Sd^a-antigen-like structures carried on core 3 are prominent features of glycans from the mucin of normal human descending colon

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This paper describes structural characterization by NMR, MS and degradative studies of mucin glycans from normal human descending colon obtained freshly at autopsy. The saccharides were mainly based on core 3 (GlcNAcβ1-3GalNAc). Among the terminal saccharide determinants Sd^a/Cad-antigen-like structures were prominent, and Lewis x, sialyl Lewis x and sulphated Lewis x were found as minor components, whereas blood group H and A antigenic determinants were absent. The saccharides were markedly different from those of mucins from colon cancers or colon cancer cell lines analysed so far, in which cores 1 and 2 are prominent features, and in which various other terminal determinants have been found, but not Sd^a/Cad.

Key words: carbohydrate, intestine, Sd^a/Cad antigen.

INTRODUCTION

Mucins constitute a heterogeneous group of usually large glycoproteins characterized by domains containing multiple O-linked glycans linked to serine and/or threonine. Intestinal mucins are mainly produced by goblet cells, but also enterocytes, and occur as soluble secreted forms, as well as membrane-bound forms [1]. The high glycosylation and extended structures give the mucins gel-forming abilities and other general physical properties which have been regarded as having a protective and electrolyte-regulative function at mucosal surfaces [2]. However, mucins also carry exact structural information carried in determinants among their glycans and in the peptide core. These determinants mediate specific binding of antibodies, pathogenic microbes and leucocytes, and may be important in host–pathogen interactions, inflammation and cancer metastasis [3–8].

O-linked glycans can be described in terms of a core and external determinants carried on the core [9]. The core consists of the monosaccharide [usually *N*-acetylgalactosamine (GalNAc)] linked to serine or threonine residues in the protein and the monosaccharide residues next to it; there are at least seven core types, e.g. cores 1 and 2 with galactose (Gal) linked by a β1-3 linkage to GalNAc, and cores 3 and 4 with *N*-acetylglucosamine (GlcNAc) linked by a β1-3 linkage to GalNAc instead. There are also many different external determinants. The external determinants can be combined with different cores in a variety of ways producing a large array of possible mucin glycan structures.

Numerous studies using antibodies and lectins as probes have suggested differences in mucin carbohydrate structures along the gastrointestinal tract, and changes upon malignant transformation [5,9]. In this way, blood group A and Sd^a/Cad determinants have been found in normal mucins but lacking in cancer mucins, whereas T-antigen, sialyl Le a and others have been found to increase in cancer. The different core types, which usually cannot be distinguished by antibody or lectin binding studies, have been indicated by a few chemical studies. Stomach mucin appears to have mainly core-1 and -2 based glycans [10,11], whereas colon mucins have mainly core 3 glycans [12,13]. In colon cancer or colon cancer cell lines an increased presence of core types 1 and 2 have been found with a decreased synthesis of core 3 [5,9].

In a previous study, the application of a combination of MS, NMR spectroscopy and degradative studies revealed an unanticipated array of core-1 and -2 based glycans with multiple sulphated Lewis x determinants in the mucin from the metastatic colon cancer cell line LS174T-HM7 [14]. We have now applied the same strategy on mucin from the descending colon freshly isolated at autopsy of accident victims. Surprisingly, this revealed blood group Sd^a determinants as a prominent feature, including major glycans with new structures having this determinant carried on core 3.

MATERIALS AND METHODS

Tissue

Eight immediate autopsy specimens of descending colon, each approx. 20 cm, were obtained from accident victims by Human Tissue Resources (Department of Pathology, University of Maryland School of Medicine, Baltimore, MA, U.S.A.) and promptly frozen at −80 °C.

Mucin preparation

The tissue specimens were thawed at 4 °C, the mucosa scraped, and the mucin was purified essentially as previously described [15]. Briefly, the mucosa samples were minced, pooled and homogenized twice with a Polytron for 2 min in 0.1 M NH_4HCO_3, 0.5 M NaCl and 0.1 mM PMSF, and centrifuged twice at 45000 ***g*** with intervening removal of floating lipids. Mucin was purified from the last supernatant by a combination of gel filtration, CsCl centrifugation and nuclease digestion.

Release of oligosaccharide alditols from mucin by alkaline/borohydride treatment

The purified mucin (38 mg) was subjected to β-elimination under reducing conditions (20 ml of 0.1 M KOH/1 M KBH_4) for 24 h at 45 °C [16]. The reaction was stopped by addition of ion-

Abbreviations used: Gal, galactose; GalNAc, *N*-acetylgalactosamine; GalNAc-ol, *N*-acetylgalactosaminitol; GlcNAc, *N*-acetylglucosamine; HPAEC, high pH anion-exchange chromatography; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; NeuAc, *N*-acetylneuraminate.

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Table 1 Sulphate content and carbohydrate composition of purified descending colon mucin and released saccharides

Abbreviation used: n.d., not determined.

			Released saccharide fractions†			
		Intact mucin*	All	FN	FI	FII
Sulphate/ carbohydrate	Yield (mg)...	38	9.4	0.94	3.0	3.2
HSO_3		0.10	n.d.	n.d.	n.d.	n.d.
NeuAc		1.20	3.20	–	1.1	2.00
GalNAc		1.05	1.80	0.37	0.35	0.70
Fuc		0.30	0.87	0.28	0.24	0.50
Gal		1.00	3.60	1.00	0.70	1.75
GlcNAc		1.00	3.20	0.78	0.70	1.40
GalNAc-ol		–	1.00	1.00	1.00	1.00
Man		< 0.10	< 0.10	0.30	< 0.10	< 0.10

* Molar ratios with Gal set as 1.
† Molar ratios with GalNAc-ol set as 1.

exchange resin (Dowex 50Wx8, $H+$ form) until the pH reached a value of 5.0. Following filtration, the filtrate was dried under vacuum and boric acid was removed by repeated evaporation with methanol. The mixture of oligosaccharide alditols was further purified by size-exclusion chromatography in 0.5 % acetic acid on a column of Bio-Gel P6 (85 cm × 2 cm; 400 mesh; Bio-Rad Laboratories, Hercules, CA, U.S.A.) eluted at 15 ml/h at 22 °C. The oligosaccharide fractions, detected by UV absorption at 206 nm and eluted at approx. 165 ml, were pooled for further fractionation and structural analysis.

Separation of neutral and acidic fractions by anion-exchange chromatography

The purified oligosaccharide alditols were fractionated on quaternary amine-bonded silica [17] using a 10 μm Micro-Pak-AX 10 column (50 cm × 0.8 cm; Varian, Walnut Creek, CA, U.S.A.) on a Spectra-Physics Model 8700 liquid chromatograph (San Jose, CA, U.S.A.) equipped with a variable wavelength UV detector (LOC Spectro Monitor D; Milton Roy, Riviera Beach, FL, U.S.A.) connected to a Spectra-Physics Model 4100 computing integrator. The solvents and gradients are given in Figure 1. Before further analysis, the acidic fractions, eluted with high salt, were desalted by size-exclusion chromatography on a Bio-Gel P2 column (90 cm × 1.6 cm; 200–400 mesh) using water as the eluent at a flow rate of 8 ml/h.

Fractionation of the neutral oligosaccharide alditol mixture on primary amine-bonded silica

The neutral oligosaccharide fraction was freeze-dried, dissolved in 40 μl of initial solvent (acetonitrile/water, 80:20, v/v) and injected on to a 5 μm Spheri-5 column (250 mm × 46 mm; Brownlee labs, Santa Clara, CA, U.S.A.) equilibrated in initial solvent. The saccharides were eluted with a linear gradient of acetonitrile/water [60:40 (v/v)] for 60 min, followed by isocratic conditions for 10 min. The flow rate was 1 ml/min and the UV absorbance was monitored at 200 nm.

Fractionation of the acidic oligosaccharide alditol mixtures by high pH anion-exchange chromatography (HPAEC)

Acidic fractions FI and FII obtained after anion-exchange chromatography were desalted, dried and dissolved in 200 μl of water, and then fractionated by HPAEC [18] on a Dionex HPLC system equipped with a Model PAD2 pulsed amperometric detector (Dionex Corp., Sunnyvale, CA, U.S.A.). The column used was a Carbopac-PA-100 (4 mm × 250 mm) pellicular anion-exchange column with a guard column (4 mm × 50 mm), and was eluted at 1 ml/min with 100 mM NaOH containing various amounts of NaOAc (see Figure 2). Each fraction collected was immediately neutralized with 30 % (v/v) acetic acid and was then desalted on a Bio-Gel P2 column as described above.

Sulphate analysis

An aliquot of native mucin (50 μg) was hydrolysed with 1 M HCl for 5 h at 100 °C [19], and the amount of sulphate released was measured by HPAEC [18].

Carbohydrate analysis

The carbohydrate compositions of the mucin and oligosaccharide alditol fractions were determined by GLC on a silicone OV 101 capillary column (25 m × 0.32 mm) following methanolysis (0.5

Table 2 Composition analysis and M S of purified fractions

The samples were analysed for carbohydrate composition by hydrolysis and HPLC, and by MALDI–TOF MS. Reactions were analysed in the positive ion mode and the number of H or Na subtracted and/or added in the pseudomolecular ions is indicated.

Fraction	*m/z*	H/Na	HSO_3	NeuAc	GalNAc	Fuc	Gal	GlcNAc	GalNAc-ol
FI-1	559.2	−H+2Na	–	1	–	–	–	–	1
FI-2	1070.1	−H+2Na	–	1	–	1	1	1	1
FI-4	720.6	−2H+Na	–	1	–	–	1	–	1
FI-5	761.8	−2H+Na	–	1	1	–	–	–	1
FI-6	761.8	−2H+Na	–	1	–	–	–	1	1
FI-7	923.6	−2H+Na	–	1	–	–	1	1	1
FI-9 A	923.3	−2H+Na	–	1	–	–	1	1	1
FI-9 B	1126.4	−2H+Na	–	1	1	–	1	1	1
FII-1	1338.3	+Na	–	2	–	1	1	1	1
FII-2	1396.2	+Na	–	2	1	–	1	1	1
FII-3	1193.0	+Na	–	2	–	–	1	1	1
FII-4	1338.2	+Na	–	2	–	1	1	1	1
FII-5	1396.2	+Na	–	2	1	–	1	1	1
FII-6	1127.7	+Na	1	1	–	1	1	1	1
FII-7	982.1	+Na	1	1	–	–	1	1	1

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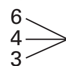
Table 3 ^{1}H-NMR chemical shifts of structural-reporter group protons for monoacidic oligosaccharide alditols of normal distal colonic mucins

◇-ol, GalNAc-ol; □, αFuc; △, αNeuAc; ■, βGal, ●, βGlcNAc; ⬙, βGalNAc; and ◇, αGalNAc. The linkage position is specified by the direction of the connecting bars as follows:



Abbreviation used: n.d., not determined.

		Chemical shift in the compound								
Residue	Proton	FI-1	FI-2	FI-4	FI-5	FI-6	FI-7A	FI-7B	FI-9A	FI-9B
GalNAc-ol	H-2	4.25	4.25	4.38	4.40	4.26	4.26	4.29	4.26	4.29
	H-3	3.84	3.98	4.06	3.87	3.99	3.99	4.01	4.01	4.01
	H-4	3.42	n.d.	3.53	3.59	n.d.	n.d.	3.55	n.d.	n.d.
	H-5	4.02	4.18	4.25	4.08	4.19	4.19	4.15	4.18	4.15
	NAc	2.057	2.034*	2.049	2.042	2.036	2.036	2.039	2.036	2.036
Gal^3	H-1	–	–	4.48	–	–	–	–	–	–
	H-2	–	–	3.57	–	–	–	–	–	–
	H-4	–	–	3.90	–	–	–	–	–	–
GlcNAc^3	H-1	–	4.65	–	–	4.61	4.64	4.62	4.66	4.64
	H-6	–	4.02	–	–	3.94	4.01	4.02	n.d.	n.d.
	NAc	–	2.066	–	–	2.080	2.078	2.080	2.0674	2.075
Gal^{3,3}	H-1	–	–	–	–	–	–	–	4.46	4.56
	H-4	–	–	–	–	–	–	–	3.92	4.10
Gal^{4,3}	H-1	–	4.45	–	–	–	4.47	4.53	–	–
	H-4	–	n.d.	–	–	–	3.93	3.96	–	–
GalNAc^3	H-1	–	–	–	5.09	–	–	–	–	–
	H-2	–	–	–	4.22	–	–	–	–	–
	H-3	–	–	–	3.92	–	–	–	–	–
	H-4	–	–	–	4.04	–	–	–	–	–
	H-5	–	–	–	4.08	–	–	–	–	–
	NAc	–	–	–	2.088	–	–	–	–	–
GalNAc^4	H-1	–	–	–	–	–	–	–	–	4.73
	NAc	–	–	–	–	–	–	–	–	2.021
Fuc^3	H-1	–	5.13	–	–	–	–	–	–	–
	H-5	–	4.82	–	–	–	–	–	–	–
	CH_3	–	1.18	–	–	–	–	–	–	–
NeuAc^3	H-3$_{ax}$	–	–	–	–	–	–	1.80	–	n.d.
	H-3$_{eq}$	–	–	–	–	–	–	2.76	–	2.68
	NAc	–	–	–	–	–	–	2.033	–	2.033
NeuAc^6	H-3$_{ax}$	1.70	1.70	1.70	1.71	1.70	1.70	–	1.70	–
	H-3$_{eq}$	2.73	2.73	2.73	2.73	2.74	2.74	–	2.74	–
	NAc	2.034	2.025*	2.034	2.035	2.033	2.033	–	2.030	–

* Assignments may have to be interchanged.

M HCl/methanol for 24 h at 80 °C), N-reacetylation and trimethylsilylation [20,21].

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS

Molecular masses of oligosaccharide alditols were measured on a MALDI–TOF instrument (Vision 2000; Finnigan-MAT, San Jose, CA, U.S.A) equipped with a 337 nm UV laser. The mass spectra were acquired in reflectron mode under 6 kV accelerating voltage and positive or negative ion detection. The sample was dissolved in water at approx. 100 pmol per μl. Sample solution (2 μl) was mixed with an equal volume of matrix solution [3-aminoquinoline (10 mg/ml) in acetonitrile/water, 80:20, v/v] and sample matrix crystallization was performed as described by Papac et al. [22]. External calibration was done with angiotensin I (molecular mass of 1296.7 Pa; Sigma).

NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, the oligosaccharide alditol fractions were exchanged twice with ^2H_2O at 22 °C and pD = 6.5. After each exchange (1 h) the materials were freeze-dried. Finally each sample was redissolved in 0.5 ml of ^2H_2O (99.96 atom % ^{2}H; CEA-Life Sciences, Paris, France). The samples were analysed at 27 °C on a Bruker DMX-600 600 MHz NMR spectrometer equipped with a triple resonance (^{1}H/^{13}C/^{15}N) self-shielded z-gradient probe head. Chemical shifts are expressed in p.p.m. downfield from sodium 4,4-dimethyl-4-silapentanoate sodium salt, but were measured by reference to internal acetone ($\delta = 2.225$ p.p.m.). The NMR spectra were interpreted by comparison with spectral data published for a large number of O-linked glycans and other relevant saccharides. Overviews are given by Kamerling and Vliegenthart [23] and in Sugabase, a database available on the World Wide Web (http://

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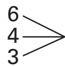
Table 4. ^{1}H-NMR chemical shifts of structural-reporter group protons for diacidic oligosaccharide alditols of normal distal colonic mucins

◇-ol, GalNAc-ol; □, αFuc; △, αNeuAc; ■, βGal; ●, βGlcNAc; ⬙, βGalNAc; and ◇, αGalNAc. The linkage position is specified by the direction of the connecting bars as follows:



Abbreviation used: n.d., not determined.

Residue	Proton	Chemical shift in compound					
		FII-1	FII-2	FII-3	FII-5	FII-6	FII-7
GalNAc-ol	H-2	4.25	4.27	4.27	4.26	4.25	4.26
	H-3	3.99	3.99	3.99	3.99	3.96	3.99
	H-4	3.53	n.d.	3.59	n.d.	3.57	3.58
	H-5	4.18	4.20	4.19	4.18	4.18	4.19
	NAc	2.027*	2.034	2.037	2.033	2.028*	2.034*
GlcNAc[3]	H-1	4.65	4.63	4.63	4.65	4.65	4.63
	H-6	n.d.	4.01	4.02	4.00	4.03	4.01
	NAc	2.066	2.075	2.075	2.069	2.067	2.076
Gal[3,3]	H-1	–	–	–	4.55	–	–
	H-3	–	–	–	4.15	–	–
	H-4	–	–	–	4.11	–	–
Gal[4,3]	H-1	4.51	4.54	4.54	–	4.56	4.58
	H-3	4.10	4.16	4.12	–	4.33	4.34
	H-4	4.04	4.12	3.96	–	4.28	4.30
GalNAc[4]	H-1	–	4.74	–	4.73	–	–
	NAc	–	2.015	–	2.022	–	–
Fuc[3]	H-1	5.13	–	–	–	5.14	–
	H-5	4.81	–	–	–	4.81	–
	CH_3	1.17	–	–	–	1.18	–
NeuAc[3]	H-3_{ax}	1.80	1.93	1.80	1.92	–	–
	H-3_{eq}	2.76	2.66	2.76	2.68	–	–
	NAc	2.036*	2.034	2.033	2.036	–	–
NeuAc[6]	H-3_{ax}	1.70	1.70	1.70	1.70	1.70	1.70
	H-3_{eq}	2.74	2.74	2.74	2.74	2.74	2.74
	NAc	2.033*	2.034	2.033	2.030	2.037*	2.032*

* Assignments may have to be interchanged.

www.boc.chem.uu.nl/sugabase/sugabase.html). Specific pertinent references are given in the Results and Discussion sections.

Permethylation analysis

The oligosaccharides were permethylated as described previously [24]. Acid hydrolysis and identification of partially methylated acetylated methyl glycosides by GLC-MS was performed as described by Fournet et al. [25].

RESULTS

Mucin (38 mg) was isolated from mucosa scrapings by a procedure that included Polytron homogenization, gel filtration, CsCl centrifugation and a final purification by gel filtration again [15]. The mucin contained 4.2 mg of protein and approx. 34 mg of carbohydrate (estimated from a hexose content of 7.4 mg). The recovery of oligosaccharide alditols by base/borohydride treatment followed by ion-exchange chromatography and desalting was 9.4 mg (approx. 28 % of total saccharides). The carbohydrate and sulphate compositions of the intact mucin and the oligosaccharide fractions are shown in Table 1. The amount of mannose was less than 10 % of the amount of Gal in the intact mucin, indicating a low abundance of N-linked glycans.

Overview of fractionation and structural characterization of oligosaccharide alditols

The oligosaccharide alditols were initially fractionated by quaternary amine anion-exchange chromatography (Figure 1) into one neutral (FN) and three acidic fractions (FI–III). Further fractionation of FN by HPLC on amino-bonded silica (results not shown) and FI–III by HPAEC (FI and FII shown in Figure 2a and 2b, FIII not shown) gave multiple subfractions. Fraction FIII was not analysed in detail, since it appeared to contain glycopeptides remaining due to incomplete release of glycans; repeated base/borohydride treatment of this fraction released glycans with the same composition, HPAEC profiles and mass spectra as fractions FI and FII.

The subfractions were collected and subjected to combinations of compositional analysis (Table 1 and results not shown), MALDI–TOF MS (Table 2) and NMR spectroscopy (Tables 3 and 4). The deduced structures are shown in Table 5.

The neutral fraction, FN, (1 mg, approx. 15 % of released glycans) produced two major subfractions (FN-1 and FN-2) with compositions and mass spectra corresponding to the disaccharide GlcNAcGalNAc-ol and the trisaccharide GalGlcNAcGalNAc-ol. Fraction FN was not analysed further. Acidic fractions FI and FII produced nine (FI-1–9) and seven (FII-1–7) subfractions

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Table 5 Proposed structures for glycans of mucin from descending colon

The amount of each glycan was established from the carbohydrate composition analysis of the isolated fractions. For the fractions containing two glycans (FI-7 and FI-9) the relative amounts of the two (A and B) were estimated from the relative intensity of specific signals in the NMR spectra.

Fraction*	Structure	Amount (μg)
FN-1[26,27]†	GlcNAcGalNAc-ol	50
FN-2[27]†	GalGlcNAcGalNAc-ol	220
FI-1[48]	NeuAcα2-6GalNAc-ol	50
FI-2[28,30]	Galβ-4(Fucα1-3)GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	80
FI-4[29]	Galβ1-3(NeuAcα2-6)GalNAc-ol	105
FI-5[29,30]	GalNAcα1-3(NeuAcα2-6)GalNAc-ol	170
FI-6[29,30]	GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	680
FI-7A[28,29]	Galβ1-4GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	210
FI-7B[47]	NeuAcα2-3Galβ1-4GlcNAcβ1-3GalNAc-ol	50
FI-9A‡	NeuAcα2-3Galβ1-3lcNAcβ1-3GalNAc-ol	70
FI-9B‡	GalNAcβ1-4(NeuAcα2-3)Galβ1-3lcNAcβ1-3GalNAc-ol	40
FII-1[30]	NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	170
FII-2‡	GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	720
FII-3[30]	NeuAcα2-3Galβ1-4GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	130
FII-5†	GalNAcβ1-4(NeuAcα2-3)Galβ1-3GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	360
FII-6[30]	HSO_3-3Galβ1-4(Fucα1-3)GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	140
FII-7‡	HSO_3-3Galβ1-4GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol	80

* Examples of reference are given for structures reported previously with NMR spectra. See also Sugabase (http://www.boc.chem.uu.nl/sugabase/sugabase.html).
† Tentative structures based on composition and MS only.
‡ Structures not reported before but strongly supported by composition, MS and NMR analysis in the present syudy.

respectively, and 15 acidic saccharide structures were deduced by detailed analysis of these subfractions (Table 5).

Core structures

Twelve structures (all except three) were based on core 3 (GlcNacβ1-3GalNAc-ol) as previously found by Podolsky [12,13]. This is seen from characteristic NMR signals from *N*-acetylgalactosaminitol (GalNAc-ol) with H-2 and H-5 at $\delta =$ 4.24–4.29 and 4.14–4.19 p.p.m. respectively, and NAc below 2.04 p.p.m. [26,27]. Three minor fractions, FI-1, FI-4 and FI-5, had other core structures easily distinguished from core 3 by characteristic signals from H-2 and H-5 of GalNAc-ol (Table 3), including core 1 (FI-4) and core 5 (FI-5).

Thirteen of the fifteen acidic structures had *N*-acetylneuraminate (NeuAc) linked by an α2-6 linkage to GalNAc-ol. This is evident from signals from NeuAc H-3_{ax} at $\delta = 1.69$–1.70 p.p.m. and from H-3_{eq} at $\delta = 2.73$–2.74 p.p.m. characteristic of the 2-6 linkage. The linkage to GalNAc-ol is shown for the core 3 structures by upfield shifts of GalNAc-ol H-2 to 4.24–4.27 p.p.m. combined with downfield shifts of H-5 to 4.17–4.19 p.p.m., and by downfield shifts of GalNAc-ol H-5 for the other structures.

Thus the main core structure of the colon mucin was GlcNAcβ1-3(NeuAcα2-6)GalNAc, and many structures were built around the tetrasaccharide Galβ1-3/4GlcNAcβ1-3(NeuAcα2-6)GalNAc extended with fucose, NeuAc and/or sulphate residues as previously described [28–30], as well as with GalNAcβ producing the Sd^a/Cad-like structures described below.

Sd^a/Cad-related determinants

The two major subfractions of FII (FII-2 and FII-5) contained structures with blood group Sd^a/Cad-like determinants: GalNAcβ1-4(NeuAcα2-3)Gal. The compositions and mass spectra indicated an additional NeuAc, GalNAc and Gal besides the residues in the sialylated core 3 described above. The NMR spectra of these fractions are shown in Figure 3. Signals from the added sialic acid are at 1.92–1.93 p.p.m. for H-3_{ax} and 2.67–2.68 p.p.m. for H-3_{eq} as found previously for the NeuAcα2-3 in Sd^a/Cad structures [31], but shifted compared with other NeuAcα2-3 residues, e.g. as in FII-3. The H-1 signal at 4.73–4.74 p.p.m. and the strongly upfield-shifted NAc signal at approx. 2.02 p.p.m. are typical of GalNAcβ; for GalNAcβ1-4Gal without NeuAc these signals are at 4.62 and 2.06 p.p.m. respectively [32]. Further evidence for linkage of GalNAc is the characteristic downfield shift of the signal of Gal H-4 to 4.11–4.12 p.p.m.

The two spectra in Figure 3 differ only slightly in signals, indicating that one contained Galβ1-4GlcNAc (from GlcNAc H-1 at 4.625 p.p.m., H-6 at 4.013 p.p.m. and NAc at 2.075 p.p.m.) and that the other contained Galβ1-3GlcNAc (from GlcNAc H-

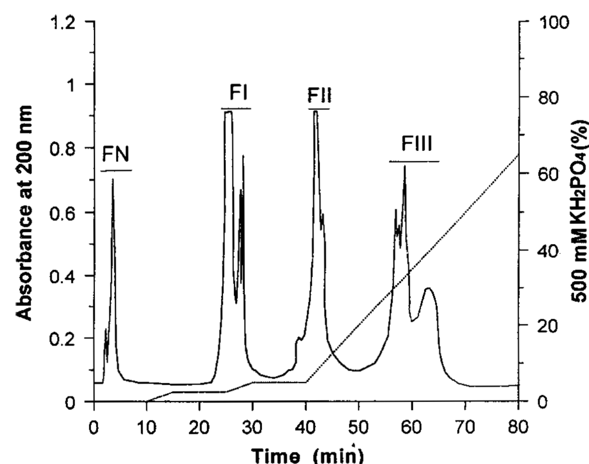

Figure 1 Charge-based fractionation of the oligosaccharide-alditols from colon mucin

The oligosaccharide alditol mixture released from the mucin by β-elimination were loaded on a quaternary amine anion-exchange column equilibrated in water and eluted with solvent containing an increasing percentage of 500 mM KH_2PO_4 (broken line). Fractions corresponding to neutral (FN), monoacidic (FI), diacidic (FII) and triacidic (FIII) saccharides were collected as indicated by the horizontal bars.

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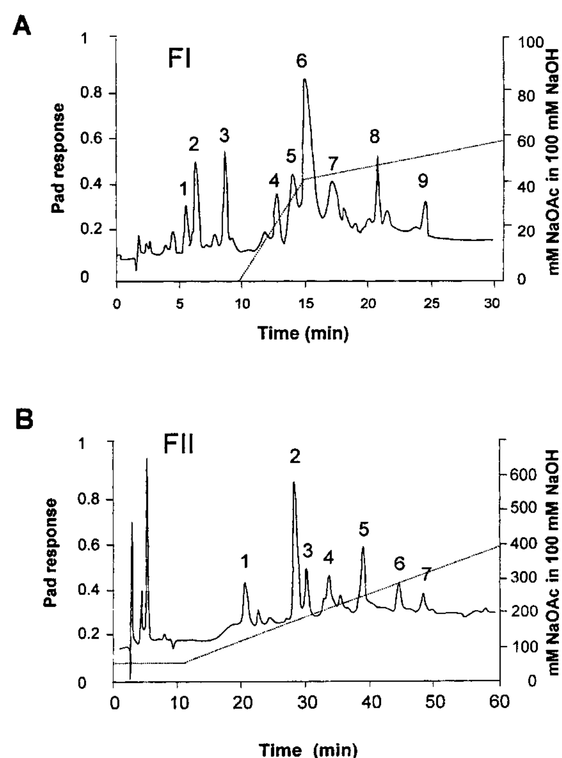

Figure 2 Fractionation of the acidic oligosaccharide fractions from colon mucin

The monoacidic fraction (FI; **a**), and diacidic fraction (FII: **b**) were further fractionated by HPAEC using increasing concentrations of NaOAc in NaOH for elution (broken lines, right-hand scale), and detected by a pulsed amperometric detector (Pad, left-hand scale).

1 at 4.652 p.p.m., H-6 at 3.999 p.p.m. and NAc at 2.069 p.p.m.). Permethylation analysis of FII-2 and FII-5 confirmed the structural assignments by NMR; the production of 3,6diMeGlcNAcNMe from FII-2 and 4,6diMeGlcNAcNMe from FII-5 agrees with the GalI-4 and GalI-3 linkages respectively.

Thus fractions FII-2 and FII-5 both contain structures with Sd^{a}/Cad-like determinants carried on a sialylated core 3 with Gal linked by a β1-4 linkage to GlcNAc for FII-2, and linked by a β1-3 linkage for FII-5. A structure similar to FII-5 (or FII-2), but without the core sialic acid, was indicated in fraction FI-9 (structure B) by similar arguments as given above.

Fucosylated structures: Lewis x, sialyl Lewis x and sulphated Lewis x

Three fractions, FI-2, FII-1 and FII-6, contained one fucose and one Gal besides the sialylated core 3, and two of the fractions, in addition, contained one NeuAc or sulphate (Table 2). In all cases the fucose was linked by an α1-3 linkage to GlcNAc as indicated by H-1 at $\delta = 5.12$–5.14 p.p.m., H-5 at approx. 4.81–4.82 p.p.m. and CH_3 at 1.17–1.18 p.p.m.; this and the linkage of Galβ1-4 to GlcNAc was further supported by signals from Gal and GlcNAc as previously discussed in detail [14].

In FII-1, NeuAc was linked by an α2-3 linkage to Gal, producing a sialyl Lewis x determinant, as indicated by signals from the NeuAc and Gal. In FII-6, sulphate was linked to the 3 position of Gal instead, producing a sulphated Lewis x determinant, as indicated by signals from Gal, especially the strongly downfield-shifted H-3 and H-4 [14].

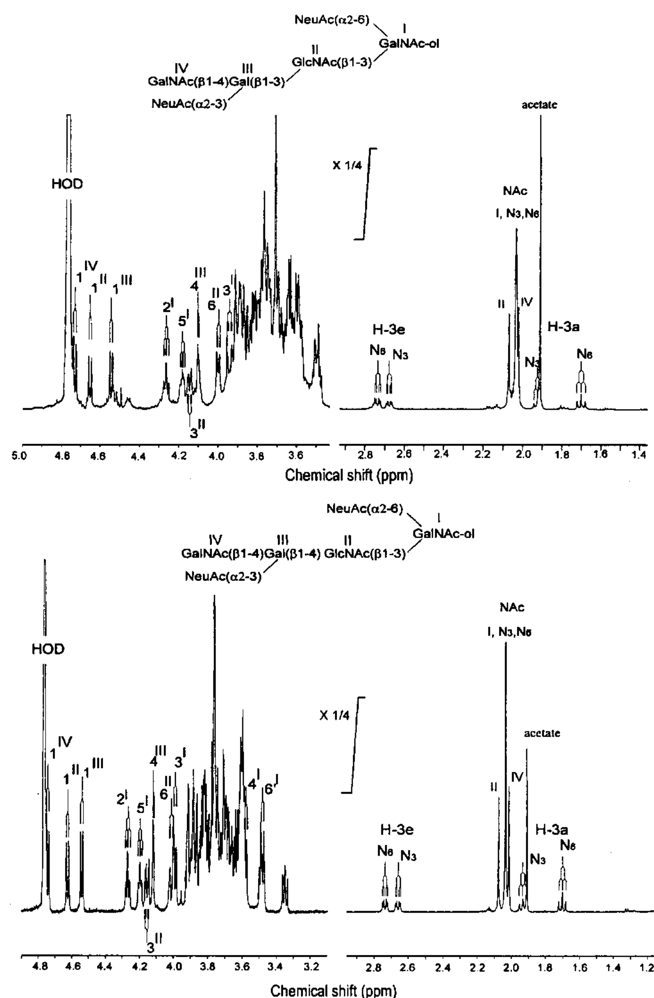

Figure 3 600 MHz ^{1}H-NMR spectra of glycans with Sd^{a}-like determinants

Spectra of fractions FII-5 (top) and FII-2 (bottom) are shown.

Other structures

The remaining structures can be regarded as incomplete versions of those described above and NMR spectra agree with those of previously known structures.

DISCUSSION

In the present paper we provide the first detailed analysis of normal colon mucin glycans by physical methods. The data agree with the only previously published detailed chemical analysis [12,13], in that almost all the structures are based on core 3 (GlcNAcβ1-3GalNAc). However we also demonstrate structures with blood group Sd^{a}/Cad determinants, GalNAcβ1-4(NeuAcα2-3)Galβ, as prominent among the colon mucin glycans, and the presence of Lewis x, sialyl Lewis x and sulphated Lewis x determinants, none of which was found in the previous study.

Podolsky [12,13] instead reported multiple large saccharides (up to decasaccharides) with the core 3 extended by linear and branched poly-*N*-acetyllactosamine-like chains capped by blood group A determinants [GalNAcα1-3(Fucα1-2)Gal] and/or NeuAc. In the present study we found no poly-*N*-acetyllactosamine-like chains, no Fucα1-2, and the only GalNAcα1-3 was found in a minor component, FI-5, as core 5. Some of

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these differences are probably due to the present use of more reliable methodology for structural analysis, such as NMR and MS. Others may be due to differences in the starting material, e.g. the use of different parts of the colon (the descending part of the colon was used in the present study, whereas the colon used was unspecified) by Podolsky; blood group A, B and H antigens have been shown to be present in proximal but absent in distal colon [5]. Although the major (over 90 %) large intestinal mucin, MUCZ, was recently shown to be insoluble without reduction [33,34], the shear force homogenization (Polytron in the present study; sonication by Podolsky and Isselbacher [35]) would have produced soluble fragments from a significant portion of it (I. Carlstedt, personal communication), and probably other mucins as well. The relatively low yield of O-glycans (28 %) is not likely to have selectively distorted the structural pattern since re-release from preserved glycopeptides by a second β-elimination cycle gave the same pattern of glycans. Hence, in all, the glycans analysed are representative of the major mucins of the large intestinal mucosa.

One surprising finding is that the Sd^a blood group antigen determinant is a major structural feature of normal human colon mucin glycans. Previously the Sd^a blood group antigen determinant has been found only as a minor component of glycans on blood cells and in the urine of 92 % of the population [36], except in the rare Cad variant where it is overexpressed on erythrocytes. In the population 4 % of individuals have weak Sd^a reactivity, and another 4 % are Sd^a negative on their erythrocytes and instead have anti-Sd^a in their serum, which is used to detect Sd^a.

The structure of the Sd^a determinant has been determined in Cad erythrocytes, where it is carried on core 1 glycans of e.g. glycophorin [31] and in Tamm–Horsfall glycoprotein of urine where it is carried on N-linked glycans [32]. Immunoreactivity with anti-Sd^a sera requires the full GalNAcβ1-4(NeuAcα2-3)Gal trisaccharide plus some, as yet unknown, feature of the next residue to which it is attached; β1-4 linkage to GlcNAc and β1-3 linkage to GalNAc produces an active structure, but β1-4 linkage to Glc does not.

Glycans, like FII-2 and FII-5 in the present study, with Sd^a/Cad determinants on core 3 have not been described previously. In FII-2 the Sd^a trisaccharide is linked by a β1-4 linkage to GlcNAc, producing a tetrasaccharide determinant similar to that found in the Tamm–Horsfall protein and shown to be active [32]. In FII-5 the Sd^a trisaccharide is bound by a β1-3 linkage to GlcNAc producing a tetrasaccharide previoulsy only found as part of some unusual gangliosides [37,38].

The prominent expression of Sd^a in human large intestine mucosa agrees with previous enzymic and immunohistochemical studies. A GalNAcβ1-4 transferase has been identified that correlates with the expression of the Sd^a antigen and can produce the Sd^a active product from an appropriate precursor, e.g. NeuAcα2-3Galβ1-4GlcNAc [39,40]. The enzyme might be encoded by the Sd^a gene, and would explain the dominant Mendelian inheritance of the Sd^a trait. Using sera from Sd^a negative individuals the antigen has been demonstrated, by immunohistochemistry and immunoabsorption [41], in colon, stomach and kidney. In colon the antigen is associated with brush borders and goblet cells, consistent with its presence in mucins.

Glycans with Sd^a/Cad-like determinants were previously indicated as minor components in large-intestine mucin from two rat strains and as prominent components in small-intestine mucin of one of the rat strains [42]. In the other rat strain the Sd^a/Cad determinants were absent in small-intestine mucin under normal conditions but transiently and strongly induced upon infection with a parasitic worm [43]. Hence, tissue-specific expression of Sd^a/Cad can be determined both by genetic and environmental factors.

Many studies suggest that mucin glycan structures in cancer cells differ from those in normal cells [5,9,44]. The present results provide a normal reference for comparison with structural analysis of colon cancer mucins. The Sd^a antigen and the corresponding GalNAc-transferase activity appear to be lost in colon cancer [39,41]; the few structural studies of mucin glycans from colon cancer or colon cancer cell lines [14,45–47] agree with this, since no Sd^a/Cad determinants were found. Other determinants increase in cancer compared with normal tissue. One is the T-antigen, Galβ1-3GalNAc, present in core 1 and core 2 glycans. The T-antigen was not found in the present analysis, but was detected in colon cancer mucins [44]. The GlcNAc-transferase responsible for core 3 synthesis decreases in activity in colon cancer, and it has been proposed that this gives the opportunity for increased synthesis of cores 1 and 2 [9]. O-acetylated sialic acids, proposed to be abundant in normal colon mucin and to decrease in cancer [39], would not have been observed in the present study since samples were treated with alkali.

In one case, the mucin glycan structures of a cancer cell line, grown as solid tumors in mice, were dramatically, and surprisingly, different from the normal mucin with almost exclusively cores 1 and 2 instead of core 3, and extensions carrying up to three sulphated Lewis x determinants [14]. Not only had the cancer cells shifted to synthesis of core 1 and core 2 instead of core 3, but they also had a quite different programme for the extensions of the core. It was proposed that the very prominent expression of sulphated Lewis x determinants in this mucin was due to selection of the cancer cells from a rare subtype of mucin-producing normal cells. The presence of sulphated Lewis x in normal colon mucin, albeit carried on a different core, supports this hypothesis.

In conclusion, the present finding indicates abundant unique glycan structures in normal descending colon mucin. This adds to the emerging view that mucin glycan structures are tissue specific [9,42] and may even vary between two mucins within the same tissue compartment (e.g. in saliva [7]). The functional implication of this is that different mucins may be recognized by different carbohydrate-binding proteins, such as bacterial adhesins and endogenous lectins (e.g. selectins binding sialyl Lewis x, [4,8]), and as a consequence may play different specific roles in host–pathogen interactions, inflammation and cancer.

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