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Distribution of O-acetylated sialomucin in the normal and diseased gastrointestinal tract shown by a new monoclonal antibody

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Abstract

***Aims*—To produce and characterise a monoclonal antibody specific for O-acetylated sialomucin and to assess its use in immunohistochemistry on a panel of normal and diseased intestinal tissue samples.**

***Methods*—Mouse monoclonal antibodies were developed following immunisation with highly purified human colonic mucin. One of these (MMM-17) showed strong binding to mucin throughout the normal colon with relative lack of binding to colon cancer tissue. The binding epitope of MMM-17 was then characterised by screening for agglutination activity against a panel of human and animal erythrocytes and by assessment of its binding to a range of normal and chemically treated slot blotted mucins. Further immunohistochemical studies were then performed on formalin fixed, normal, and diseased human intestinal samples.**

***Results*—Binding of MMM-17 to slot blotted human colonic mucin was reduced by 38 (SD 14%) (n = 4) by alkali treatment of the mucin, sequential alkali and sialidase treatment completely abolished binding. Sialidase treatment alone, however, caused only an 11 (11%) reduction in binding. MMM-17 failed to agglutinate any human, rabbit, rat or mouse erythrocytes. These findings were compatible with specificity of MMM-17 for sialomucins O-acetylated at the C-7 or C-8 positions on the sialic acid. Strong staining by MMM-17 was found in all goblet cells throughout all 40 normal colonic and rectal samples studied, but staining was absent in seven of 13 colorectal carcinomas. Normal duodenum (n = 16) and normal ileum (n = 3) all showed occasional positive goblet cells. The normal gastric antral mucosa was generally negative B MMM-17, but in all of 15 cases of gastritis with intestinal metaplasia the metaplastic glands were strongly positive for MMM-17.**

***Conclusion*—Monoclonal antibody MMM-17 has specificity for O-acetylated sialomucins and its binding depends both on the position of O-acetylation and on the adjacent oligosaccharide structure. Preliminary studies using the antibody on archival tissue samples support the previous reports of reduced O-acetylation in colon cancer demonstrated by indirect histochemistry and show the neo-formation of O-acetylated sialomucin in intestinal metaplasia in the stomach.**

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Human colonic mucin is heavily sialylated. This increases the charge of the mucin and is important in conferring resistance to degradation by glycosidases as sialic acid needs to be removed by sialidases before exoglycosidases can further degrade the oligosaccharides.[1] Much of the sialic acid in the human colon is O-acetylated at the C7, 8, or 9 positions[2-4] which considerably increases its resistance to sialidase.[5,6]

The O-acetylation of colonic sialomucins has been shown histochemically before by showing that, whereas colonic mucin is normally only lightly stained by the periodic acid Schiff (PAS) procedure, prior treatment with alkali, which will remove O-acetyl groups, causes a large increase in PAS staining.[7] Biochemical confirmation of this evidence has been provided by comparative histochemical and biochemical investigations.[8] Further investigations using histochemical[3,9] and biochemical[6,10] techniques have shown that normal colonic mucus contains di-O-acetylated (C8,C9 and C7,C9) or tri-O-acetylated (C7, C8,C9) sialomucins. Although O-acetylation at C4 was inferred from histochemical techniques,[7] subsequent biochemical studies have failed to show C4 O-acetylated sialic acids in human mucins[11] and this reflects the difficulty in interpreting the ingenious but complex histochemical tests for O-acetylation.

Decreased O-acetylation of sialomucins is a common early feature of malignant and premalignant epithelial disorders, particularly in the colon. It has been demonstrated, using both histochemical and biochemical techniques, in colorectal cancer,[10,12] in ulcerative colitis,[3,13] and in colonic adenoma.[14]

The availability of an antibody with specificity for O-acetylated sialomucin would therefore have considerable potential for the study of changes in mucin in malignant and premalignant gastrointestinal disease. Several monoclonal antibodies to normal[15-17] and neoplastic[18,19] colonic mucin have been developed, but it is not clear whether any of these have specificity for O-acetylated sialomucins.

Methods

PREPARATION OF MUCIN

A 10 cm length of macroscopically normal colon (at least 10 cm remote from the tumour) was obtained at hemicolectomy for carcinoma of the colon, opened, washed thoroughly in physiological saline and the luminal surface scraped with a glass slide. The scrapings were sonicated in physiological saline with 8 × 15 second bursts using an MSE ultrasonicator at No 4 power setting (Fisons, Loughborough, England), followed by centrifugation at 110 000 × *g* for 90 minutes. The supernatant fluid was collected, dialysed extensively against deionised water, lyophilised and reconstituted in 0·1M TRIS/HCl (pH 8·0). This crude mucin was injected using a 200 μl sample loop on to a 10 mm × 300 mm Superose 6 gel filtration column (Pharmacia, Uppsala, Sweden)[20] and eluted using 0·1M TRIS/HCl (pH 8·0) at 0·25 ml/minute with continuous optical density monitoring at 280 nm and collection of 30 × 1 ml fractions. The fractions comprising the initial optical density peak were pooled and shown to contain mucin, free from non-mucin glycoproteins or proteins, as judged by the absence of low density glycoprotein on caesium chloride density gradient centrifugation and the absence of lower molecular weight glycoproteins detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis and PAS staining.[20]

IMMUNISATION AND FUSION

A Biozzi/BALB/c F1 mouse was immunised subcutaneously with about 1 μg highly purified colonic mucin in saline mixed with an equal volume of Freund's complete adjuvant and subsequently boosted intravenously two and four months later with a similar amount of mucin. By this time the mouse was shown by ELISA to be producing antibody to mucin so it was boosted once more and killed four days later by ether anaesthesia and the spleen removed for fusion. Fusion with NS-1 myeloma was done by standard polyethylene glycol procedure[21] and supernatant fluids from proliferating clones screened for anti-mucin antibody by ELISA.

ELISA FOR DETECTION OF ANTI-MUCIN ANTIBODY

ELISA plates (Dynatech, Billingshurst, Sussex) were coated with human colonic mucin, prepared as before, about 10 μg protein/ml in 100 μl 0·05M carbonate/bicarbonate buffer (pH 9·5), by incubation at 4°C for 16 hours. The plates were washed in 0·01M phosphate buffered saline (pH 7·4), blocked with 125 μl 0·5% bovine serum albumin (BSA) and 0·5% Tween 20 in PBS (PBS/BSA/Tween) for 2 hours at 37°C, washed three times with 0·05% Tween 20 in PBS (PBS/Tween) and 100 μl culture supernatant fluid added for 2 hours at 37°C. The plates were then washed three times with PBS/Tween, 100 μl 1 in 500 peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako, High Wycombe, England) added and incubated for 2 hours at 37°C. After five washes with PBS/Tween, peroxidase activity was detected using 100 μl o-phenylene diamine(OPD)/hydrogen peroxide as substrate (0·4 mg/ml OPD, 0·4 μl/ml 30% hydrogen peroxide in 0·05M phosphate/citrate buffer, pH 5·0); after 6 minutes at room temperature the reaction was stopped by the addition of 100 μl 4M sulphuric acid and the absorption at 490 nm read with a CLS microplate reader (Cambridge Life Sciences, Cambridge, England).

To study the ability of bovine submaxillary mucin and its desialylated derivative (desialylated by treatment with 0·05M sulphuric acid for 1 hour at 80°C) to inhibit binding of antibody to human colonic mucin, ELISA plates were coated with purified mucin as described above, blocked with PBS/BSA/Tween, and then 50 μl of 1 in 5 culture supernatant fluid was added followed immediately by 50 μl potential inhibitor and the standard ELISA procedure continued.

ERYTHROCYTE SCREENING

Agglutination of human erythrocytes covering a range of known blood group antigens including Lewis, Kidd, Duffy, Rh, Kell, P, MN, Lutheran, Xg and cord cells was tested. Erythrocytes were incubated with a 1 in 10 culture supernatant fluid containing anti-mucin antibody under a range of conditions: in 0·9% NaCl for 1 hour at 4°C, 20°C, or 37°C in the presence of albumin 20 mg/ml for 1 hour at 20°C, and in 0·85% NaCl for 20 minutes or 1 hour at 37°C. Agglutination was also tested against human group O and cord erythrocytes following T activation by incubation for 30 and 60 minutes at 37°C with *Clostridium perfringens* neuraminidase (Sigma, Poole, England) 0·1 units in phosphate buffered saline (pH6·8). Agglutination was also tested against BALB/c and C57B1 mouse, Wistar rat and New Zealand Rabbit red blood cells. As a positive control another anti-mucin monoclonal antibody (CAM 17·1) with confirmed anti-blood group activity[22] was used in parallel.

HISTOLOGICAL TECHNIQUES

Immunohistochemical staining was performed on 145 formalin fixed, paraffin wax embedded human tissues samples chosen to represent a wide range of normal, inflamed, and neoplastic tissues. Sections (5 μm) were dewaxed and then immersed in 0·03% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, followed by incubation with normal sheep serum for 20 minutes to block non-specific binding. Sections were incubated for 30 minutes at room temperature with culture supernatant fluid containing antibody to mucin at 1 in 100 in PBS (pH7·8) followed by rinsing in PBS. Peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako, High Wycombe, England) 1 in 50 for 30 minutes was applied, washed off, and bound antibody visualised using 3-amino-9-ethyl carbazole.

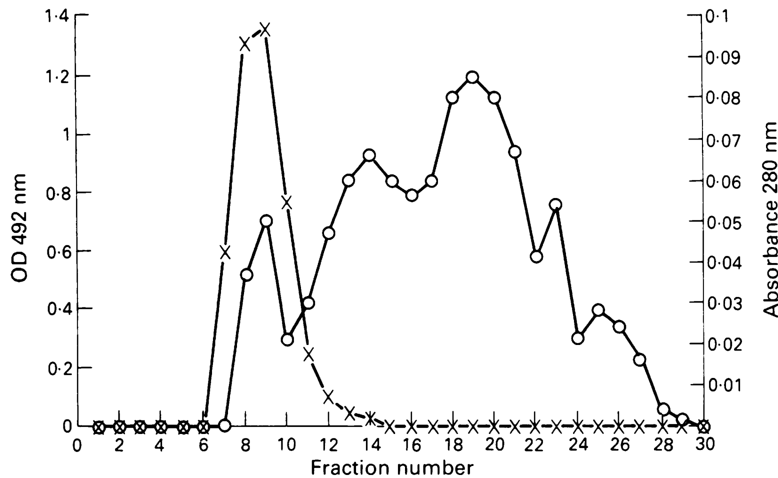

Figure 1 Gel filtration (Superose 6) of crude extract obtained by ultrasonication of mucosal scraping from a resected normal human colon. X = binding activity of MMM-17: ELISA OD 492 nm; O = absorbance at 280 nm.

SAPONIFICATION, SIALIDASE TREATMENT, AND MILD ACID HYDROLYSIS OF SLOT BLOTTED MUCINS

Aliquots (100 μl) of highly purified mucin were blotted on to nitrocellulose paper using a slot blot manifold (Hoeffer Scientific, Newcastle-under-Lyme, England) and flushed through with 400 μl PBS. The paper was then incubated in either PBS or 0·1M NaOH for 1 hour at 20°C, rinsed three times with PBS, and blocked with PBS/BSA/Tween. The blots were then rinsed with PBS, washed twice in 0·1M acetate buffer (pH 5·0), and then incubated overnight at 37°C in 2 ml acetate buffer alone or containing 1 unit of *C perfringens* neuraminidase (Sigma, Poole, England). The blots were then washed three times with PBS/Tween, incubated with antibody containing culture supernatant fluid diluted 1 in 5 with PBS, for 2 hours at 20°C, washed three times with PBS/Tween and incubated with 1 in 400 peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako, High Wycombe, England) for 2 hours. After four washes in PBS/Tween, chloronaphthol substrate solution (1 ml chloronaphthol 3 mg/ml in methanol, 4 ml PBS, and 5 μl 30% hydrogen peroxide) was added and colour allowed to develop for 6 minutes, after which blots were rinsed three times each in PBS and distilled water and then dried. The intensity of staining of slot blots was measured with a densitometer (Hoeffer, San Francisco, USA) linked to a chart recorder and the heights of the peaks recorded. In some experiments the blots were stained using 1 in 200 peroxidase conjugated *Limax flavus* agglutinin (LFA) (E-Y San Mateo, California, USA) or wheat germ agglutinin (WGA) (Sigma, Poole, England) for 4 hours. Substrate incubation was as for the antibody incubation.

For mild acid hydrolysis blots were treated with 0·05M sulphuric acid for 1 hour at 80°C and then blocked and stained as described before. Immunoblotting was also performed on samples of bovine (BSM) and equine submaxillary mucin (ESM) (kindly supplied by Dr A P Corfield, Department of Medicine, University of Bristol, England). As an alternative procedure mucin was also incubated in solution with either 0·1M NaOH or 0·05M

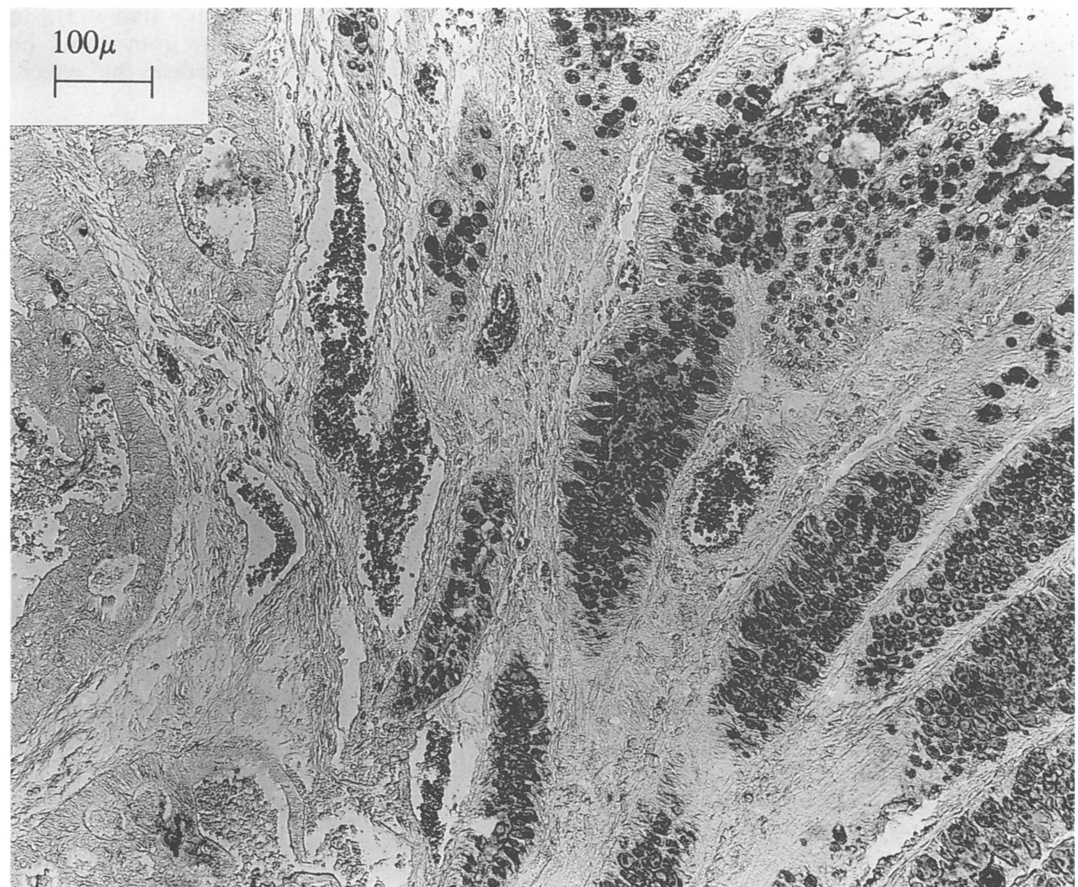

Figure 2 Colonic carcinoma and adjacent normal mucosa (formalin fixed) stained with MMM-17 and peroxidase labelled anti-mouse Ig. Strong staining of all normal goblet cells with absence of staining of the carcinoma.

H_2SO_4, before blotting on to nitrocellulose and proceeding to incubation with MMM-17 as described before.

Results

Screening of 50 culture supernatant fluids from fusion products yielded a culture, MMM-17, which when expanded produced an antibody with affinity for purified colonic mucin as demonstrated by ELISA. Screening with IgM and IgG subclass specific anti-immunoglobulins showed the antibody to be IgG1. Monitoring of gel filtration fractions from crude mucin applied to a Superose 6 column showed that the antibody reacted with the initial mucin fractions (fig 1) with no reactivity with lower molecular weight material.

The only normal mucosa which consistently showed strong positive staining was the colon (n = 36) in which all goblet cells were stained without exception (fig 2). These included nine samples of ascending and descending colon from patients with a range of known ABO blood groups and secretor status. Gall-bladder columnar epithelium stained consistently albeit less strongly (n = 4). Mucin producing cells in other sites, including nasal mucosa (n = 2), submandibular salivary gland (n = 3), normal gastric antrum (n = 7), duodenal (n = 16) and ileal (n = 3) mucosa, were either unstained or showed weak focal positivity (table 1). Consistent positive staining was observed in metaplastic epithelium in the stomach (n = 15) and oesophagus (n = 3) (fig 3). Seven out of 13 carcinomata of the colon or rectum showed complete lack of staining (fig 2); in the remaining six occasional goblet cells were positive. These results are summarised in table 1.

No agglutination of human, Wistar rat, BALB/c and C57B1 mouse or New Zealand rabbit erythrocytes was observed under any of the conditions tested.

Mild acid hydrolysis of blotted normal human colonic mucin almost abolished 82 (SD 15) % reduction binding of MMM-17 (fig 4) and caused more than a 95% reduction in binding of LFA, although WGA binding was slightly increased (table 2), suggesting that there was no significant removal of mucin from the nitrocellulose. When mucin was incubated with dilute acid before application on to the nitrocellulose the ability to bind MMM-17 was similarly abolished, with no effect on WGA binding, again indicating that the effect of the acid was not simply to remove the mucin from the nitrocellulose.

When blots were treated with sialidase alone there was no significant reduction in MMM-17 binding (11 (11%) reduction), although LFA binding was significantly reduced as expected (69 (14%) reduction) (table 2). Alkali treatment caused a definite reduction (39 (14%) reduction) in binding of MMM-17 and alkali treatment followed by sialidase caused complete loss of binding of MMM-17 (fig 4). Alkali treatment of blots caused some increase in binding of WGA (table 2), again showing that there had been no loss of mucin from the blot; whereas it roughly doubled (113 (20%) increase) the binding of LFA, suggesting that this lectin is specific for non-O-acetylated sialic acids (table 2). When mucin was treated with alkali before application to the nitrocellulose the binding ability for MMM-17 was reduced, but not abolished (50 (10%) reduction at 1 hour) (table 3). Increasing the incubation time from 1 to 2 hours did not substantially increase the effect of alkali on MMM-17

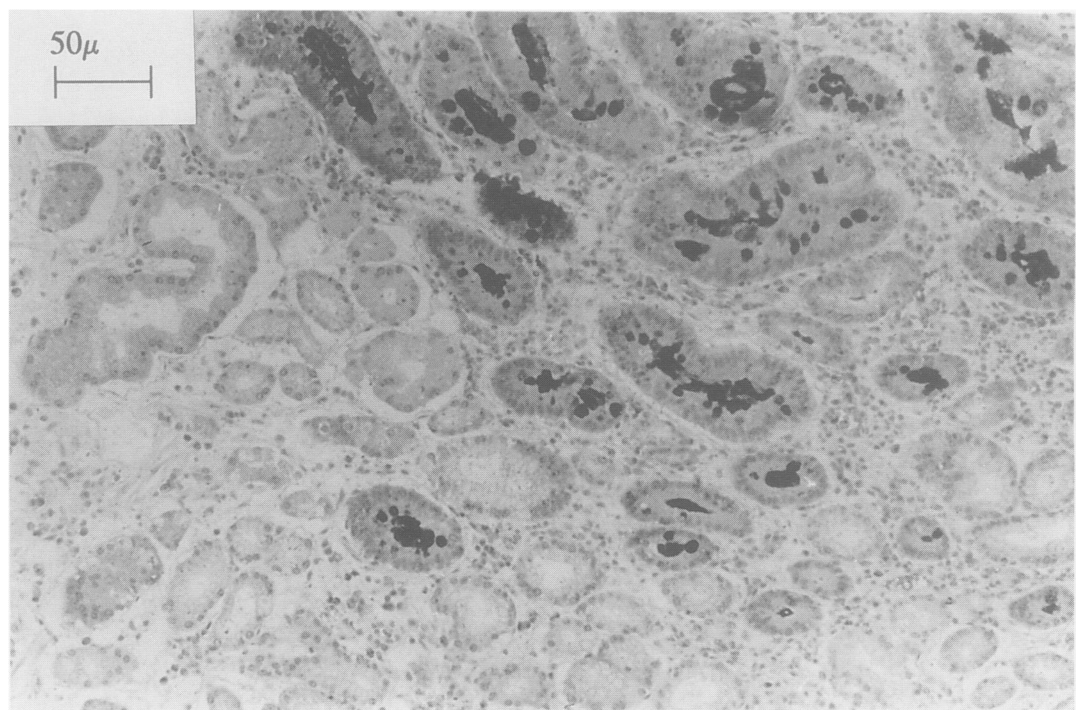

Figure 3 Gastric antral mucosa (formalin fixed) stained with MMM-17 and peroxidase labelled anti-mouse Ig. Glands which show intestinal metaplasia are strongly stained in the goblet cells and secreted mucin while the normal glands are not stained.

Table 1 Immunohistochemistry with MMM-17 of formalin fixed human intestine

Site	Nature	Staining: Positivity	Staining: Intensity	Site of staining
Oesophagus	Oesophagitis	3/3	+ + +	Squamous epithelium Goblet cells in metaplasia
	Adenocarcinoma	0/1		
Stomach	Normal	1/7	+	Columnar cells
	Gastritis with intestinal metaplasia	15/15	+ + +	Goblet cells
	Gastritis NOS	0/2		
	Adenocarcinoma	1/5	+	Adenocarcinoma cells
Duodenum	Normal	16/16	+	Goblet cells 12/16 Columnar cells 10/16
	Duodenitis	1/1	+	Goblet cells
Ileum/ jejunum	Normal	3/3	+	Goblet cells 2/3 Columnar cells 1/3
	Inflammation	1/1	+	Goblet cells
	Adenocarcinoma	0/1		
Colon	Normal	36/36	+ + +	Goblet cells
	Colitis (UC)	1/1	+ + +	Goblet cells
	Adenocarcinoma	3/7	+	Occasional goblet cells
Rectum	Normal	4/4	+ + +	Goblet cells
	Adenocarcinoma	3/6	+	Patchy in goblet cells

Table 2 Effect of treatment of slot blotted mucin with acid, alkali or sialidase on its ability to bind lectins

Treatment of mucin	% control lectin binding: LFA	WGA
Sialidase	31 (14)	ND
Acid	<5	116 (13)
Alkali	213 (20)	127 (49)

Results are mean (SD) of at least two experiments with triplicate slot blots of mucin. ND = not done.

Table 3 Time course of effect of alkaline treatment of mucin solution on its subsequent ability to bind MMM-17 when slot blotted

Time	Binding*
0	100 (5)
0·5	64 (4)
1·0	53 (6)
2·0	50 (2)

Binding expressed as a percentage of the binding of mucin incubated for zero time before slot blotting. Mean (SD) of quadruplicate blots.

binding (table 3).

MMM-17 showed no binding to equine submaxillary mucin but bound strongly to bovine submaxillary mucin. Alkali treatment of the bovine submaxillary mucin resulted in a substantial reduction in binding (76 (9%) reduction) with complete loss of binding with subsequent sialidase treatment as was found with human colonic mucin. Similarly, acid treatment considerably reduced or abolished (at least 80% reduction) binding of MMM-17 to BSM.

The effect of bovine submaxillary mucin on the ability of MMM-17 to bind to colonic mucin was investigated with an ELISA inhibition technique, where it was found that BSM inhibited such binding by 60% at 1 μg/ml; desialylated BSM was much less effective, requiring a concentration of 10 μg/ml for comparable inhibition (fig 5). Equine submaxillary mucin, which does not bind MMM-17, did not inhibit the binding of the antibody to colonic mucin.

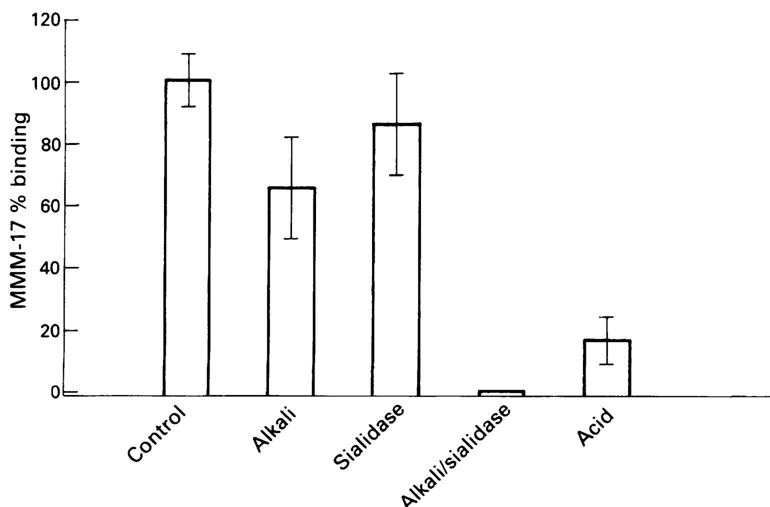

Figure 4 Effect of treatment with alkali (0·1M NaOH), sialidase (1 unit), alkali followed by sialidase, and acid (50mM H_2SO_4) on MMM-17 binding to blots of colonic mucin, as revealed with peroxidase labelled anti mouse Ig. Mean (SD); four triplicate assays.

Discussion

The monoclonal antibody MMM-17 is specific for human colonic mucin and is effective for use in immunohistochemistry on fixed tissue.

Prevention of MMM-17 binding to mucin by prior mild acid hydrolysis of the mucin suggests that its binding epitope contains sialic acid, fucose, or ester sulphate, all of which would be removed by this process. The finding that there is only partial reduction in MMM-17 staining following alkali treatment, whereas the combination of alkali and sialidase results in total loss of binding, suggests that the epitope involves those sialic acids or sialylated oligosaccharides which are O-acetylated, but that binding is not totally dependent on the O-acetyl groups themselves. The additional finding that sialidase alone has no effect on binding implies that only those sialic acid residues that are O-acetylated are involved in binding and that they differ from those that are not O-acetylated, either in the nature of their linkage to the oligosaccharide side chain or the nature of the side chain to which they are linked. The lack of agglutinating activity for human erythrocytes is consistent with specificity for O-acetylated sialomucin as human erythrocyte sialic acids are not O-acetylated.[11 23] Rabbit and rodent red blood cells are known to contain sialic acid residues O-acetylated at the C-9 position,[23] so the lack of agglutination of these cells implies that MMM-17 does not recognise mono-9-O-acetylated sialic acid, as found on these cells.

The binding pattern of MMM-17 to human colonic mucin and bovine submaxillary mucin together with the inhibition of binding to human colonic mucin by BSM shows that MMM-17 binds to a determinant present on both these mucins. Bovine submaxillary mucin contains C9 and probably C7 and C8 mono-O-acetylated sialic acids, C7,9 and C8,9 di-O-acetylated sialic acids and C7,8,9 tri-O-acetylated sialic acids whereas equine submaxillary mucin contains

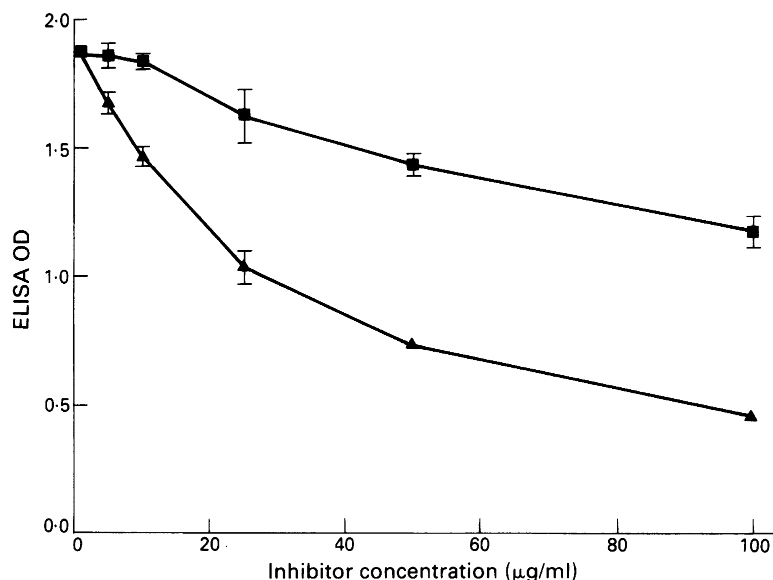

Figure 5 Inhibition of binding of MMM-17 to solid phase human colonic mucin by normal and desialylated bovine submaxillary mucin as assessed by ELISA. ▲ = normal bovine submaxillary mucin; ■ = desialylated bovine submaxillary mucin.

sialic acids that are predominantly O-acetylated at C4 or C9.[11] The lack of binding of MMM-17 to equine submaxillary mucin suggests that it probably has no affinity for C4 or C9 O-acetylated sialomucins.

There is a tendency for the O-acetyl group to migrate from the C7 position to the C9 position[11] so that the C7 and C8 mono-O-acetyl sialic acid residues are likely to be uncommon in stored tissue samples. It can therefore be suggested that MMM-17 has an affinity for di-O-acetylated (C8, C9 or possibly C7, C9) or tri-O-acetylated (C7, C8, C9) sialomucins. As either the nature of the linkage of the sialic acid to the side chain or the nature of the sugar(s) to which it is linked probably affect the binding of the antibody, further characterisation would require extensive chemical synthetic procedures to produce the appropriate di-O-acetyl and tri-O-acetyl sialic acids for binding inhibition studies.

The considerably increased LFA binding of mucin following alkali treatment suggests that LFA has specificity for non-O-acetylated sialic acids. This is consistent with the fact that periodate oxidation of the C-7-8-9 side chain of sialic acid has been shown to reduce binding of LFA[24] which implies that the C-7-8-9 side chain is involved in binding. Therefore, acetylation of any part of this side-chain might be expected to interfere with binding. Further studies (data not shown) have also shown that acid treatment of mucin which removes non-O-acetylated sialic acid very efficiently abolishes LFA binding, but subsequent alkaline treatment regenerates the ability to bind LFA, presumably due to de-O-acetylation of O-acetylated sialic acids which are resistant to mild acid hydrolysis.

This is, we believe, the first description of a monoclonal antibody with affinity for some types of O-acetylated sialomucins. Monoclonal antibodies have been produced against O-acetylated sialic residues on gangliosides[25 26] but these have not been shown to react with sialomucins,[25] probably because sialomucins and gangliosides have different configurations of the oligosaccharide side chain.

The striking positivity seen with MMM-17 in all 15 of the gastric tissue samples with intestinal metaplasia samples studied shows that the sialylated mucin known to be secreted by these cells[27] has a similar O-acetylation to that of normal colonic mucin. The colorectal tumours that have been studied so far with this antibody have shown either a complete lack of MMM-17 staining or only patchy staining, thus supporting the reduction in O-acetylation of colon cancer sialomucin that has previously been shown using indirect histochemical techniques.

There is considerable interest in the alterations in sialylation that may occur in mucin and cell surface glycoproteins in malignant disease and there is evidence that such alterations may correlate with altered metastatic potential.[5 28 29] The role of changes in O-acetylation of tumour sialic acids is unknown but deserves further study. Reduction in O-acetylation of sialic acids has been reported in colon cancer,[10 12 14] adenomatous colonic polyps,[14] and in ulcerative colitis,[13 30] but these studies have all relied on indirect histochemical techniques which can sometimes be difficult to interpret. Monoclonal antibodies specific for O-acetylated sialomucins should facilitate further studies of these changes.

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