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Altered expression of mucins throughout the colon in ulcerative colitis

J E Smithson, A Campbell, J M Andrews, J D Milton, R Pigott, D P Jewell

Abstract

***Background/Aims*—Regional differences in the biology of the colonic epithelium may determine the extent of involvement by ulcerative colitis. Novel monoclonal antibodies (MAbs) were used in this study to investigate regional heterogeneity in the colonic mucosa.**

***Methods*—MAbs generated using a method of tolerisation against common antigens in the proximal colon and distal colon were used for immunoperoxidase staining, comparative histochemistry, immunoblotting, and slot-blot analysis.**

***Results*—The colon specific MAbs 5F1 (IgG_3) and 6G4 (IgM) stained goblet cell contents throughout the normal distal colon but staining was markedly reduced in the proximal colon ($p<0\cdot0001$). In the distal colon of patients with ulcerative colitis, whether quiescent or actively inflamed, reactivity was reduced compared with controls ($p<0\cdot05$, $p<0\cdot001$ respectively). By contrast, an overall increase in staining was seen in the uninflamed proximal colon in ulcerative colitis compared with controls ($p<0\cdot02$). Comparative staining with high iron diamine and biochemical analyses indicated that MAb 6G4 was reactive with mucin bearing sulphate or *O*-acetylated sialic acid groups, or both.**

***Conclusions*—Regional differences in the staining characteristics of normal colonic mucin have been shown using novel monoclonal antibodies. The pattern of mucin expression throughout the colon in ulcerative colitis is altered even in the absence of histological changes.**

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Keywords: colonic mucin, monoclonal antibodies, ulcerative colitis.

Gastroenterology Unit, Radcliffe Infirmary, Oxford
J E Smithson
J M Andrews
D P Jewell

Nuffield Department of Cellular Pathology, John Radcliffe Hospital, Oxford
A Campbell

University Department of Medicine, Liverpool
J D Milton

British Biotech Ltd, Watlington Road, Oxford
R Pigott

Correspondence to:
Dr J E Smithson,
Department of Medicine,
Selly Oak Hospital,
Raddlebarn Road,
Birmingham B29 6JD.

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In ulcerative colitis the rectum is nearly always affected, while the proximal extent of colonic involvement is variable. At presentation, approximately 50% of patients will have distal disease (limited to the recto-sigmoid), 25% will have left sided colitis (extending no further than the splenic flexure), and the remainder will have subtotal or pancolitis.[1–4] Proximal extension of disease previously limited to the distal colon may occur in up to 30% of patients after 20 years of follow up.[3 5] The factors responsible for this striking regional variation in disease susceptibility in the colon remain unknown and disease progression within individual patients cannot be predicted. The develoment of markers for identifying patients at risk of subsequent disease extension could prove to be of considerable benefit in long term treatment.

Various mechanisms have been proposed to account for the distribution of ulcerative colitis in the colon. It has been argued that the epithelial expression of a 40 kilodalton 'autoantigen', which co-localises with IgG and activated complement increases towards the rectum.[6] Alternatively, it has been suggested that ulcerative colitis is an 'energy deficiency disease', as metabolism of the major fuel substrate, butyrate, seems to be abnormal even in quiescent mucosa.[7 8] The proportionately greater metabolic reliance on butyrate by isolated epithelial cells of the left colon compared with the proximal colon[9] provides a possible mechanism for regional differences in disease susceptibility. Others have argued that ulcerative colitis arises as a result of a specific mucin abnormality[10] possibly leading to deficient epithelial resistance to noxious luminal constituents, which increase in concentration along the colonic axis.[11] A more recent study has implicated the neurovascular supply of the colon in determining disease extent.[12]

Common to these hypotheses is the concept that the distribution of ulcerative colitis arises as a consequence of inherent regional differences in the biology of the colon. To investigate further regional heterogeneity of the colonic epithelium, novel murine monoclonal antibodies (MAbs) with differential reactivity between the mucosa of the proximal and distal colon have been generated. A technique of 'tolerisation' using cyclophosphamide was used because this has been shown to produce MAbs that discriminate between closely related tissues.[13] MAbs raised in this way[14] have been used to demonstrate regional differences in the colonic expression of secretory component[15] and carcinoembryonic antigen.[16] This study describes the findings with novel MAbs reactive with colonic mucin.

Methods

Preparation of immunogens from normal colonic mucosa

Mucosal biopsy specimens were obtained from the caecum and rectum of five subjects without inflammatory bowel disease who were undergoing routine colonoscopy. Ten biopsy specimens from each site were transferred into separate Petri dishes containing ice cold phosphate buffered saline (PBS). After mincing with scalpel blades, the tissue suspensions were passed through a sequence of fine bore needles (19G to 23G) to exclude large tissue

fragments. Between each step, the suspensions were agitated by hand and washed in ice cold PBS followed by centrifugation at 1000 *g* for five minutes. The resulting pellets were resuspended in 0·5 ml aliquots of PBS and stored at −70°C for later use. Each aliquot contained approximately 10^6 cells.

Production of monoclonal antibodies using cyclophosphamide tolerisation

Table I shows the schedule for immunisation and tolerisation of a single Balb/c mouse. MAbs were then produced by standard methods. Splenocytes were fused with SP2/0 mouse myeloma cells at a ratio of 5:1 using polyethylene glycol 1500. After pelleting at 200 *g* the cells were suspended in Dulbecco's modified Eagle medium (Gibco) containing HAT (hypoxanthine 100 mM, aminopterin 0·1 mM, and thymidine 1·6 mM) and recombinant interleukin 6, 1 ng/ml (R & D Systems, Europe). The suspension was transferred to 96 well plates with refeeding at seven days. Twelve days after fusion, supernatants were screened for anti-colon activity by indirect immunohistochemistry on frozen sections of normal colon. Hybridoma colonies were then subcloned twice by limiting dilution and grown in bulk to produce supernatants for subsequent study. Monoclonal antibody isotypes were determined using a Sigma Immunotype kit (ISO-1).

Immunohistochemistry

For frozen sections, tissue obtained at endoscopy or surgery was mounted in OCT compound (Tissue-Tek) from which 5 μm sections were transferred to microscopic slides (Shandon). After air drying for one hour, slides were stored at −70°C. Sections were fixed in acetone for 10 minutes before use. For formalin fixed tissue, 5 μm sections of routinely processed paraffin wax embedded archival samples were dewaxed in xylene, immersed in citrate buffer (0·01 M, pH 6·0), and heated in a domestic microwave oven for 10 minutes (800 W) followed by medium power (400 W) for 15 minutes. Endogenous peroxidase activity was blocked with methanol/2% hydrogen peroxide. For subsequent immunostaining, undiluted hybridoma supernatants were incubated with tissue sections for between 30 minutes and 16 hours. Peroxidase conjugated rabbit antimouse Ig (P260, Dako) 1/100 in 33% normal swine serum in TRIS buffered saline (TBS) was applied for 30 minutes after washing in TBS. Negative controls were provided by incubating with secondary antibody alone. Slides were developed in a solution of diaminobenzidine, 75 mg in 150 ml TBS, with 450 μl H_2O_2 added just before use. After counterstaining with haematoxylin, slides were dehydrated in alcohol, cleared in xylene, and mounted in DPX mountant (BDH Laboratory Supplies).

TABLE I *Immunisation protocol*

Day	*Procedure*
0	IP PC
3	IP cyclophosphamide 1 mg
21	IP PC
24	IP cyclophosphamide 1 mg
42	IP DC
56	IP DC
70	IV boost DC
72	Animal sacrificed for fusion

Tolerisation to the proximal colonic immunogen (PC) followed by immunisation with the distal colonic immunogen (DC) was carried out in four mice. This regimen would be expected to generate MAbs that react preferentially with distal colonic antigens. The reverse procedure was performed in the fifth animal. IP (intraperitoneal), IV (intravenous).

Specificity of immunoperoxidase staining

A variety of frozen sections were used to examine the specificity of observed staining. Normal extracolonic tissues included stomach (5), duodenum (6), terminal ileum (1), liver (3), pancreas (1), skin (4), kidney (1), and lung (1). Colonic controls included Crohn's disease (10), cancer (5), and Shigella colitis (1).

Comparison between immunoperoxidase staining in normal subjects and patients with ulcerative colitis

Fourteen subjects with normal colonic histology undergoing routine colonoscopy were compared with ulcerative colitis patients. These comprised people with quiescent or actively inflamed proximal (12, 5) and distal (13, 13) colonic mucosa respectively. Active inflammation in ulcerative colitis was defined by a departmental pathologist if an infiltrate of acute inflammatory cells was present. Slides were blinded and assessed by two independent observers (JS and AC). Scores were assigned on a semiquantitative scale to reflect the depth of crypt staining: 0 (trace or absent mucin staining); ++ (staining of goblet cells in upper parts of crypts only); ++++ (staining of goblet cells extending to lower parts of crypts throughout section). For each section the two scores were averaged and analysed by Mann-Whitney U test (two tailed) using Minitab software for Macintosh.

Comparative histochemistry

To establish whether there was a relation between monoclonal antibody staining patterns and colonic histology in patients with inactive ulcerative colitis (15), parallel haematoxylin and eosin stained frozen sections were examined. Comparisons were also made between immunostaining and high iron diamine/alcian blue (HID/AB) staining (method described by Spicer[17]) on parallel formalin fixed sections from normal subjects (4) and patients with ulcerative colitis (7) and Campylobacter colitis (1). (HID and AB distinguish sulphated and non-sulphated sialomucins respectively). Semi-quantitative scores were assigned to HID/AB stained sections using the same method as before.

Western blotting

Lysates of normal rectal mucosa were prepared by homogenising snap frozen colonoscopic

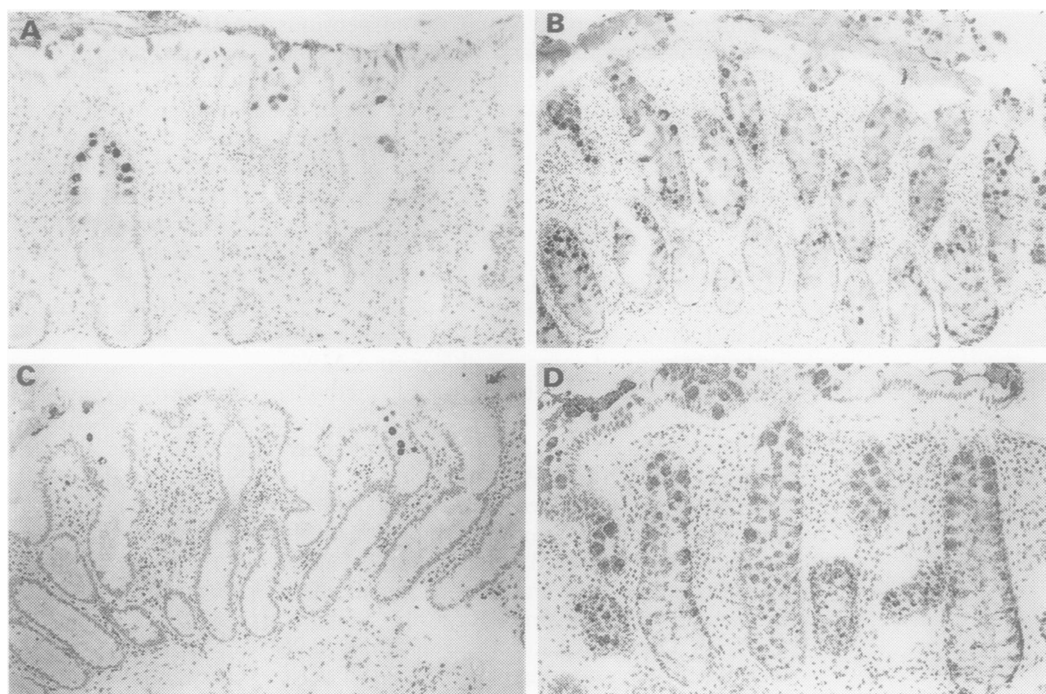

Figure 1: Immunoperoxidase staining of paired frozen sections of normal mucosa from proximal and distal colon. Dense staining is seen throughout the crypts of the distal colon (B, D) compared with proximal (A, C) with MAbs 5F1 (upper pair) and 6G4 (lower pair).

biopsy specimens in ice cold lysis buffer (TBS with Nonidet P-40 1%, polymethylsulphonylfluoride 100 μg/ml, leupeptin 5 μg/ml, and pepstatin 5 μg/ml). Equal volumes of gel loading buffer (100 mM TRIS/Cl, pH 6·8, 4% sodium dodecyl sulphate, 20% glycerol and 0·05% bromophenol blue, with and without dithiothreitol 200 mM) were added to the lysates, which were stored at –20°C before use. Protein content was measured using a Pierce BCA assay kit. For SDS-PAGE, lysates were loaded (20 μg protein/lane) and resolved by electrophoresis in a 7·5% acrylamide gel. After electrophoretic transfer of proteins, Millipore-P membranes were incubated with 5% Marvel milk powder in wash buffer (0·9% NaCl, 100 mM TRIS/Cl pH 7·5, 0·1% Tween 20) to block non-specific binding. Incubation then followed with MAb (diluted 1/5) and peroxidase conjugated rabbit antimouse Ig (Dako P260, 1/1000), each for 30 minutes. Wash buffer was used for antibody dilutions and for three washes between staining steps. Isotype matched murine MAbs non-reactive with colonic mucosa were used as negative controls. Bound protein conjugates were detected by chemiluminescence (ECL, Amersham) and molecular weights were estimated by comparison with biotinylated standards (BioRad, Broad Range).

Effect of chemical treatments on antibody binding to colonic mucin

The methods are described in detail elsewhere.[18] Quadruplicate samples of purified mucin from normal colonic mucosa were blotted on to nitrocellulose membrane (Schleicher & Schuell) using a slot-blot manifold (Hoeffer Scientific, Newcastle under Lyme, England) and probed with MAbs after treatment with either *C perfringens* neuraminidase (Sigma) 0·5 U/ml in 0·1 M acetate buffer pH 5·0 for 12 hours at 37°C, 0·05 M sulphuric acid for two hours at 80°C or 0·1 M NaOH for one hour at room temperature. The change in antibody binding after each treatment was determined by measuring peak heights with a densitometer (Hoeffer Scientific, San Francisco).

Results

MAbs 5F1 and 6G4, monoclonal antibodies that react preferentially with normal distal colonic mucosa

From five fusions, 49 MAbs with anti-colon activity were identified of which seven displayed regional differences in staining of normal colonic mucosa. The data presented in this report relate to two of these, MAbs 5F1 (IgG_3) and 6G4 (IgM). Both antibodies stained goblet cell contents uniformly throughout the crypts of the normal distal colon whereas in the proximal colon staining was limited to the upper crypts or was absent altogether (Fig 1). Staining of sections from biopsy specimens obtained at intervals around three normal colons showed that the depth of

TABLE II *MAb staining of normal extracolonic tissues*

Tissue (n)	*MAb 5F1*	*MAb 6G4*
Terminal ileum (1)	Negative	Occasional goblet cells
Duodenum (6)	Negative	Trace staining of epithelial cytoplasm
Stomach (5)	Negative	Negative
Pancreas (1)	Negative	Negative
Liver (3)	Negative	Negative
Skin (4)	Negative	Negative
Kidney (1)	Negative	Tubular epithelium
Lung (1)	Negative	Negative

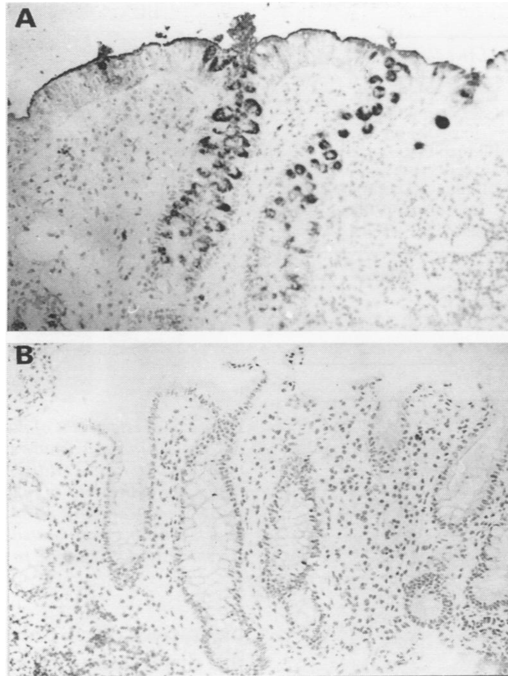

Figure 2: Altered staining patterns in ulcerative colitis with MAbs 5F1 and 6G4. Compared with normal mucosa, staining was increased in some cases of ulcerative colitis in the proximal colon (A, quiescent, MAb 6G4) and decreased in the distal colon (B, quiescent, MAb 5F1).

crypt immunoreactivity increased steadily in a distal direction. Both antibodies were highly specific for colonic mucosa compared with other tissues (Table II).

Staining of ulcerative colitis mucosa by MAbs 5F1 and 6G4 compared with controls

In ulcerative colitis, staining of distal sections was significantly reduced compared with normal controls, both in active and quiescent disease. By contrast, in the proximal colon of patients with ulcerative colitis, staining was increased in several cases, both actively inflamed and uninvolved or quiescent (Fig 2). Semiquantitative staining scores are shown in Figure 3. There was 84% concordance between the scores assigned by the two observers. Reduced staining was also seen in Crohn's colitis, especially in cases of active inflammation and in colonic cancers (Table III).

Comparison between MAb staining and histology in quiescent ulcerative colitis

Table IV shows MAb staining scores and histology of non-inflamed mucosa from patients with ulcerative colitis. In the proximal colon, scores greater than the median expected in normal mucosa were found with both antibodies in five of 12 cases, of which three had no histological evidence of involvement by ulcerative colitis. By contrast, in the distal colon, scores were less than would be expected with both antibodies for normal mucosa in eight of 13 cases, all of which displayed some histological abnormality. Hence there was no consistent relation between histology and MAb staining.

Comparison between MAb 6G4 staining and high iron diamine/alcian blue staining

After microwave heating of formalin fixed sections, MAb 6G4 gave staining of a similar pattern and quality to that obtained with frozen sections. Immunostaining with MAb 5F1 was unsuccessful. The semiquantitative scores assigned to MAb 6G4 and high iron diamine staining of parallel sections from patients with ulcerative colitis (7) and infectious colitis (1) in different states of inflammation corresponded exactly in 17 of 20 cases. Examples are illustrated in Figure 4. By contrast, there was agreement in staining scores for MAb 6G4 and alcian blue in only six of 20 cases. However it is possible that the alcian blue stain was obscured in some cases by the dense high iron diamine precipitate present in the same section.

Biochemical characterisation of the antigens reactive with MAbs 5F1 and 6G4

On probing western blots of normal colonic mucosa MAb 6G4 yielded a broad band of at least 200 kDa, consistent with a mucus glycoprotein (Fig 5). MAb 5F1 failed to react with both dithiothreitol reduced and non-reduced blots. Table V summarises the results of the slot blot analysis for MAb 6G4. The data show that binding was unaffected by sialidase, abolished by alkali and reduced by mild acid treatment. MAb 5F1 failed to react with mucin blots from three separate patients.

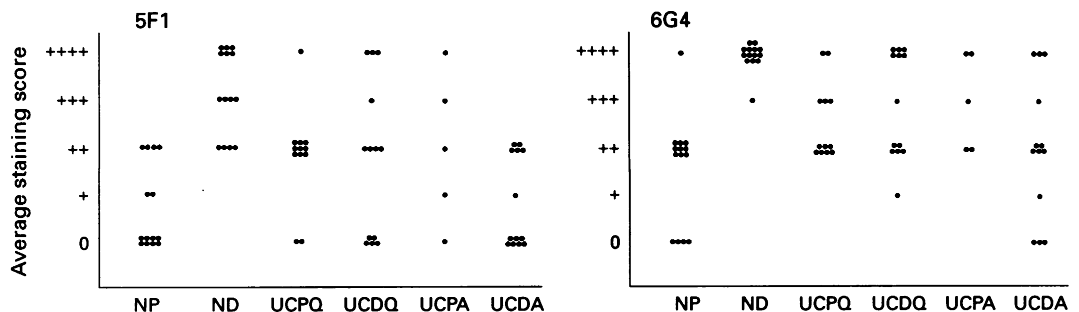

Figure 3: Immunoperoxidase staining scores for MAbs 5F1 and 6G4 in ulcerative colitis patients compared with controls. NP (normal proximal colon), ND (normal distal colon), UCPQ (quiescent/non-inflamed UC proximal colon), UCDQ (quiescent UC distal colon), UCPA (actively inflamed UC proximal colon), UCDA (actively inflamed UC distal colon). MAb 5F1: NP v ND $p<0.0001$; NP v UCPQ $p<0.01$; ND v UCDQ $p<0.05$; ND v UCDA $p<0.0001$. MAb 6G4: NP v ND $p<0.0001$; NP v UCPQ $p<0.02$; NP v UCPA $p<0.05$; ND v UCDQ $p<0.01$; ND v UCDA $p<0.001$. All other comparisons not significant.

TABLE III *MAb staining in other colonic diseases*

Tissue (segment, activity, n)	*MAb 5F1*	*MAb 6G4*
CD (P, I, 1)	Upper crypts only	Upper crypts only
CD (D, I, 4)	Reduced compared with normal controls	Less than normal controls in two cases
CD (D, A, 5)	All negative	Reduced or absent
Shigella colitis (D, A, 1)	Negative	Upper crypts only
Colon cancer, junctional mucosa (5)	Reduced compared with normal controls, negative in three cases	All reduced compared with normal controls
Colon cancer, tumour (5)	All negative	Weak in one case

CD: Crohn's disease; P: proximal colon; D: distal colon; I: inactive; A: actively inflamed.

TABLE IV *Relation between MAb staining and histology in inactive ulcerative colitis*

Proximal colon			*Distal colon*		
Histology	*MAb 5F1*	*MAb 6G4*	*Histology*	*MAb 5F1*	*MAb 6G4*
Normal	++	++	Normal	++++	++++
Normal	++	++	AD	0	++
Normal	++	++	AD	++	++
Normal	++	+++	AD	+++	++++
Normal	++	+++	AD, CI	0	+
Normal	++	+++	AD, CI	0	++
CI	0	++	AD, CI	0	++
CI	++	++++	AD, CI	++	++
AD	++	++	AD, CI	++++	++++
AD	++++	++++	AD, MD	++++	++++
MD, CI	++	++	AD, MD, CI	0	++
AD, MD, CI	0	++	AD, MD, CI	++	+++
			AD, MD, CI	++	++++

AD: architectural distortion; CI: chronic inflammatory cell infiltrate; MD: mucin depletion.

Discussion

In this study two murine monoclonal antibodies (5F1 and 6G4) were generated that were highly specific for the colon and displayed clear regional differences in staining of normal colonic mucosa. Both reacted with goblet cell contents and free luminal mucin, suggesting that they recognised epitopes located on mucus glycoproteins, or on products co-secreted with mucin. Both displayed gradients of reactivity, not only within colonic crypts but also along the colonic axis in a distal direction. In ulcerative colitis, reduced staining was found in the distal colon irrespective of disease activity when compared with normal controls. The reduction in staining was not specific to ulcerative colitis, occurring also in cases of infectious colitis, Crohn's colitis, and colon cancer. By contrast, a significant overall increase in staining was found in the proximal colon of patients with ulcerative colitis compared with controls.

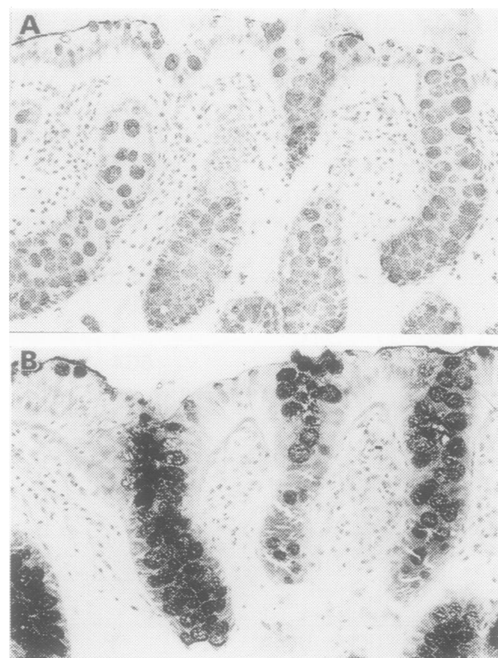

Figure 4: Staining of paired sections by MAb 6G4 and high iron diamine. Formalin fixed sections of normal rectum from the same person stained with MAb 6G4 (A) and high iron diamine/alcian blue (B). The pattern and density of crypt staining is similar with both methods.

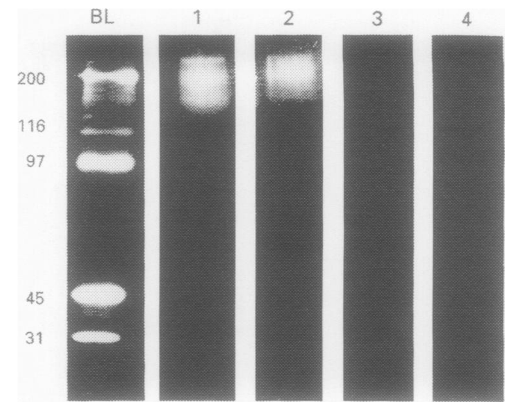

Figure 5: Western blot of normal rectal mucosa probed with MAb 6G4. Lane 1 (non-reduced lysate of normal rectal mucosa) and lane 2 (dithiothreitol reduced lysate) incubated with MAb 6G4. Lanes 3 and 4 show the same samples probed with control IgM supernatant. Ladder (BL) on left contains biotinylated standards indicating molecular weight (kDa). Exposure time five seconds.

A group of three murine MAbs with a similar staining profile has recently been described.[19] The antibodies reacted with 'large intestinal mucin antigen' (LIMA), were colon specific, and showed a clear proximal to distal gradient of staining that was reduced in cases of colon cancer. Systematic examination of cases of inflammatory bowel disease was not undertaken although reduced staining was noted in some instances. Full biochemical characterisation of the presumed mucin borne antigens was not described.

While the identity of the epitope recognised by MAb 5F1 has not been elucidated here, for MAb 6G4 the data are consistent with binding to moieties that bear ester sulphate or *O*-acetylated sialic acid residues, or both. No firm conclusions can be drawn regarding the nature of the antigen recognised by MAb 5F1 because this antibody failed to react both with mucin slot blots and western blots. This lack of reactivity may have resulted from changes in epitope configuration on binding to the membranes used in these experiments. Nevertheless, the similarity between the staining profiles obtained with MAb 5F1 and MAb 6G4 may

TABLE V *Effects of chemical treatments on reactivity of MAb 6G4 with slot blots of normal colonic mucin*

Treatment	*Control*	*Treated*	*Treated/control (%)*
Sialidase*	13·25 (0·95)	13·75 (0·75)	103·8
Alkali	6·5 (0·29)	0	0
Mild acid hydrolysis	14·25 (2·2)	7·0 (0·8)	49·1

Numbers refer to mean (SD) densitometry readings for four samples tested in each experiment. **C perfringens* neuraminidase.

indicate that they recognise closely related antigens.

The immunohistochemical data reported here are also consistent with binding of sulphated or *O*-acetylated sialomucins, or both, by MAbs 5F1 and 6G4. Increased expression of sulphomucins and *O*-acetylated sialomucins in the normal distal colon compared with the proximal colon has been suggested by biochemical, metabolic, and histochemical studies.[20–23] In ulcerative colitis, it seems that mucosal expression of these types of mucin is decreased, even in the absence of acute inflammation.[20–23] Interestingly, increased incorporation of radiolabelled sulphate in colonic glycoproteins of the unaffected proximal colon has been reported in patients with ulcerative colitis,[24] a finding that is supported by the immunohistochemical data in this study. Other workers have described monoclonal antibodies that are thought to recognise *O*-acetylated sialomucin (MMM-17[18] and PR.3A5[25]) and sulphomucin (91·9H[26]). However, regional differences in staining of the normal colon and mucin expression in ulcerative colitis have not been explored using these antibodies.

Whether an abnormality of mucin is of primary importance in the aetiology of ulcerative colitis remains controversial. Specific depletion of a mucin subclass ('Fraction IV') has been found in ulcerative colitis by chromatographic techniques.[10 27] Although these data have been challenged[28] it is of interest that a subclass defect has also been reported in unaffected identical twins of patients with ulcerative colitis.[29] This finding supports the notion that a genetic susceptibility to ulcerative colitis may be expressed at the level of the colonic epithelial cell. The biochemical nature of Fraction IV has not been characterised, but a study using monoclonal antibodies suggested that this mucin subclass was expressed mainly in the distal colon.[30]

In functional terms, a mucin abnormality in ulcerative colitis may render the colonic epithelium vulnerable to attack by microorganisms and their products. It is thought that both sulphation and *O*-acetylation reduce mucin degradation by bacterial enzymes.[11 31 32] Thus abnormalities of sulphation or *O*-acetylation in ulcerative colitis may result in a loss of mucus barrier function and exposure of the mucosa to luminal agents that promote or perpetuate inflammation. The typical disease distribution of ulcerative colitis might then be explained, either because of increasing concentrations of bacterial glycosidases in a distal direction, or because mucin abnormalities in ulcerative colitis may be region specific. In this study, relatively deficient expression of mucins reactive with MAbs 5F1 and 6G4 was shown in the distal colon of patients with ulcerative colitis even in the absence of inflammation. However, immunoreactivity was also increased in several cases in the proximal colon in both quiescent and active disease. These findings do not therefore suggest a simple reduction in the expression of certain mucin species in ulcerative colitis. Instead they are consistent with abnormal epithelial metabolism or disturbed kinetics of mucin synthesis affecting the colon to a variable extent, irrespective of disease activity. It remains to be seen whether increased immunoreactivity with MAbs 5F1 and 6G4 in the proximal colon of patients with distal ulcerative colitis will prove to be a useful marker for identifying those people, perhaps a genetically distinct subgroup, who are at risk of disease extension.

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