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Altered oligosaccharide expression in ulcerative colitis with increasing grades of inflammation

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Ulcerative colitis is an idiopathic chronic inflammatory condition of the large bowel associated with abnormalities of mucin synthesis and secretion. In the present study, glycans were identified in 45 formalin-fixed, paraffin-embedded tissue samples from patients with ulcerative colitis. The tissue samples represented a spectrum of inflammation from chronic quiescent disease to severe inflammation. Thirteen biotinylated lectins, directed against a range of sialyl, fucosyl and *N*-acetylgalactosaminyl sequences, were applied using an avidin–peroxidase revealing system. The results were assessed semiquantitatively for a number of cellular sites. The expression of all sialyl sequences was increased in absorptive cells and in goblet cells and the expression of α2–6-linked sialyl sequences was enhanced in proportion to the degree of inflammation, while α2–3-linked sialyl sequences were diminished in more severe inflammation. The binding of *N*-acetylgalactosaminyl-directed lectins was increased in the Golgi apparatus, while there was a reduction in the expression of α-fucosyl sequences in severe degrees of inflammation. This suggests that there is an increased biosynthetic rate for sialyl residues in all stages of disease with a reduction in α2–3-linked sialyl and fucosyl sequences in severe inflammation, and a shift from stored *N*-acetylgalactosaminyl sequences in goblet cells to an earlier form in the Golgi apparatus. The changes in sialyl sequences are a feature of ulcerative colitis even in quiescent disease and may be related to its aetiology and early pathogenesis, while most of the other changes reflect the severity of the disease and are probably part of its later pathogenesis or of induced reactive changes.

Keywords: colon, histochemistry, lectins, rectum, ulcerative colitis

Introduction

Ulcerative colitis is a chronic inflammatory condition of the large bowel of unknown aetiology, associated with abnormalities of mucin production. These abnormalities relate to both the intracellular synthesis and extracellular secretion of colonic mucins. Normal colonic mucin contains a mixture of sulphated and sialylated types, whereas in ulcerative colitis there is a relative decrease in sulphation, resulting in an apparent increase in sialomucins, as determined by standard tinctorial methods for mucin detection [1]. More detailed information on glycan expression in colonic mucin may be obtained *in situ* by the use of lectin histochemistry. Previous studies employing lectin histochemistry have described the patterns of lectin binding in variable degrees of inflammation [1–12], and many of these groups have also described the changes seen in association with the precancerous state of dysplasia in these tissues [1, 2, 4–7, 11, 13]. The most frequently used lectin has been that of the peanut, while the horse gram and gorse lectins have also been assessed by most investigators. This study evaluates the binding patterns obtained with a panel of 13 biotinylated lectins with specificities directed mainly against fucosyl, sialyl and

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N-acetylgalactosaminyl residues at non-reducing termini of glycans, using an avidin–peroxidase revealing system, in tissue from 45 patients with chronic ulcerative coitis showing a range of degree of inflammatory activity. These lectins were chosen in an attempt to reproduce previous reports, particularly with regard to fucosylated and *N*-acetylgalactosaminylated sequences, but also, by using more recently available lectins directed against sialyl sequences, to demonstrate *in situ* the glycoconjugates responsible for the apparent increase in sialomucin expression noted previously in ulcerative colitic mucosa.

Materials and methods

Biopsies of colon and rectum from 45 patients with a history of ulcerative colitis were obtained at colonoscopy. There were 24 female patients (mean age 40.4 years, range 29–55) and 21 male patients (mean age 42.3, range 27–63). They had been symptomatic for between 3 months and 16 years and the majority had been treated with either sulphasalazine or mesalazine. Tissues were fixed in 10% neutral buffered formalin for at least 24 h, after which they were routinely processed and embedded in paraffin wax. Haematoxylin and eosin-stained sections were examined to determine disease activity, according to standard criteria. This resulted in 14 cases in which there were features of chronic quiescent disease (group 1) and three further groups of tissues which, in addition to features of chronicity, showed increasing degrees of neutrophil infiltration, mucin depletion and mucosal ulceration of mild (14 cases, group 2), moderate (13 cases, group 3) and severe (four cases, group 4) extent. No cases with dysplasia were studied.

Sections 4 μm thick were cut and mounted on poly-L-lysine-coated slides. Lectin histochemistry was carried out using the method of Jones *et al.* [14] using a carefully selected panel of lectins (see Table 1 for major sugar specificities and references [15–32]). The sections were dewaxed in xylene, rinsed in absolute ethanol and treated for 30 min with absolute methanol containing 0.4% (v/v) HCl and 0.5% (v/v) hydrogen peroxide to inactive endogenous peroxidase. After rinsing in water and washing in Tris-buffered saline (TBS), pH 7.6 (50 mM Tris–HCl, 0.15 M NaCl), for 15 min (three changes), sections were incubated for 30 min at room temperature with 10 μg/ml biotinylated lectin in TBS plus 1 mM $CaCl_2$, pH 7.6 (TBSC). Washing for 15 min in TBSC (three changes) was followed by incubation in 5 μg/ml

Table 1. Lectins used in this study and their specificities

Abbreviation	Source	Major specificity	Reference
SNA	*Sambucus nigra* (elderberry bark)	NeuNAcα2,6Gal/GalNAc	15
LFA	*Limax flavus* (slug)	NeuNAc	16, 17
MAA	*Maackia amurensis*	NeuNAcα2,3Galβ1-	18
PNA	*Arachis hypogaea* (peanut)	Galβ1,3GalNAcα1- > Galβ1,4GlcNAcβ1-	19, 20
UEA-1	*Ulex europaeus* (gorse)	L-Fucα1,2Galβ1,4GlcNAcβ1-	21, 22
LTA	*Tetragonolobus purpureus* (lotus)	α-L-fucosyl terminals (especially where clustered)	23
BSA-$1B_4$	*Griffonia simplificolia*	Galα1,3Galβ1,4GlcNAcβ1-	24
PSA	*Pisum sativum* (garden pea)	α-D-mannose in non-bisected bi-/triantennary, complex *N*-linked sequences	23, 25
SBA	*Glycine max* (soybean)	Terminal GalNAcα1- > Galα1-	26, 27
DBA	*Dolichos biflorus* (horse gram)	GalNAcα1,3(L-Fucα1,2)Galβ1,3/4GlcNAcβ1-	28, 29
VVA	*Vicia villosa* (hairy vetch)	GalNAcα1-Ser/Thr and GalNAcα1,3Galβ1-	30
WFA	*Wisteria floribunda*	GalNAcα1,6Galβ1- > GalNAcα1,3Galβ1-	29, 31
HPA	*Helix pomatia* (snail)	GalNAc	32

avidin–peroxidase (Sigma) in 0.125 M Tris–HCl pH 7.6, 0.347 M NaCl [33] at room temperature for 1 h, then sections were washed in three changes of TBS over 15 min. Sites of lectin binding were visualized using 0.05% (w/v) diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical, Gillingham, UK) in TBS with 0.015% (v/v) hydrogen peroxide for 5 min at room temperature. Sections were then washed, counterstained with methyl green, dehydrated, cleared and mounted in XAM (Gurr, BDH, Poole, UK).

As a negative control, sections were incubated in TBSC in place of the lectin. Sections were also treated with 0.1 units of neuraminidase (Sigma type VI from *Clostridium perfringens*) in 0.2 M acetate buffer, pH 5.5, containing 1% $CaCl_2$, for 1 h at 37°C before incubation in PNA. Positive control material included normal colonic tissues, which demonstrated previously described features [34].

Results

The results of lectin binding were assessed using a semiquantitative scoring method in which 0 represented an absence of binding, 1+ weak focal staining, 2+ diffuse weak staining, 3+ moderate reactivity and 4+ strong intense binding. Sites of cellular localization assessed were the superficial cell membrane (MEMB), the apical cytoplasm (APEX), the supranuclear region (SN), which has been shown in previous ultrastructural studies to correspond to the Golgi apparatus [35, 36], the mucin goblets of epithelial cells (GC), the acellular connective tissue stroma (STR) and, where appropriate, the endothelial cell lining of blood vessels (BV) and the membranes of red blood cells (RBC). Variations between binding at the crypt base (CB) and the crypt surface (CS) were also noted in the tables. Comparison was made with the results obtained in a previously described study of normal left-sided colonic and rectal mucosa [34]. Differences of more than one category in the various tissue compartments are highlighted in the tables by shading. The detailed results are shown in Tables 2–4.

The following specific features are notable.

SNA binding was seen in the apical–membranous region and mucin goblets of cells throughout crypts of ulcerative colitic cases. This was greater at crypt surfaces, and binding was increased in relation to the degree of active inflammation (Figure 1). Staining was generally increased in comparison with normal mucosa.

Weak LFA binding to the apical region and superficial membrane of cells was noted, with more marked binding in goblet cells. This was slightly greater at crypt surfaces and was in excess of that seen in normal mucosa. In the more severe degrees of inflammation, there was a decrease in apical and goblet cell staining. In half of the cases, vascular endothelial reactivity was observed.

There was mild apical–membranous and goblet cell staining with MAA, which was more intense at crypt bases (Figure 2). Staining intensity was slightly greater than in normal mucosa and there was a decrease in goblet cell staining in severe degrees of inflammation.

Very little staining was seen with PNA before neuraminidase treatment, as in normal mucosa. In the more severe degrees of inflammation, there was mild reactivity with membranes and the Golgi apparatus. After neuraminidase pretreatment, PNA reactivity was similar to that seen before removal of sialyl residues, except for enhanced stromal binding (Figure 3).

No epithelial staining was noted with UEA-1 and only one tissue showed vascular endothelial staining, although 40% of tissues demonstrated binding to red blood cell membranes.

LTA reactivity was seen in the superficial membranes of cells at the base of crypts, as in normal mucosa (Figure 4). There was a diminution in this membranous staining in the most severe degrees of inflammation, and in milder degrees of inflammation there was mild apical cytoplasmic staining.

No epithelial staining was seen with BSA-$1B_4$. Two cases showed diffuse endothelial staining, while 20% of tissues demonstrated staining of erythrocyte membranes.

PSA binding was seen in the membranous regions, Golgi apparatus and mucin goblets of cells throughout the mucosa, more so at the crypt surface (Figure 5). This was similar to the pattern seen in normal mucosa, and there was a mild increase in membranous reactivity in severe degrees of inflammation.

SBA staining was noted in the Golgi apparatus and mucin goblets of cells and was greater at crypt surfaces (Figure 6). Binding to the Golgi apparatus was not seen in normal mucosa and goblet cell reactivity was decreased in more severe degrees of inflammation.

DBA binding was seen in the membranous region of cells, particularly in the upper crypts, in milder degrees of inflammation, as in normal

Table 2. Results of staining with lectins directed against sialyl sequences

Lectin category	Tissue compartments (see text for abbreviations)							
	MEMB	APEX	SN	GC	CB	CS	STR	BV[a]
SNA								
N	1	1	1	0	1	1	0	0
1	**2**	2	0	2	3	3	3	0
2	**4**	2	0	3	2	3	4	0
3	**4**	2	0	3	3	4	4	0
4	**4**	2	0	3	3	4	3	0
LFA								
N	1	1	0	2	1	2	0	0
1	2	2	0	2	1	2	2	4/14
2	2	1	0	2	1	2	2	9/14
3	2	**0**	0	2	1	2	2	7/13
4	2	**0**	0	**1**	0	2	1	2/4
MAA								
N	1	1	0	1	1	1	0	0
1	2	2	0	2	2	1	0	0
2	2	2	0	2	2	1	0	0
3	2	1	0	0	2	0	0	0
4	2	2	0	0	2	1	0	0
PNA								
N	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	**1**	0	0	1	0	0
3	0	0	**1**	0	0	0	0	0
4	**2**	0	**1**	0	1	1	0	0
PNA + NA								
N	NA							
1	0	0	0	0	0	0	2	0
2	0	0	**1**	0	1	1	2	0
3	**1**	0	**1**	0	1	1	2	0
4	**2**	0	**1**	0	2	2	2	0

[a]Number of cases demonstrating endothelial reactivity.
Disease catgories: N, normal left-sided colon and rectum (23 cases), as per previous paper [34]; 1, quiescent chronic colitis (14); 2, mild active chronic colitis (14); 3, moderate active chronic colitis (13); 4, severe active chronic colitis (4).
Bold entries represent distinct changes between categories.
NA, results of staining in normal mucosa not available.

mucosa. Goblet cell reactivity was similar to that of normal mucosa in lesser degrees of inflammation, but was much diminished in severe inflammation.

VVA reactivity was noted in goblet cells, especially in the upper crypts, in milder degrees of inflammation, similar to that of normal mucosa. Membranous staining which was present in normal mucosa was not seen in ulcerative colitic mucosa. In severe inflammation, there was slight binding to the Golgi apparatus (Figure 7).

There were binding sites for WFA in membranous regions, the Golgi apparatus and mucin goblets of cells in all degrees of inflammation, particularly in crypt surfaces, although there was a slight diminution in reactivity in the most severely inflamed cases. No comparison with normal mucosa was possible with this lectin. Endothelial staining was observed in 13% of cases.

HPA staining was noted in the Golgi apparatus, mucin goblets and membranous regions of cells, mainly at crypt surfaces. Membranous staining was

Table 3. Results of staining with lectins directed against fucosylated sequences

Lectin category	Tissue compartment (see text for abbreviations)							
	MEMB	APEX	SN	GC	CB	CS	BV[a]	RBC[b]
UEA-1								
N	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0/14	8/14
2	0	0	0	0	0	0	1/14	5/14
3	0	0	0	0	0	0	0/14	4/13
4	0	0	0	0	0	0	0/4	1/4
LTA								
N	2	0	0	0	2	0	0	0
1	2	**2**	0	0	2	0	0	0
2	2	**2**	0	0	2	0	0	0
3	**1**	0	0	0	1	0	0	0
4	**0**	0	0	0	0	0	0	0
BSA-1B_4								
N	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0/14	1/14
2	0	0	0	0	0	0	0/14	3/14
3	0	0	0	0	0	0	1/13	3/13
4	0	0	0	0	0	0	1/4	1/4
PSA								
N	2	1	1	2	2	2	0	0
1	2	0	1	3	2	3	0	0
2	2	0	2	3	2	3	0	0
3	**3**	0	2	3	2	3	0	0
4	**3**	0	2	3	2	3	0	0

[a]Number of cases demonstrating endothelial reactivity.
[b]Number of cases demonstrating erythrocyte membrane reactivity.
Disease categories: N, normal left-sided colon and rectum (23 cases), as per previous paper [34]; 1, quiescent chronic colitis (14); 2, mild active chronic colitis (14); 3, moderate active chronic colitis (13); 4, severe active chronic colitis (4).
Bold entries represent distinct changes between categories.

slightly greater in more severe degrees of inflammation, while goblet cell reactivity was reduced in the same groups (Figure 8). Endothelial and red blood cell membrane staining was observed in 44% of cases.

Discussion

The results summarized in Tables 2–4 show that there are some differences between the expression of glycans on normal and colitic tissues, even in quiescent disease, while other differences appear only in moderate or severe disease and are absent from the quiescent or milder forms. These changes in glycosylation affect both absorptive epithelial and goblet cells, but show several differences between them.

The changes noted in absorptive epithelium with SNA imply that there was an increase in the expression of *N*-acetylneuraminic acid (NANA) α2,6Gal/GalNAc- at the cellular surface and a shift of the precursor pool from the Golgi apparatus to apical cytoplasmic vesicles, suggesting an increased biosynthetic rate. The other sialyl-specific lectins showed much smaller changes, indicating that other categories of sialyl terminal (such as NANAα2,3Gal-) were much less affected. Goblet cells expressed elevated levels of NANAα2,6Gal/GalNAc- in all forms of ulcerative colitis, but NANAα2,3Gal-, while increased in mild disease, is lost in more severe forms. No previous studies

Table 4. Results of staining with lectins directed against *N*-acetylgalactosaminylated sequences

Lectin category	Tissue compartment (see text for abbreviations)							
	MEMB	APEX	SN	GC	CB	CS	BV[a]	RBC[b]
SBA								
N	1	0	0	2	1	2	0	0
1	0	0	2	2	2	2	0	0
2	0	0	2	**0/3**[c]	2	3	0	0
3	0	0	2	**0**	2	2	0	0
4	0	0	2	**0**	2	2	0	0
DBA								
N	2	1	0	3	2	3	0	0
1	2	0	0	4	3	4	0	0
2	1	0	0	**1/4**[c]	2[c]	3[c]	0	0
3	**0**	0	1	**2**	1	2	0	0
4	**0**	0	0	**0**	0	0	0	0
VVA								
N	2	0	0	2	2	2	0	0
1	0	0	0	2	1	2	0	0
2	0	0	0	2	2	2	0	0
3	0	0	**2**	**0**	2	2	0	0
4	0	0	**2**	**0**	1	2	0	0
WFA								
N	NA							
1	2	0	2	4	3	4	4/14	0
2	3	0	2	4	3	4	2/14	0
3	2	0	3	3	3	4	0/13	0
4	3	0	2	3	2	3	0/4	0
HPA								
N	NA							
1	0	0	2	3	2	3	4/14	4/14
2	1	0	2	**0/4**[c]	2	3	3/14	3/14
3	1	0	2	**0**	2	3	6/13	6/13
4	1	0	3	**1**	2	2	3/4	3/4

[a]Number of cases demonstrating endothelial reactivity.
[b]Number of cases demonstrating erythrocyte membrane reactivity.
[c]Separation of category 2, roughly eight positive and six negative.
Disease categories: N, normal left-sided colon and rectum (23 cases), as per previous paper [34]; 1, quiescent chronic colitis (14); 2, mild active chronic colitis (14); 3, moderate active chronic colitis (13); 4, severe active chronic colitis (4).
Bold entries represent distinct changes between categories.
NA, results of staining with normal mucosa not available.

have examined the expression of glycans binding these lectins in ulcerative colitic mucosa.

The saccharide GalNAcα1,3Gal- (SBA- and VVA-reactive) was present in goblet cells in quiescent ulcerative colitis and might have been slightly elevated above normal, but was lost with increasing severity of the disease. The saccharide GalNAcα1,6Gal- (WFA-reactive) appeared to be abundant, but was slightly diminished in severe disease. The site of expression of GalNAcα1,3Gal- changed with the severity of the disease, there being a retention of the saccharide in the Golgi apparatus in some severe cases, while GalNAc-α1,6Gal- did not change. A similar pattern of

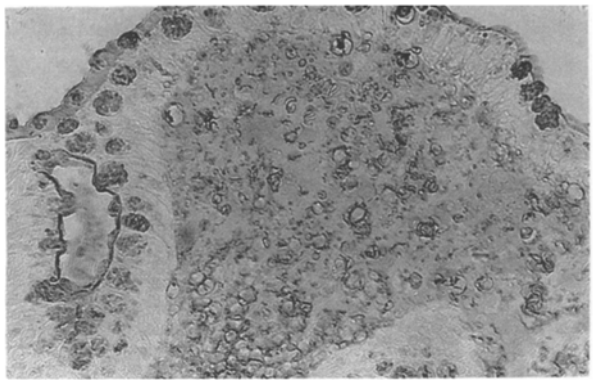

Figure 1. Binding of SNA at crypt surface in moderately active ulcerative colitis demonstrating goblet cell and membranous staining. —— represents 22.2 μm.

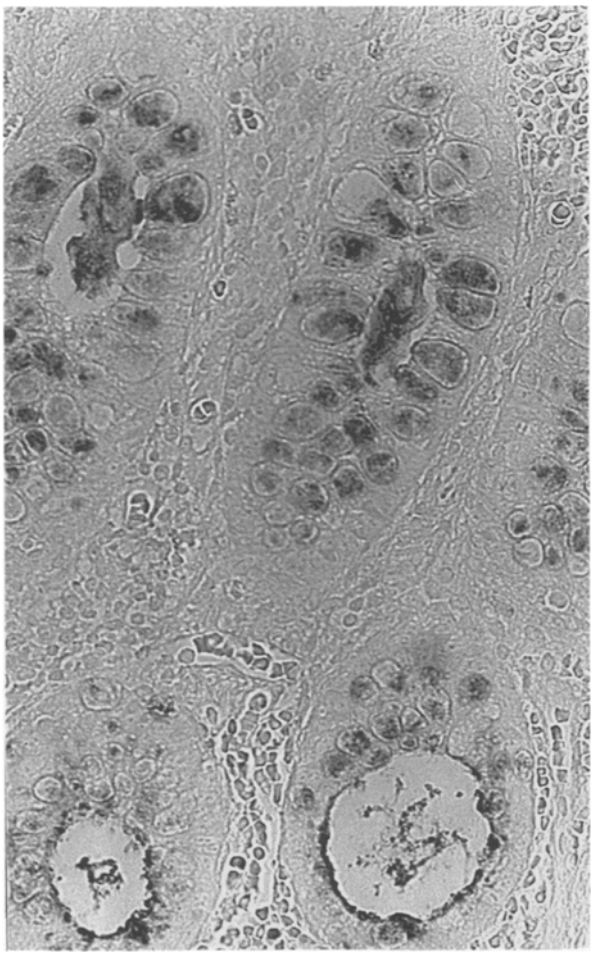

Figure 2. High-power view of crypt base with MAA showing goblet cell (above) and mild membranous staining (below). —— represents 11.1 μm.

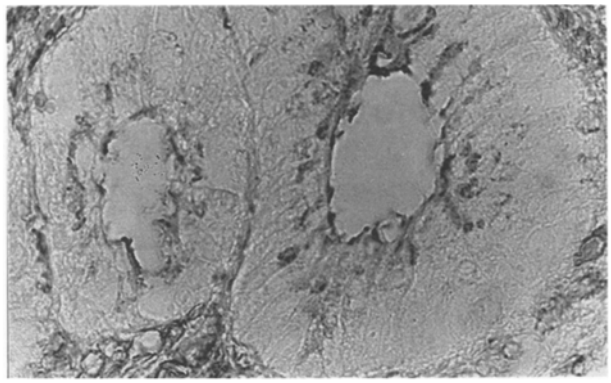

Figure 3. Peanut lecting binding after neuraminidase pretreatment of severely inflamed mucosa in which there is mild Golgi apparatus and membranous staining. —— represents 5.6 μm.

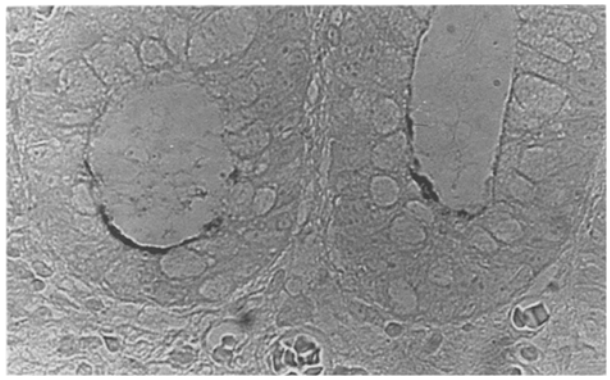

Figure 4. Membranous staining at base of crypts with LTA. —— represents 11.1 μm.

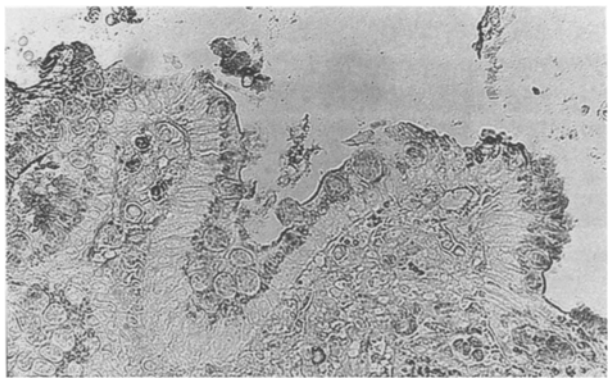

Figure 5. PSA binding to membrane, supranuclear region and goblet cells. —— represents 22.2 μm.

expression was seen by Yoshioka *et al*. [10], who demonstrated reduced goblet cell staining and increased Golgi apparatus reactivity for HPA, with an increase in SBA reactivity in goblet cells.

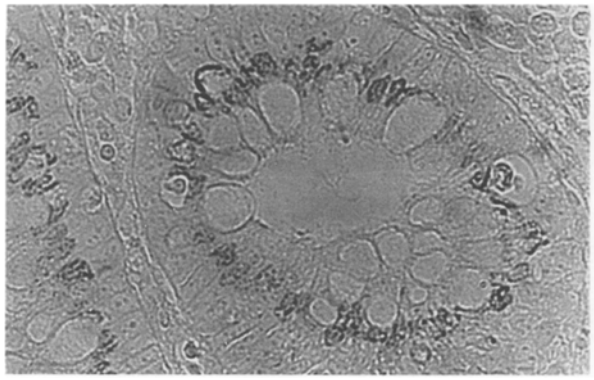

Figure 6. Supranuclear reactivity with SBA in moderately inflamed mucosa with absence of goblet cell binding. —— represents 5.6 μm.

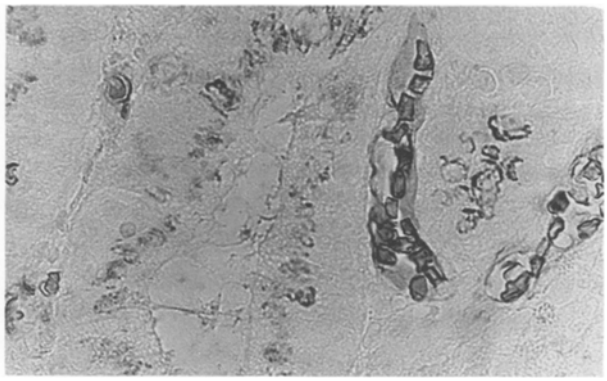

Figure 8. Supranuclear and weaker goblet cell and membranous staining (to the left) with HPA as well as endothelial and red cell membrane reactivity in adjacent stroma (to the right). —— represents 11.1 μm.

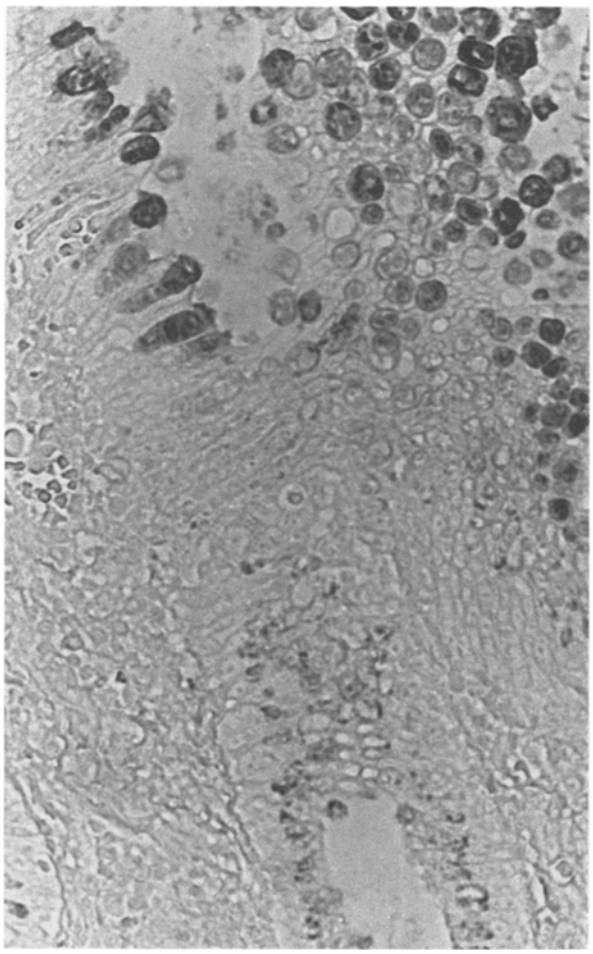

Figure 7. VVA binding to supranuclear region towards crypt base (below) with goblet cell reactivity at crypt surface (above) in severely inflamed mucosa. —— represents 11.1 μm.

Boland *et al.* [2] demonstrated diminished SBA binding in goblet cells, particularly in long-standing disease and in dysplasia.

Peanut lectin reactivity in the supranuclear region of absorptive epithelial cells in mild to severe disease and the epithelial surface in severe disease was probably binding largely to Gal-β1,3GalNAcα1-. The failure of neuraminidase to increase epithelial staining with PNA suggested that very little of this was becoming sialylated, but other sugars were probably being added because the supranuclear stain strongly suggests that there was a biosynthetic pool of this saccharide in the Golgi apparatus which only reached the surface in an unaltered form in severe disease. Two groups have shown increased apical and supranuclear staining in ulcerative colitis with PNA [10, 11], while other groups have demonstrated increased reactivity with the Golgi apparatus alone [2, 7, 8, 12, 13]. Cooper *et al.* [7] found that supranuclear reactivity was greatest in the most severe grades of inflammation. Ryder *et al.* [12] showed that, using both frozen sections and paraffin-embedded tissues and both direct immunoperoxidase and avidin–biotin peroxidase methods, normal left-sided colonic tissues expressed PNA binding sites in the supranuclear region in 30% of cases and that ulcerative colitic mucosa demonstrated increased (65%) supranuclear reactivity. Those patients with increased PNA binding also showed an enhanced crypt cell proliferative response to peanut lectin *in vitro*. Goblet cells in ulcerative colitic mucosa appeared to lack Galβ1,3GalNAcα1-, or any simple sialylated derivative of it, in the present study, although

two previous studies have reported such a finding [6, 7].

Some LTA-reactive fucosyl residues were being lost as the disease increased in severity. Similar effects were seen with DBA staining, which was diminished in mild active disease and lost in more severe forms. However, unlike LTA, DBA also stained goblet cells, being increased over normal in quiescent disease, but progressively diminishing in all forms of active disease. Three groups have shown increased goblet cell binding with DBA in ulcerative colitis compared with normal mucosa [3, 8, 10], and other investigators have reported diminished goblet cell staining in increasing grades of inflammation [2, 4, 9], as confirmed in the present study. A possible explanation is as follows: DBA binds to GalNAcα1,3(Fucα1,2)Galβ1,3/4GlcNAcβ1- and requires both the terminal residues of *N*-acetylgalactosamine and fucose for a strong interaction. The progressive loss of binding in goblet cells could, therefore, arise from an increasing failure of transfer of *N*-acetylgalactosamine, since the related saccharide GalNAcα1,3Galβ1- also appeared to be lost, as shown by the loss of VVA and WFA binding (see above). In the absorptive epithelial cells, though a contribution from the loss of *N*-acetylgalactosamine may have occurred, a loss of the 2-fucosyl residues, perhaps in a family of related glycans, might explain the loss of stain with both DBA and LTA. The glycan Fucα1,2Galβ1,4GlcNAcβ1-, which binds to UEA-1, was always absent in the current study, although others have shown increased goblet cell [3, 6, 8, 10] and supranuclear [10] reactivity with UEA-1 in ulcerative colitis. Though LTA can bind to the fucosyl residue of -GlcNAcβ1,4(Fucα1,6)GlcNAcβ1- core sequences of some *N*-glycans, this is unlikely to be a contributory factor here, because PSA requires such a residue, in complex, biantennary, non-bisected *N*-glycans, for strong binding. It bound to structures unstained by LTA (such as goblet cells) and its staining of the membranes of absorptive epithelial cells shifted in the opposite direction to that of LTA, with increasingly severe disease. The exact nature of the glycan bound by LTA remains unclear.

Hence, changes in NANAα2,6Gal/GalNAc-expression on both goblet and absorptive epithelial cells are a feature of ulcerative colitis and are present even in quiescent disease. They may, therefore, be closely related to the aetiology or early pathogenesis of the disease, or to early reactive changes which are not morphologically evident. Most of the other changes identified here reflect the severity of the disease and so arise either from its later pathogenesis or from the reactive changes that it induces. Most of these effects cannot be attributed simply to changes in crypt or villous length, with attendant alterations in the sizes of functionally and biochemically different cellular populations, as the data in the CB and CS columns of Table 2–4 indicate. For example, there are no normal compartments which show the intensity of staining with SNA found in the tissues from colitic patients, or the lack of stain with DBA seen in severe disease.

This survey has focused on saccharides particularly associated with the non-reducing termini of both *O*- and *N*-linked glycans, and it offers no information on the relative abundances of different subsets of *N*-glycans or on core sequences. The finding in this study of variations in the terminal sequences of outer chains with sialyl-, galactosyl- and fucosyl-specific lectins, which correspond to the specificities for the Lewis and sialyl-Lewis blood group antigens, does indicate that further analysis of terminal sequences should be rewarding, particularly in the light of previous findings with carbohydrate-associated antigens in colorectal carcinoma [37, 38] and ulcerative colitis [38, 39].

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