	146.13 ± 14.12	103.67 ± 13.58	<0.04
Ser	29.62 ± 2.71	22.90 ± 2.77	NS
Thr	181.63 ± 18.33	118.50 ± 17.18	<0.02
Tyr	0.79 ± 0.18	0.61 ± 0.17	NS
Val	14.69 ± 1.61	15.00 ± 1.89	NS

The statistical significance of the differences between control and UC results was tested using the Mann–Whitney U test. NS not significant.

Table 5. Ratio of serine threonine and proline in mucin glycopolypeptide and predicted from mucin gene *MUC1–7* tandem repeats. The molar proportion of serine and threonine relative to proline (=1.0), were calculated from the values given in the literature for the tandem repeat sequences of each *MUC* gene. The values measured for control and UC cases are the averages given in Table 2. The percentage of the total was the sum of all three amino acids as a proportion of the total amino acid complement.

Sample	*Serine*	*Threonine*	*Proline*	*% of total*	*Ref.*
Glycopolypeptide					
Control	0.2	1.2	1	74	–
Ulcerative colitis	0.2	1.1	1	66	–
Mucin genes					
MUC1	0.4	0.6	1	50	[36]
MUC2	0	2.8	1	83	[37]
MUC2[a]	0.2	1.5	1	100	[37]
MUC3	5.0	7.0	1	76	[38]
MUC4	1.5	2.0	1	56	[39]
MUC5B[b]	1.0	4.3	1	66	[40]
MUC5C	2.0	4.0	1	88	[40]
MUC6	1.1	1.9	1	63	[41]
MUC7	0.5	0.6	1	74	[42]

[a]Imperfectly conserved repeat (example); [b]Most common degenerate sequence.

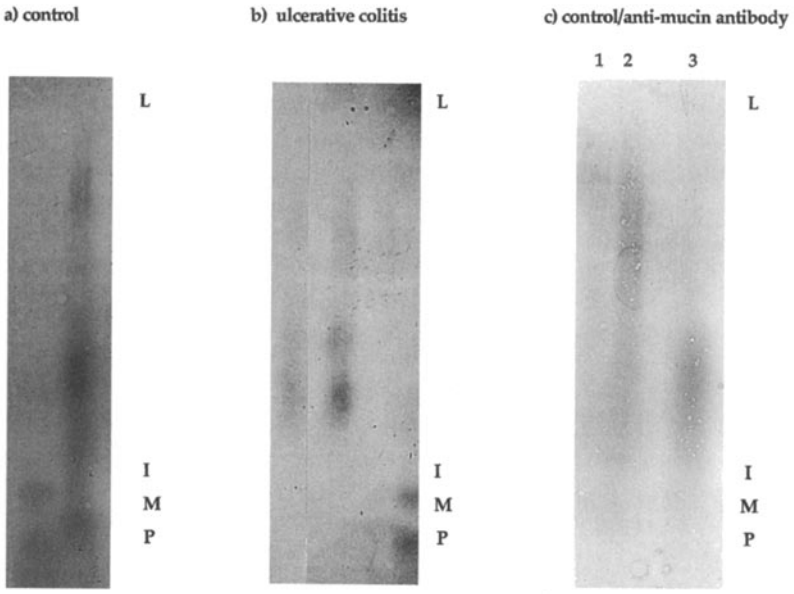

Figure 6. Vacuum blots after electrophoresis on 1% agarose gels of Sepharose CL 4B V_0 mucin fractions after reduction and alkylation. The positions of the loading wells L and the markers IgM, I; myosin, M and phosphorylase b, P are shown. The markers were used to determine relative migration positions and not molecular weights. a) Control mucin samples stained with WGA showing 4 bands. b) UC mucins with apparent reduction or loss of the fastest migrating band. c) Control mucin fractions from CsCl density gradient centrifugation, 1, 1.35 g ml^{-1}, 2, 1.41 g ml^{-1}, 3, >1.50 g ml^{-1} stained with anti-human colon mucin antibody PR3A5.

compared with other sites. In all groups large enough for analysis $p > 0.01$, except for sigmoid [^{35}S] secreted, transverse [^{3}H] and [^{35}S] cellular; descending [^{3}H] and [^{35}S] cellular and [^{3}H] secreted where $p > 0.005$ (Fig. 7b).

Discussion

This work demonstrates qualitative and quantitative differences in the major MUC2 colonic mucins synthesized by the mucosa from European patients with severe ulcerative colitis. Previous studies have implicated defects in the glycosylation of mucins in this inflammatory disease using metabolic labelling to detect these changes in biopsy tissue samples [19–21, 24]. The present study demonstrates the validity of metabolic labelling in organ culture by comparing radiolabelled mucins with those prepared directly from tissue samples obtained from the same patient at the same time. The behaviour of biopsies in the culture system adopted shows continued cell viability ([^{3}H]-thymidine incorporation) and the expected pattern of mucin labelling with time in secreted and cellular soluble fractions (Fig. 1), while histological analysis confirmed that no abnormalities arise in these biopsies within the 24 h incubation period routinely used.

The introduction of dual labelling techniques with [^{35}S]-sulfate and D-[^{3}H]-glucosamine has enabled a rapid qualitative assessment of labelled purified mucin to be made. The mucin content of the Sepharose CL 4B V_0 fraction used here to assess the mucin quality and turnover was analysed further by density gradient centrifugation, protease digestion, reduction and alkylation and proteoglycan degrading enzymes, β-elimination and agarose gel electrophoresis and found to be composed of at least 90% mucin in both control and UC samples. These analyses are well established as standard techniques for the confirmation of mucin structure [2, 34].

The dual isotope technique with [^{35}S]-sulfate and [^{3}H]-glucosamine has been used in previous studies where changes in mucin sulfation were found [5, 21]. Others have confirmed [20] and refuted [19] a loss of mucin sulfation in UC. The variation in experimental conditions [19, 20, 24] prompted the more extensive analysis of the mucin fractions reported here. The metabolic labelling results (Table 1, Figs 1 and 2) clearly show the sulfate depletion in UC mucins, and this is also apparent in the mucin oligosaccharides removed by β-elimination (Fig. 4). Chemical analysis of the pronase digested mucin glycopolypeptide derived from the same patients confirms a significant reduction in sulfate whether related to dry weight or carbohydrate content (Table 5). This strongly suggests that the sulfate depletion observed in the metabolic labelling experiments is genuine.

Examination of the metabolically labelled products for differences between control and UC native mucins showed the depletion of a fast migrating component in UC samples on agarose gel electrophoresis. The nature of this component is under further study. Density gradient

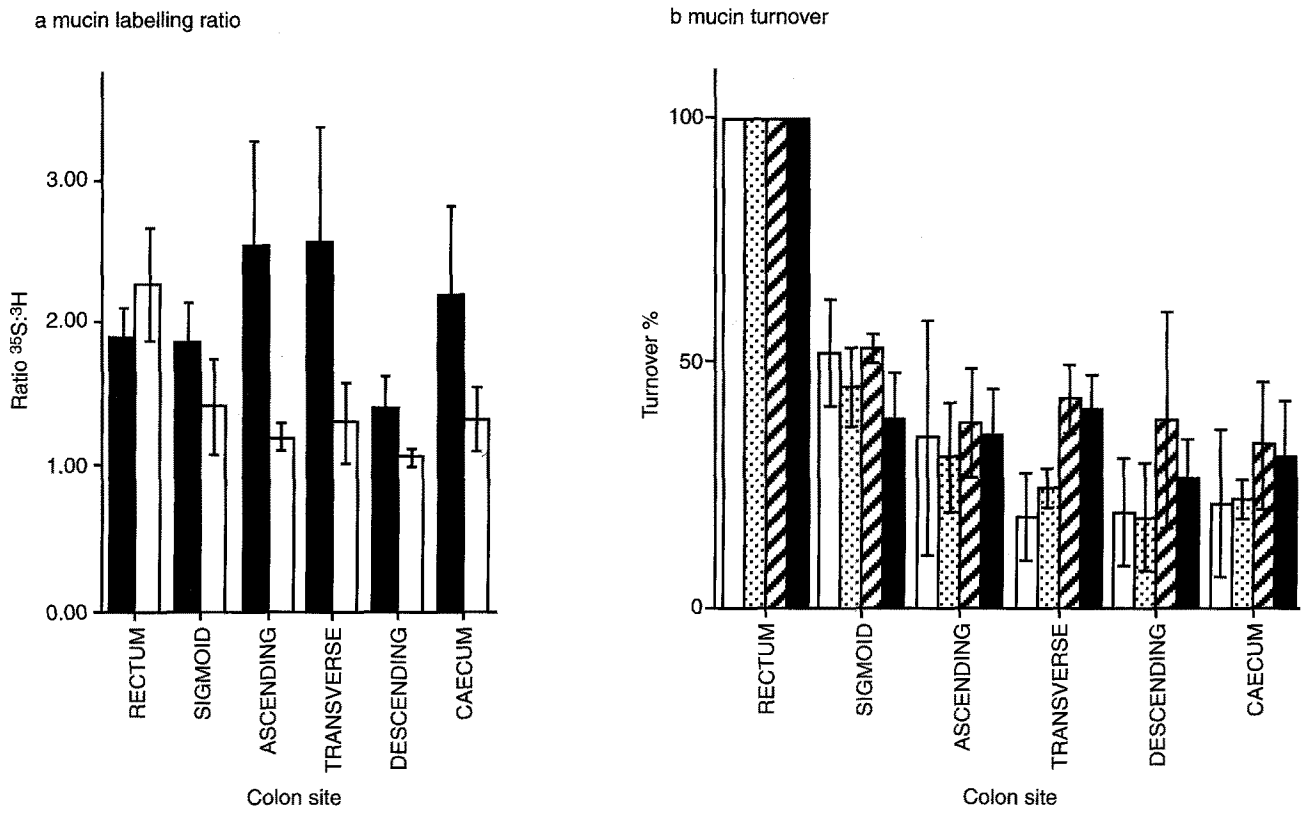

Figure 7. Mucin characteristics at sites throughout the normal colon. Mucosal explants from different sites through the colon were incubated under standard conditions with [^{35}S]-sulfate and D-[^{3}H]-glucosamine. The mucins were isolated as the V_0 fraction on Sepharose CL 4B and results are expressed as the mean ± SD. The number of samples analysed in each case was rectum, 10; sigmoid, 5; ascending, 2; transverse, 3; descending, 3; caecum, 2. a) The ratio of [^{35}S]:[^{3}H] for cellular, ■ and secreted □. No significant difference was observed between the cellular mucin samples. A significant difference to the rectum was found for the secreted samples at sigmoid, transverse and caecum $p > 0.01$ and for ascending and descending where $p > 0.001$. b) The turnover of control cellular [^{35}S] □, and [^{3}H] ■, control secreted [^{35}S] _, and [^{3}H] ■ is shown as a percentage relative to the rates found in the rectum as [^{35}S] dpm per μg DNA and [^{3}H] dpm per μg DNA given in Table 1. Comparison of the rates in all groups large enough for analysis with the rectum showed significantly lower values with $p > 0.01$ except for sigmoid [^{35}S] secreted, transverse [^{3}H] and [^{35}S] cellular; descending [^{3}H] and [^{35}S] cellular and [^{3}H] secreted where $p > 0.005$.

centrifugation of the native mucins revealed two patterns present in both normal and disease groups but with no disease related differences. Examination of the reduced mucin subunits by this technique may improve identification of mucin changes in the future.

Glycosylation changes in UC have been reported in other studies using histochemical and lectin [3, 4] methods. The results of the present biochemical study show modification of mucin glycosylation in UC. Mucin oligosaccharides have shorter chains in UC (Figs 5 and 6). Changes in sialic acid and sulfate content also occur in UC, as most oligosaccharides elute in the V_0 fraction of BioGel P6 due to the high negative charge. This can be seen from the shift in elution profile after acid hydrolysis (Fig. 5c,d). A high Gal:GlcNAc ratio for fractions P1-P5 shows that these oligosaccharides in control and UC mucins still contain Gal-GlcNAc repeat, backbone structures, as reported by Podolsky, [12]. Significant changes in GlcNAc and sialic acid found in fractions P1, 2, 3 and 5 are associated with UC, together with a smaller proportion of neutral oligosaccharides, compared with previous work [12]. The large size and complexity of colonic mucin oligosaccharides [12] makes detailed analysis of individual oligosaccharides impractical for studies with a series of patients. Preparation of larger amounts of oligosaccharides from individual cases and the application of sophisticated separation and analytical techniques are required to provide evidence of defined structural differences.

The amino acid analysis of the mucin glycopolypeptide (Tables 4 and 5) was investigated in order to assess the complement of *MUC* gene peptide associated with the major mucins isolated. The proportion of serine, threonine and proline in the glycopolypeptides derived

from pronase digestion of the mucins should approximate to the amounts of these amino acids in the tandem repeat motifs identified in the *MUC* gene oligonucleotide sequences [14]. The ratios measured show a homology with the values predicted for MUC2 mainly due to the low amount of serine (Table 5). This suggests that MUC2 may be the main colonic mucin, as already reported [15, 16]. There was no significant difference between control and UC mucins, in agreement with *in situ* hybridization analysis for *MUC* gene expression in normal and UC tissues [35]. Direct demonstration of *MUC2* gene sequence in control and UC mucins was not provided in this study and analysis with anti-MUC antibodies after deglycosylation is needed to confirm the above findings.

Examination of the nature of mucins at different sites in the large intestine showed higher sulfation of secreted mucin in the rectum relative to the rest. There was no variation in the cellular mucin sulfation and the ratio was the same as the rectal secreted mucins (Fig. 7a). Histochemical analysis has shown a difference in the location of intracellular goblet cell sulfated mucins, with the upper crypts containing more sulfation than the lower regions in the proximal colon [3, 4]. In contrast, the distal colon and rectum showed sulfation in both the lower crypts and throughout the crypt.

The general trend of these results indicates an increase in the amount of mucin sulfation from proximal to distal colon [4]. Metabolic labelling illustrates the selective secretion of a more highly sulfated mucin population in the rectum, but no significant change in the cellular mucins which would correspond to the mucin detected histochemically. However, it is not possible to correlate the dual labelling, qualitative result with the histochemical data using e.g. high iron diamine/ Alcian blue stain because an index of the relative level of each stain for mucin cannot be achieved. The use of agarose gel electrophoresis may help resolve the question of multiple mucin types at the different colonic sites.

In contrast to metabolic labelling methods it is not possible to measure either secretion or turnover of mucins using histological techniques. Mucin turnover in the rectum was higher than any other site in the colon under the culture conditions employed in agreement with other workers [20].

In conclusion, the results of this study demonstrate properties of the mucins isolated from control and UC colon and that the dual metabolic labelling method is a valuable and simple approach to the assessment of colonic mucosal mucin synthesis and secretion. The work confirms the sulfate depletion in Europeans with severe colitis using chemical analysis and suggests that the main mucin is expressed as MUC2 and that this is not altered by UC.

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References

1. Allen A, Hutton DA, Pearson JP, Sellers LA (1990) In *The Cell Biology of Inflammation in the Gastrointestinal Tract.* (Peters TJ ed.) pp. 113–25 Hull: Corners Publications.
2. Forstner JF, Forstner GG (1994) In *Physiology of the Gastrointestinal Tract.* (Johnson LR ed.) pp. 1245–83. New York: Raven Press.
3. Jass JR, Roberton AM (1994) *Pathology International* **44**: 487–504.
4. Filipe MI (1989) In *Gastrointestinal and Oesophageal Physiology.* (Whitehead R ed.) pp. 65–89 Edinburgh: Churchill Livingstone.
5. Corfield AP, Warren BF, Bartolo DCC, Wagner SA, Clamp JR (1992) *Br J Surg* **79**: 1209–12.
6. McCormick DA, Horton LWL, Mee AS (1990) *J Clin Pathol* **43**: 143–46.
7. Podolsky DK, Isselbacher KJ (1983) *J Clin Invest* **72**: 142–53.
8. Raouf A, Parker N, Iddon D, Ryder S, Langdon-Brown B, Milton JD, Walker R, Rhodes JM (1991) *Gut* **32**: 1139–45.
9. Irimura T, Wynn DM, Hager LG, Cleary KR, Ota DM (1991) *Cancer Res* **51**: 5728–35.
10. Hutchins JT, Reading CL, Giavazzi R, Hoaglund J, Jessup JM (1988) *Cancer Res* **48**: 483–89.
11. Corfield AP, Wagner SA, Clamp JR, Kriaris MS, Hoskins LC (1992) *Infect Immunol* **60**: 3971–78.
12. Podolsky DK (1985) *J Biol Chem* **260**: 8262–71.
13. Kuhns W, Jain RK, Matta KL, Paulsen H, Geyer R, Baker MA, Brockhausen I (1994) *Glycobiology* **4**: 722.
14. Gendler SJ, Spicer AP (1995) *Annu Rev Physiol* **57**: 607–34.
15. Chang S-K, Dohrman AF, Basbaum CB, Ho SB, Tsuda T, Toribara NW, Gum JR, Kim YS (1994) *Gastroenterology* **107**: 28–36.
16. Tytgat KMAJ, Büller HA, Opdam FJM, Kim YS, Einerhand AWC, Dekker J (1994) *Gastroenterology* **107**: 1352–63.
17. Pullan RD, Thomas GAO, Rhodes M, Newcombe RG, Williams GT, Allen A, Rhodes J (1994) *Gut* **35**: 353–59.
18. Corfield AP, do Amaral Corfield C, Wagner SA, Warren BF, Mountford RA, Bartolo DCC, Clamp JR (1992) *Biochem Soc Trans* **20**: 95C.
19. Morita H, Kettlewell MGW, Jewell DP, Kent PW (1993) *Gut* **34**: 926–32.
20. Raouf AH, Tsai HH, Parker N, Hoffman J, Walker RJ, Rhodes JM (1992) *Clin Sci* **83**: 623–26.
21. Probert CSJ, Warren BF, Perry T, Mackay EH, Mayberry JF, Corfield AP (1995) *Gut* **36**: 696–702.
22. Clamp JR, Fraser G, Read AE (1981) *Clin Sci* **61**: 229–34.
23. Clamp JR, Gough M (1991) *Clin Chim Acta* **203**: 243–48.
24. Corfield AP and Paraskeva C (1993) In *Glycoprotein Analysis in Biomedicine, 14,* (Hounsell EF ed.) pp. 211–32 Totowa: Humana Press.

25. Thornton DJ, Howard M, Devine PL, Sheehan JK (1995) *Anal Biochem* **227**: 162–67.
26. Clamp JR, Bhatti T, Chambers RE (1971) *Methods Biochem Anal* **19**: 229–344.
27. Thornton DJ, Holmes DF, Sheehan JK, Carlstedt I (1989) *Anal Biochem* **182**: 160–64.
28. Vavasseur F, Dole K, Yang J, Matta KL, Corfield AP, Myerscough N, Paraskeva C, Brockhausen I (1994) *Eur J Biochem* **222**: 415–24.
29. Bradford M (1976) *Anal Biochem* **72**: 248–54.
30. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) *Anal Biochem* **28**: 350–56.
31. Reuter G, Schauer R (1994) *Methods Enzymol* **230**: 168–99.
32. Bidlingmeyer BA, Cohen SA, Tarvin TL (1984) *J Chromatog* **336**: 93–104.
33. Silvestri LJ, Hurst RE, Simpson L, Settine JM (1982) *Anal Biochem* **123**, 303–9.
34. Carlstedt I, Sheehan JK, Corfield AP, Gallagher JT (1985) *Essays in Biochemistry* **20**: 40–76.
35. Myerscough N, Warren BF, Gough M, Corfield AP (1995) *Biochem Soc Trans* **23**: 536S.
36. Gendler SJ, Lancaster CA, Taylor J, Duhhig T, Peat N, Burchell J, Pemberton L, Lalani E, Wilson D (1990) *J Biol Chem* **265**: 15286–93.
37. Gum JR, Hicks JC, Toribara NW, Lamport DTA, Kim YS (1989) *J Biol Chem* **264**: 6480–87.
38. Gum JR, Hicks JW, Swallow DM, Lagace RL, Byrd JC, Lamport DTA, Siddiki B, Kim YS (1990) *Biochem Biophys Res Commun* **171**: 407–15.
39. Porchet N, Nguyen VC, Dufosse J, Audie JP, Guyonnet-Duperat V, Gross MS, Denis C, Gand P, Bernheim A, Aubert JP (1991) *Biochem Biophys Res Commun* **175**: 414–22.
40. Dufosse J, Porchet N, Audie JP, Guyonnet Duperat V, Laine A, Van-Seuningen I, Marrakchi S, Degand P, Aubert JP (1993) *Biochem J* **293**: 329–37.
41. Toribara NW, Roberton AM, Ho SB, Kuo W-L, Gum E, Hicks JW, Gum JRJ, Byrd JC, Siddiki B, Kim YS (1993) *J Biol Chem* **268**: 5879–85.
42. Bobek LA, Tsai H, Biesbrock AR, Levine MJ (1993) *J Biol Chem* **268**: 20563–69.