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Two glycosylation alterations of mouse intestinal mucins due to infection caused by the parasite *Nippostrongylus brasiliensis*

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The glycosylation alterations of mouse small intestinal mucins during a 12-day infectious cycle caused by the parasite *Nippostrongylus brasiliensis* have been studied. The guanidinium chloride insoluble mucins were isolated at day 0 to 12 from the small intestine of infected and non-infected C57BL/6 mice. The *O*-linked oligosaccharides were released by reductive β-elimination from the mucins and separated into neutral, sialylated and sulfated fractions. All fractions were analyzed by monosaccharide composition analysis and the neutral oligosaccharides were structurally characterized by gas chromatography/mass spectrometry. Two oligosaccharides containing blood group H-type epitopes (Fucα1-2Gal-) were transiently expressed with a maximum at day 6. Additional oligosaccharides with the common structure HexNAc-Gal-3GalNAcol were transiently induced with a maximum at day 10. Northern blot analysis on total RNA showed a transient expression at day 4–6 of the *Fut2* gene encoding a Fucα1-2 fucosyltransferase, probably responsible for the detected blood group H-type epitopes. Comparisons with the corresponding infection in rat studied previously, revealed structurally different alterations, although occurring as transient events in both species. Both showed an induced blood group-type transferase halfway through the infection (a blood group A transferase in rat) and an induced transferase adding a terminal GalNAc (to a sialic acid- containing epitope in rat) towards the end of the infection. These differences between closely related species suggest rapid evolutionary alterations in glycosyltransferase expression.

Keywords: **mucin, *O*-glycosylation, oligosaccharides, parasite infection, fucosyltransferase**

Abbreviations: **GalNAcol, *N*-acetylgalactosaminitol; HexNAc, *N*-acetylhexosamine; Hex, hexose; HexNAcol, *N*-acetylhexosaminitol; GC, gas chromatography; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ESI, electrospray ionization.**

Introduction

A mucous layer covers the epithelial cells of the gastrointestinal tract and functions as a barrier against intruding pathogens and at the same time allowing the passage of nutrients. A high turnover rate of both epithelial cells and the mucous gel efficiently removes invading microbes and other foreign substances. Mucins are glycoproteins produced by goblet cells and they comprise the main component of the mucous gel. The carbohydrate part of the mucins consists of *O*-linked oligosaccharides, which constitute 50–80% of the total mucin mass. Mucin oligosaccharides are attached to Ser or Thr of the protein backbone and are together with Pro often found in tandem repeat sequences typical of mucins. These highly glycosylated repeats are referred to as mucin domains and are protease resistant. Thirteen human mucin (MUC) genes have been identified and are fully or partially sequenced, including the recently identified MUC17 [1–3]. In addition, mucins from other species have been identified and sequenced. The major gel forming mucin of the small and large intestine is MUC2 in human and Muc2 in rat and mouse. These mucins form multimers of several MDa size and are recovered in the guanidinium chloride insoluble mucin fraction [4].

Transient alterations in the glycosylation of mucins have been found in rats during an infection caused by the intestine-dwelling parasite *Nippostrongylus brasiliensis* [5,6]. The amount of NeuGc-containing oligosaccharides decreased compared to NeuAc-containing oligosaccharides and the Sd^a/Cad-type blood group antigen (NeuGc(Ac)α2-3(GalNAcβ1-4)Galβ1-) appeared. The *Abo* blood group A glycosyltransferase gene was also transiently induced [6]. This

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suggests that the *N. brasiliensis* infection could be used as a model system for studies on the modulation of glycosylation by the expression of specific glycosyltransferases during an infection. These studies in the rat have now been extended to the mouse. Interestingly, the alterations found were different from the rat as they showed a transient induction of oligosaccharides with fucosylated epitopes linked α1-2, due to the transient induction of the Fut2 fucosyltransferase. A transient induction of an epitope with the core saccharide HexNAc-Gal-3GalNAcol also appeared during the infection cycle.

Materials and methods

Infection of mice by the nematode *N. brasiliensis* and isolation of the small intestinal insoluble mucin complex

C57BL/6 mice (M&B, Denmark) were subcutaneously infected with *N. brasiliensis* third-stage larvae [7]. Two different series of infection experiments were included in this study. For the first infection study (infection 1), 4 mice were included in each group and mucosa was collected at day 0, 2, 4, 6, 8, 10, 12 and 14 of the infection. The day 0 animals were not infected and were used as a control group. For infection 2, 10 mice per group were used and mucosa was collected at day 0, 3, 6, 8, 10 and 12 of the infection. The mice were sacrificed by decapitation after isofluoran anaesthesia and the mucosa from the small intestine was scraped off and the material from each stage of infection was pooled and stored at -20°C. The mucosa was homogenized in 6.0 M guanidinium chloride as described previously [4]. The pellets obtained after centrifugation were extracted 4 times in 6.0 M guanidinium chloride and solubilized by reduction and alkylation [4]. The obtained insoluble mucin fractions (mainly Muc2) were dialyzed against water and lyophilized. The amounts of insoluble mucins were quantified by gravimetry and by suction fixed volumes through a nitrocellulose membrane (0.025 μm) in a slot blot apparatus (Schleicher and Schuell, Dassel, Germany). The membrane was subjected to periodate oxidation followed by staining with the Schiff's reagent [8]. The membrane was captured by a video camera and the intensity quantified by an imaging software (Image Access, MicroMacro, Stockholm, Sweden).

Release and fractionation of oligosaccharides

Oligosaccharides were released from the insoluble mucins (infection 1: 0.5–1 mg, infection 2: 6–20 mg) by reductive β-elimination (1 M sodium borohydride) under alkaline conditions (0.05 M potassium hydroxide) [4]. Collected oligosaccharides were desalted and separated into three subfractions containing neutral, sialylated and sulfated oligosaccharides, respectively [9]. The separation was performed on a DEAE-Sephadex A-25 column and the neutral fraction was eluted with dry methanol. The sialylated oligosaccharide alditols were converted to methyl esters by on-column derivatization of the carboxyl groups by methyl iodide and eluted with dry methanol. The methyl esters were converted to *N*-methyl amides by methylamine in methanol. The sulfated species were eluted with pyridinium acetate and desalted by gel chromatography (Sephadex G-10).

GC, GC-MS, MALDI-TOF-MS and ESI-MS analysis of oligosaccharide fractions

The neutral oligosaccharides were permethylated by methyl iodide under alkaline conditions and analyzed by high-temperature GC and GC-MS [9]. For analysis by capillary GC, a Hewlett-Packard 5890 series II gas chromatograph equipped with an on-column injector was used (hydrogen as carrier gas, linear gas velocity of 75 cm/s). For GC-MS, the same type of GC was interfaced to a JEOL SX-102A mass spectrometer (JEOL, Tokyo, Japan). Fused silica capillary columns coated with 0.03 μm of cross-linked PS264 (Fluka, Buchs, Switzerland), 10 m of length and inner diameters of 0.25 mm were used. The oligosaccharides were dissolved in ethyl acetate (1 μl) and injected on-column at 80°C (1 min). The temperature was raised linearly (10°C/min) to 350°C where it was kept for 5 min. The conditions for the mass spectrometer were: interface temperature, 350°C; ion source temperature, 350°C; electron energy, 70 eV; trap current, 300 μA; acceleration voltage, 10 kV; mass range scanned, m/z 100–1600; total cycle time, 1.8 s; resolution, 1200 ($m/\Delta m$, 10% valley definition); electron multiplier, 1.3 kV.

The sialic acid-containing oligosaccharides were permethylated and analyzed by high-temperature GC and GC-MS [9]. *N*-methyl amide derivatives of sialylated oligosaccharides were analyzed by MALDI-TOF-MS and nano-ESI-MS [10,11]. The desalted sulfated oligosaccharides were analyzed by MALDI-TOF-MS and nano-ESI-MS [10,12].

Monosaccharide composition analysis

Mucin oligosaccharide alditols from the neutral, sialylated and sulfated monosaccharides were prepared and analyzed as described [13]. Sialic acid analysis was performed on the sialylated oligosaccharides, which had been converted to methyl esters. The methyl ester groups were reverted to carboxylic acids by saponification in 0.05 M NaOH (40°C, 2 h) before acidic hydrolysis in 0.125 M HCl (80°C, 1 h). *N*-acetyl neuraminic acid (NeuAc) and *N*-glycolyl neuraminic acid (NeuGc) were analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with muramic acid as internal standard. The column (Carbopac PA10, 4 × 250 mm, Dionex Corp., Sunnyvale, CA, USA) with a guard column (Carbopac PA10, 4 × 50 mm) was eluted with a gradient of 0.1 M sodium hydroxide in 1.0 M sodium acetate/0.1 M sodium hydroxide at a flow of 1.0 ml/min.

Northern blot analysis

Total RNA was purified from mucosal scrapings of a 5-cm section of the central part of the small intestines of

non-infected mice and mice infected by *N. brasiliensis* [14]. The collected mucosa was homogenized in a cold Dounce homogenizer with guanidinium thiocyanate (2.5–6.25 ml) with 1% mercaptoethanol. 10% (w/v) laurylsarcosinate (0.05 volumes) was added, followed by additions of 2 M potassium acetate pH 5.5 (125–315 μl) and 1 M acetic acid (200–500 μl) and the solution was mixed. The RNA was precipitated by addition of room temperature 99.5% EtOH (1.875–4.7 ml) and the samples were shaken and frozen in −20°C for a minimum of two hours. The solutions were centrifuged at 10,000 × g, +4°C, 20 min. The pellets were dissolved in 7.5 M guanidinium chloride with 10 mM dithiothreitol (1.25–3.1 ml). The RNA was precipitated by addition of 2 M potassium acetate pH 5.5 (63–156 μl) and room temperature 99.5% EtOH (0.625–1.6 ml) and frozen in −20°C for a minimum of two hours. Centrifugation and precipitation was repeated once. After the final centrifugation step, the RNA was dissolved in 20 mM EDTA (0.625–1.6 ml). Chloroform:n-butanol (4:1) (1.25–3.1 ml) was added and the samples were shaken on ice for 5 min. The solutions were centrifuged at 2,000 rpm, +4°C, 10 min, to get a two-phase system. The lower phase was extracted twice by addition of 20 mM EDTA (0.5–1.25 ml), 5 min. shaking and centrifuging at 2,000 rpm, +4°C, 10 min. The supernatants from the centrifugation steps were pooled and 3 M sodium acetate pH 7 (0.1 volumes) and room temperature 99.5% EtOH (2.5 volumes) were added and frozen in −20°C for a minimum of two hours. The solution was centrifuged at 10,000 × g, +4°C, 10 min. The pellet was washed in 70% EtOH, centrifuged as above, dried by air and dissolved in RF water (300 μl). Approximately 10 μg of total RNA was electrophoresed, blotted and probed with ^{32}P-labeled Fut2 probe (Rediprime, Amersham Biosciences) as previously described [15]. The Fut2 probe was a 214 bp DNA fragment from mouse Fut2 open reading frame specifically reacting with the Fut2 transcript [16]. The 18 S ribosomal RNA bands (∼1.9 kb) visible on the gel were used as standards for quantification.

Results

Increased amounts of mouse small intestinal mucins

Mice (C57BL/6) were subcutaneously infected with the parasite *N. brasiliensis*. The parasite migrates via the lymphatic system to the lungs, is swallowed and establishes itself in the small intestine, where it starts to lay eggs. The number of goblet cells increases and mucins accumulate within the cells. These are coordinately emptied at day 12–13 and the parasites are expelled [17]. The mucosa was scraped off from the small intestines of infected and non-infected mice from infection series 1 and 2. Day 0 animals were used as normal controls and shown to have similar amounts of mucus, as well as identical *O*-glycans, as observed in several experiments on wild-type C57BL/6 mice [18]. Quantification by gravimetry revealed that the amounts reached their maximum at day 6 and 10, with a dip at day 8 (Figure 1). Mucins insoluble in 6 M guanidinium chloride were partly purified and quantified by gravimetry and periodic acid-Schiff (PAS) staining of mucins (from infection 2) adsorbed to a nitrocellulose membrane. Both these measurements gave a maximum at day 6 and 10 of the infection (Figure 1), showing that the amount of insoluble mucins per intestine followed the same pattern as the amount of mucosa.

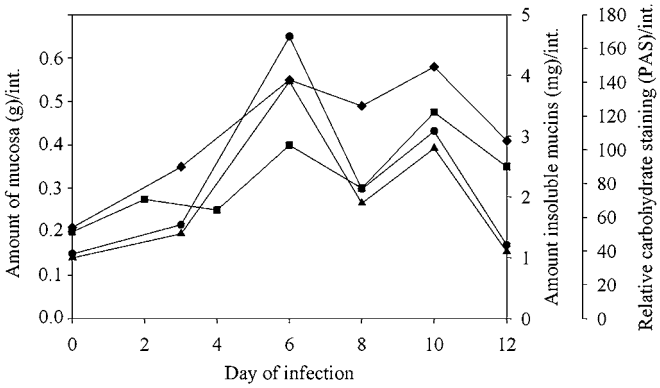

Figure 1. Amount of mucosa and insoluble mucins isolated from the small intestine of C57BL/6 mice during an infection cycle induced by *N. brasiliensis*. The amount of collected mucosa from infection 1 (■) and infection 2 (♦) and partly purified insoluble mucins from infection 2 (▲) were quantified by gravimetry. The relative amount of partly purified insoluble mucins from infection 2 was also quantified by PAS (periodic acid-Schiff) staining of carbohydrates (●). The amounts were calculated per intestine (int.).

Release and analysis of neutral oligosaccharides

To analyze the glycosylation alterations, the *O*-linked oligosaccharides were released from the insoluble mucins collected from mice at different stages of the parasite infection. The released oligosaccharides were separated into three subfractions containing neutral, sialylated and sulfated compounds, respectively, using ion-exchange chromatography [9]. The GalNAc residues at the reducing end were converted to GalNAcol by sodium borohydride after the release. The molar ratios of Fuc, GlcNAc, GalNAc and Gal relative to GalNAcol in each fraction are presented in Table 1. No major alterations of individual monosaccharide levels were detected for any of the subfractions. The average length of the neutral oligosaccharide chains was 4 residues and the molar ratio of oligosaccharide chains of the three subfractions showed that the proportion of neutral chains was not significantly altered during the infection cycle (Figure 2).

The neutral oligosaccharides were permethylated and further analyzed by high-temperature GC and GC-MS. Eight oligosaccharides with up to four sugar residues could be identified (Table 2). The mass spectra were interpreted as previously described, allowing the assignment of not only the oligosaccharide branches attached to the GalNAcol, but also their localization to the C3 or C6 carbons [19,20]. The oligosaccharides were based on both core 1 (Galβ1-3GalNAc-) and core 2 (Galβ1-3(GlcNAcβ1-6)GalNAc-) structures and lactosamine

Table 1. Monosaccharide composition of oligosaccharides released from small intestinal insoluble mucins of non-infected C57BL/6 mice and mice infected with *N. brasiliensis* (infection 2)

	Day 0	*Day 3*	*Day 6*	*Day 8*	*Day 10*	*Day 12*
Neutral oligosaccharides[1,2]						
Fuc per GalNacol[3]	0.2	0.2	0.3	0.3	0.2	0.3
GlcNAc per GalNacol	0.9	0.9	0.7	0.7	0.7	0.7
GalNAc per GalNAcol	0.4	0.4	0.4	0.4	0.4	0.4
Gal per GalNAcol	1.4	1.2	1.2	1.3	1.3	1.5
Average chain length[4]	4.2	4.1	3.8	4.0	4.0	4.6
Monosaccharides (mole%)[5]	38	37	35	38	34	29
Sialylated oligosaccharides[1,2]						
Fuc per GalNacol[3]	1.1	1.5	0.7	0.9	0.9	0.9
GlcNAc per GalNAcol	2.8	2.7	2.2	2.2	2.3	2.1
GalNAc per GalNAcol	2.9	2.7	2.6	2.5	2.4	2.4
Gal per GalNAcol	4.6	5.0	4.7	4.3	4.0	4.0
NeuAc per GalNAcol	0.9	0.8	0.5	2.1	2.2	1.1
Average chain length[4]	13.3	13.7	11.9	13.0	12.7	12.6
Monosaccharides (mole%)[5]	11	8	48	15	13	21
Sulfated oligosaccharides[1,2]						
Fuc per GalNacol[3]	0.5	0.5	0.6	0.6	0.6	0.5
GlcNAc per GalNAcol	2.9	2.5	1.9	2.1	1.9	2.2
GalNAc per GalNAcol	2.2	1.6	1.0	1.5	1.2	1.3
Gal per GalNAcol	5.0	5.0	3.1	4.3	3.6	4.2
Average chain length[4]	12.3	11.3	8.0	10.2	8.8	10.4
Monosaccharides (mole%)[5]	51	54	17	47	53	50

[1]Mean values of two analyses.
[2]Oligosaccharides were subfractionated into neutral, sialylated and sulfated species.
[3]Fucose levels relative to the linkage sugar GalNAcol, which was reduced during oligosaccharide release from the protein backbone. Corresponding calculations were performed for GlcNAc, GalNAc, Gal and NeuAc levels.
[4]Average monosaccharide residue chain length, based upon measured molar amounts of Fuc, GlcNAc, GalNAc, Man and Gal relative to GalNAcol. Addition of NeuAc for sialylated species.
[5]Percentage molar amounts of oligosaccharides distributed in the neutral, sialylated and sulfated fractions.

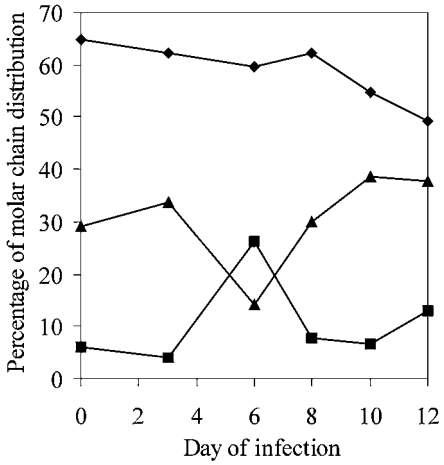

Figure 2. Molar distribution of the small intestinal insoluble C57BL/6 mice mucin oligosaccharide chains between the neutral, sialylated and sulfated subfractions from different stages of the infection. The molar amounts of GalNAcol (one per chain) were calculated from the monosaccharide composition analysis for the neutral fraction (♦), sialylated fraction (■) and sulfated fraction (▲). The results are mean values of two analyses on the oligosaccharides from infection 2.

Table 2. Sequences of neutral oligosaccharides expressed on insoluble mucins purified from small intestine of non-infected C57BL/6 mice and mice infected by *N. brasiliensis*. Structural characterization by GC-MS

No.	*Neutral oligosaccharides[1]*	*Molecular mass[2] (Da)*
N1.1	GalNAcol	307.2
N2.1	Gal-3GalNAcol	511.3
N3.1	Fuc-Gal-3GalNAcol	685.4
N3.2	Gal-3**(GlcNAc-6)**GalNAcol	756.4
N3.3	HexNAc-Gal-3GalNAcol	756.4
N4.1	Fuc-Gal-3**(GlcNAc-6)**GalNAcol	930.5
N4.2	Gal-3**(Gal-4GlcNAc-6)**GalNAcol	960.5
N4.3	HexNAc-Gal-3**(GlcNAc-6)**GalNAcol	1001.5

[1]The saccharides marked in bold are located on C6 of GalNAcol. The following assumptions have been made: hexose residues are Gal, deoxyhexose residues are Fuc, *N*-acetylhexosamine linked to GalNAcol is GlcNAc and *N*-acetylhexosaminitol residues are GalNAcol.
[2]Permethylated oligosaccharides, calculated monoisotopic mass.

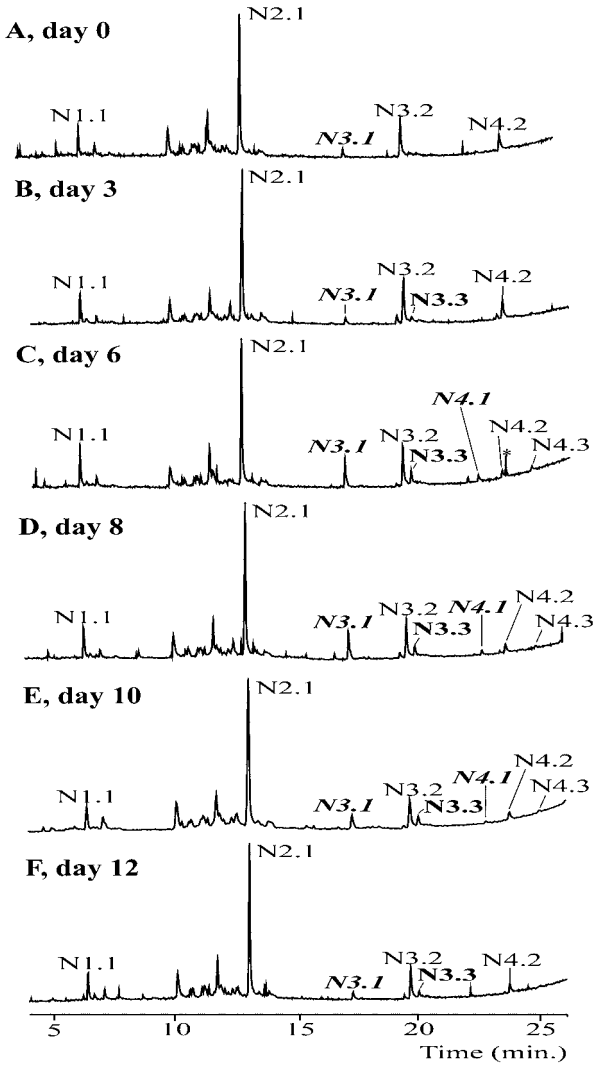

Figure 3. Total ion mass chromatograms from GC-MS analysis of permethylated neutral oligosaccharides released and purified from C57BL/6 mice small intestinal insoluble mucins at different stages of the *N. brasiliensis* infection (infection 2). The oligosaccharides were dissolved in ethyl acetate and injected at 80°C (1 min). The temperature was raised linearly (10°C/min) to 350°C (5 min). Panel A shows oligosaccharides from non-infected mice (day 0) and panel B–F shows oligosaccharides from infected mice on day 3, day 6, day 8, day 10 and day 12 of the infection, respectively. The transiently induced oligosaccharides are marked (***N3.1, N4.1***) and (**N3.3**). The peak marked by * is due to siloxane from the stationary phase.

type 2 chain (Galβ1-4GlcNAc-) attached to C6 of GalNAcol. The profiles of the neutral oligosaccharides as revealed by GC-MS are shown in Figure 3. At a first examination, the pattern of the oligosaccharide peaks appeared rather similar during the infection cycle. However, a more detailed comparison of the chromatograms revealed transient inductions of at least three oligosaccharide components: Fuc-Gal1-3GalNAcol (structure N3.1), HexNAc-Gal1-3GalNAcol (structure N3.3)

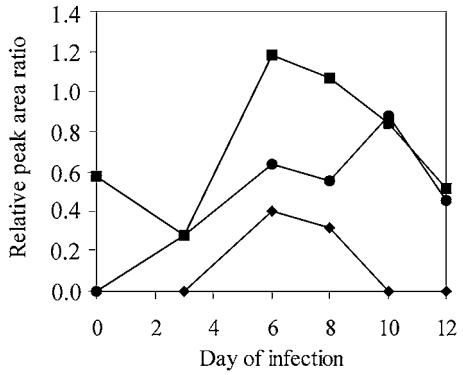

Figure 4. Ratios of the peak area of three induced oligosaccharides and GalNAcol during the parasite infection (infection 2) of C57BL/6 mice small intestine. The peak areas were determined from GC analysis. Fuc-Gal-3GalNAcol (component N3.1 in Figure 3 and Table 2) per GalNAcol (■), HexNAc-Gal-3GalNAcol (component N3.3 in Figure 3 and Table 2) per GalNAcol (●) and Fuc-Gal-3(GlcNAc-6)GalNAcol (component N4.1 in Figure 3 and Table 2) per GalNAcol (♦).

and Fuc-Gal1-3(GlcNAc-6)GalNAcol (structure N4.1). Another HexNAc-containing oligosaccharide, HexNAc-Gal1-3(GlcNAc-6)GalNAcol (structure N4.3) also appeared to be induced during the infection cycle, although it was present in small amounts. The relative induction levels were calculated as the quote of the peak areas for three of these oligosaccharides to that of GalNAcol at each stage of the infection (Figure 4). The two Fuc-containing oligosaccharides (N3.1 and N4.1) had maximal expression levels at day 6–8, whereas the HexNAc-terminating one (N3.3) had the highest expression at day 10 of infection.

The general fragmentation features of the permethylated oligosaccharides analyzed are illustrated by the mass spectrum of the Gal-3(Gal-4GlcNAc-6)GalNAcol saccharide (structure N4.2) in Figure 5A. The identity of the N4.2 compound was deduced from the B_i fragment ions at *m/z* 219 (terminal Hex-) and *m/z* 464 (Hex-HexNAc-). The ion at *m/z* 422 arises from a diagnostic C4-C5 cleavage of the GalNAcol chain, while the *m/z* 725 ion arises from the cleavage of the Hex residue at C3 from the GalNAcol chain when GalNAcol is substituted with a Hex-HexNAc sequence at C6. An intense fragment ion at *m/z* 182 is diagnostic for a type 2 chain (Galβ1-4GlcNAc-) or a terminal GlcNAc, i.e. a free C3 of a GlcNAc [4,20,21]. This fragment ion is the most intense one of N4.2, strongly suggesting that the C6 branch is a type 2 chain.

The mass spectrum of the induced oligosaccharide HexNAc-Gal-3GalNAcol (structure N3.3) is presented in Figure 5B. The fragment ion at *m/z* 667 originates from the C4-C5 cleavage of the GalNAcol chain with a C3 substituted HexNAc-Hex- as suggested by the *m/z* 260 ion from a terminal HexNAc. The *m/z* 182 ion is relatively small in this spectrum, indicating that N3.3 is more likely to have a terminal GalNAc than a terminal GlcNAc, according to the discussion above.

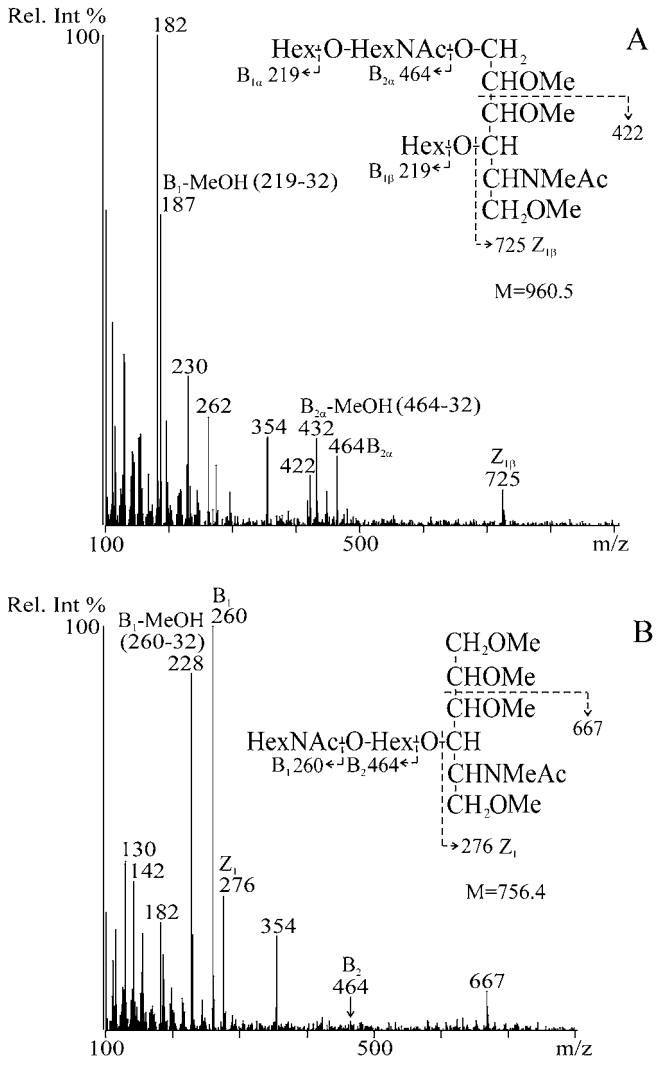

Figure 5. Mass spectra of permethylated oligosaccharides from GC-MS analysis. (A) The oligosaccharide Gal-3(Gal-4GlcNAc-6)GalNAcol from the analysis of oligosaccharides of non-infected C57BL/6 mice (day 0, infection 2). The spectrum is from the component detected at 23.9 min. (N4.2) in the chromatogram of Figure 3A (sequence N4.2 in Table 2). (B) The oligosaccharide HexNAc-Gal-3GalNAcol from the analysis of oligosaccharides of infected C57BL/6 mice (day 6, infection 2). The spectrum is from the component detected at 19.5 min. (N3.3) in the chromatogram of Figure 3C (sequence N3.3 in Table 2). Rel. Int %, relative intensity in % of the most intense ion.

Analysis of sialylated and sulfated oligosaccharides

The monosaccharide composition analysis of the sialic acid- and sulfate-containing oligosaccharides displayed the typical mucin oligosaccharides Fuc, GlcNAc, GalNAc and Gal, for which the molar ratios relative to GalNAcol were calculated (Table 1). The molar ratios of NeuAc to GalNAcol were calculated for the sialylated oligosaccharides (Table 1). No significant alterations of monosaccharide levels were detected for the sialylated and sulfated oligosaccharides. The average chain lengths for the sialylated and sulfated glycan chains were approximately 13 and 10 residues respectively, with some alterations during the infection cycle (Table 1). The sulfated oligosaccharides were normally more abundant than the sialylated ones during the infection. However, in the middle of the infection at day 6, there was a shift and the sialylated chains dominated over the sulfated ones (Figure 2 and Table 1). The structures of the sialic acid and sulfate-containing oligosaccharides were not determined due to small amounts and more importantly their large sizes and thus high heterogeneity.

Northern blot analysis of fucosyltransferase Fut2

The enzymatic background of the induced fucosylation was addressed by northern blot analysis with a specific probe against the murine α1,2-fucosyltransferase Fut2 [16] (Figure 6). This α1,2-fucosyltransferase catalyzes the transfer of Fuc to the terminal Gal residue of a glycoconjugate and is thus a likely candidate for the final biosynthesis of the structures N3.1 and N4.1. The specific Fut2 probe hybridized to an mRNA transcript with an estimated size of 3.3 kb, which is the reported size of the Fut2 gene [16]. The strongest band was observed on day 6 of the infection, while day 4 and 8 showed a weaker binding of the Fut2 probe to the RNA. Day 10 gave a very weak signal and day 0 and 12 showed no binding at all. Comparison of the rRNA 18 S bands revealed that approximately the same amounts of total RNA was analyzed, with slightly higher amounts of the day 8 and 12. These results indicated a transient induction of the Fut2 fucosyltransferase during the parasite infection cycle. This was in accordance with the upregulation of oligosaccharides carrying the blood group H-type epitope as revealed by GC-MS. The Fut2 fucosyltransferase is therefore suggested to be involved in the induction of blood group H-type epitopes during the parasite infection.

Discussion

The glycosylation pattern of the neutral oligosaccharides of mouse mucins have been investigated during an infection caused by the parasite *N. brasiliensis* and several glycosylation alterations were found. Two oligosaccharides carrying a terminal blood group H-type epitope were transiently induced and the Fut2 fucosyltransferase is suggested to be responsible for this upregulation. Additional oligosaccharides with the common structure HexNAc-Gal-3GalNAcol were also transiently induced during the infection cycle.

The *N. brasiliensis* infection model has been used for studies of mucosal dynamics in the intestinal tract of mice and rats. We have previously reported increased amounts of the mucins and the Muc2 mucin in rats infected with *N. brasiliensis* [5,6]. In those studies, the amounts of purified mucins peaked at day 12, just before expulsion of the parasite at day 13, whereas this study showed a bimodal pattern with maximum peaks of mucosa and insoluble mucins at both day 6 and 10 of the infection. The majority of parasites were probably expelled a

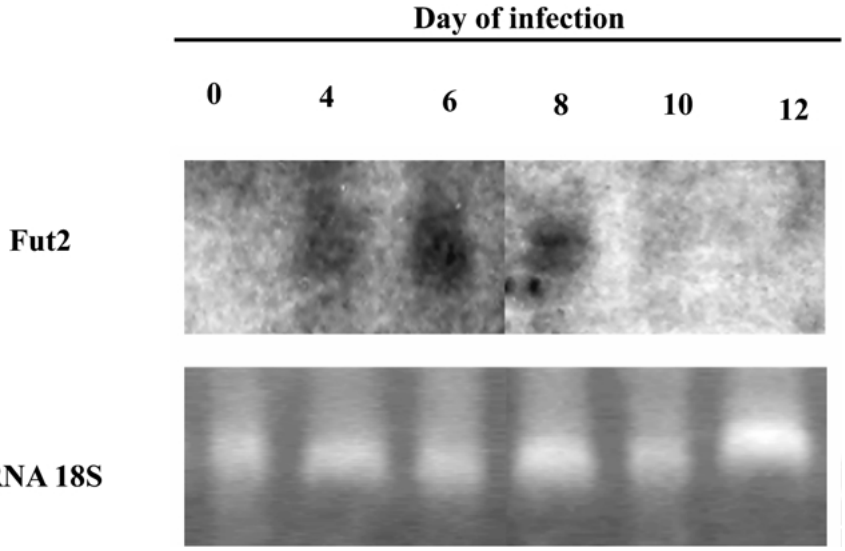

Figure 6. Northern blot analysis of the expression of the Fut2 transcript in C57BL/6 mice small intestinal tissue during the parasite infection. Total RNA (10 μg) isolated from mice small intestinal mucosa from different stages of the parasite infection were separated by agarose gel electrophoresis, blotted and probed with a 214 bp long ^{32}P-labeled Fut2 probe. Day 0, 4 and 6 RNA from infection 1. Day 8, 10 and 12 RNA from infection 2. One single transcript with an estimated size of 3.3 kb was detected.

little earlier in the C57BL/6 mice compared to the rats as the mucin amount and glycosylation alterations were almost back to normal at day 12 and the mice had fully recovered at day 14 (not shown). In the previous rat study, individual rats could be analyzed due to their larger size, but the amount of mucosa obtained from an individual mouse was not sufficient. Due to this, up to 10 mice were used in each group and the mucosa samples had to be pooled. This is less of a problem since inbred mice were used which eliminates genetic variability in between the animals.

O-linked oligosaccharides from purified insoluble mucins were released by alkaline hydrolysis and structurally characterized. It was possible to resolve eight neutral oligosaccharide peaks and their structures were determined by GC-MS. The core structures of the oligosaccharides were core 1 (Galβ1-3GalNAc-) and core 2 (Galβ1-3(GlcNAcβ1-6)GalNAc-). The single GalNAcol together with the disaccharide Gal-3GalNAcol and the trisaccharide Gal-3(GlcNAc-6)GalNAcol were the most abundant neutral species. All the identified compounds have previously been described in mouse [18].

Two specific glycosylation alterations were revealed in the infected mice. The first one, an induced fucosylation of the mouse mucins, was demonstrated by the structural characterization of mucin oligosaccharides by high-temperature GC-MS. The mass spectra showed that Fuc was linked to Hex, assumed to be Gal from the sugar analyses. This combined information suggested the following two structures: Fuc-Galβ1-3GalNAcol and Fuc-Galβ1-3(GlcNAc-6)GalNAcol. These epitopes have a sequence similar to the blood group H epitope, suggesting that the Fuc could be linked Fucα1-2 to the Gal. The expression of these epitopes peaked at day 6–8 as revealed by GC-MS (Figure 4). Mice have been shown to have three different Fucα1-2 transferases, all with distinct tissue-specific expression patterns [16]. Out of these, the Fut2 enzyme is the most likely candidate as it is similar to the human secretor (FUT2) enzyme present in the intestine. Northern blot analysis with a probe specific to the murine Fut2 fucosyltransferase revealed that the Fut2 enzyme is transiently upregulated during the *N. brasiliensis* infection. The suggestion that the Fut2 enzyme was responsible for the fucosylation found in this study is in accordance with the predicted specificity for the mouse Fut2 glycosyltransferase [16]. The Fut2 message was first detected at day 4 and the expression peaked at day 6. This is earlier than the product was found, a phenomenon also observed in the rat study [6] where the expression of the blood group A glycosyltransferase peaked 3–4 days before the maximum levels of blood group A epitopes found on the mucins. This is probably due to the storage of the gel-forming mucins in the goblet cells and their slow turnover [22].

The second induced oligosaccharide epitope HexNAc-Gal was found on two oligosaccharide sequences; HexNAc-Gal1-3GalNAcol (N3.3) and HexNAc-Gal1-3(GlcNAc1-6)-GalNAcol (N4.3). Both these spectra had only a small *m*/*z* 182 ion peak (shown for N3.3 in Figure 5B) which indicates that the HexNAc- is not a GlcNAc, as this ion normally is the base peak of spectra from compounds with terminal GlcNAc [4,20,21]. One could thus suggest that the terminal HexNAc is a GalNAc. The *Helix Pomatia* lectin, reacting with terminal α-GalNAc, had its maximum reactivity with the purified mucins at day 6, before the peak of the HexNAc-Gal epitope (not shown). This suggests that the potential terminal GalNAc was not attached via an α-linkage, but instead via a β-linkage. This is very interesting as a corresponding glycan epitope (NeuAcα2-3(GalNAcβ1-4)-Galβ1-3GalNAcol), in this case sialylated, was found to be

induced on rat mucins during a *N. brasiliensis* infection [5]. One could thus suggest that the epitope induced in mice has the corresponding structure, but lacking sialic acid; GalNAcβ1-4Galβ1-3GalNAcol. The glycosyltransferase responsible for the addition of the GalNAcβ1-4 sugar was not identified in the rat, nor has it been possible to perform any experiment to identify the enzyme responsible for the biosynthesis of the mouse epitope as none of these enzymes have been cloned and characterized.

Glycosylation alterations could be part of defense mechanisms against infection at mucosal surfaces. Altered fucosylation of intestinal glycoconjugates of germ-free mice exposed to specific intestinal bacteria have raised the hypothesis that the microbes are directly involved in the up-regulation of glycosyltranferases, supposedly for their own benefit [23]. Another theory is that glycosylation alterations can be part of a general defense mechanism utilized by the host and affecting mucins and other glycoconjugates present in the intestinal mucosa. The latter general defense hypothesis is supported by the observed binding of pathogens to glycans [24] and the large diversity of glycan structures between species and individuals [25]. The evolution and biological role of this diversity for the protection of a species have been discussed previously [26]. Other infection models have shown the same phenomenon with induced fucosylation in mice small intestine, also pointing towards a general response mechanism [27,28]. The induced amount of mucosa and insoluble mucins and the transient inductions of different oligosaccharide epitopes could all be parts of a preprogrammed response to an intestinal infection.

When comparing the results from the studies of the transient glycosylation alterations caused by the *N. brasiliensis* infection in rat and mouse [5,6], remarkable parallels are found. The first event occurring about halfway through the infection was the formation of blood group-type epitopes by the addition of an α-GalNAc residue in the rat and an α-Fuc in the mouse. Rats carry blood group H epitopes that are thus converted to A, whereas the mouse have precursor β-Gal epitopes that are converted to blood group H. The enzymes induced, the α-GalNAc transferase encoded by the *Abo* gene [6] and the α-fucosyltransferase encoded by the *Fut2* gene are different, but there seems to be a common denominator in their regulation. The second event in the rat is the addition of a GalNAcβ1-4 residue to a sialylated epitope creating the Sd^a/Cad-like epitope NeuAc(Gc)α2-3(GalNAcβ1-4)Galβ1-3GalNAcol. The formation of this epitope is similar to the appearance of the GalNAc-Gal1-3GalNAcol epitope in the mouse as described here. Both these epitopes appear late in the infection with a peak at day 12 for the rat and at day 10 for the mouse.

To summarize, there are interesting similarities, but also distinct differences, in the transient alterations of mucin glycosylation in mouse and rat. This suggests that there are common reasons and mechanisms behind these spatial and infection triggered events, suggesting an evolutionary advantage for the host. The reasons for this are currently not understood.

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