Identification of transient glycosylation alterations of sialylated mucin oligosaccharides during infection by the rat intestinal parasite *Nippostrongylus brasiliensis*

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The sialylation of the oligosaccharides from small-intestinal mucins during a 13-day infectious cycle was studied in Sprague–Dawley rats with the parasite *Nippostrongylus brasiliensis*. Sialic acid analysis and release, permethylation and analysis by GC-MS of the sialylated oligosaccharides isolated from the 'insoluble' mucin complex revealed a relative decrease (4–7-fold) of *N*-glycolylneuraminic acid compared with *N*-acetylneuraminic acid just before parasite expulsion. Northern blots showed that this effect was due to the decreased expression of a hydroxylase converting CMP-*N*-acetylneuraminic acid into CMP-*N*-glycolylneuraminic acid. Analysis of other rat strains showed that this parasite infection also caused the same effect in these animals. Detailed analysis of infected Sprague–Dawley rats revealed four sialylated oligosaccharides not found in the uninfected animals. These new oligosaccharides were characterized in detail and all shown to contain the trisaccharide epitope NeuAc/NeuGcα2-3(GalNAcβ1-4)Galβ1 (where NeuGc is *N*-glycolyl neuraminic acid). This epitope is similar to the Sd^a- and Cad-type blood-group antigens and suggests that the infection causes the induction of a GalNAcβ1-4 glycosyltransferase. This model for an intestinal infection suggests that the glycosylation of intestinal mucins is a dynamic process being modulated by the expression of specific enzymes during an infection process.

Key words: O-glycosylation, mass spectrometry, Muc2, parasite infection, sialic acid.

INTRODUCTION

The gastrointestinal tract is covered by protective mucus that must fulfil many requirements in order to be an effective barrier against the hostile milieu in the lumen of this tract, but at the same time allow nutrients and other smaller molecules to traverse it. The underlying epithelial cells are a target for infecting microbes and viruses, and in this respect the mucus serves at least as a physical barrier. Continuous damaging of the mucosal layer demands a system with constant renewal of the mucus in order to maintain the protective layer. In the intestine, the majority of the mucus is produced by the goblet cells interspersed between the enterocytes.

The protein matrix in the mucus is made up mainly of mucins. These are highly glycosylated proteins, with up to 80% of their weight being from O-linked oligosaccharides, i.e. oligosaccharides linked via GalNAc to serine or threonine. The elongation of the GalNAc, on both C-3 and C-6, with galactose, GlcNAc, fucose, sialic acid and sulphate groups gives enormous potential variation to mucin glycosylation (reviewed in [1]).

Cloning and sequencing of mucin genes have revealed that the potential sites for glycosylation are located in long sequences rich in the amino acids serine and threonine together with proline. These 'mucin domains' are now considered as a prerequisite for identifying a protein as a mucin or a mucin-like molecule. Up until now, nine human mucin genes have been identified and fully or partly sequenced ([2,3] and reviewed in [4,5]), in addition to mucins identified from other species. The gel-forming property of some of these mucins is believed to be due to their capability of forming intermolecular disulphide bridges. Indeed, studies of the porcine submaxillary mucin have revealed that the organization of cysteines, localized in their N- and C-termini, allows for multimerization of mucin subunits [6]. Among those gel-forming mucins are the human MUC2 and rat Muc2 found as 'insoluble' mucin complexes in the large and small intestine [7–10].

The glycosylation of mucins and underlying epithelial-surface glycosphingolipids and glycoproteins may be the target for adhesins found on microbes and it has been shown that bacteria can interact with glycoconjugates (reviewed in [11]). However, only a few examples have been shown illustrating that microbes are actually capable of altering the glycosylation in such an interplay [12]. Altered glycosylation could be of benefit for an invading microbe in order to create a more effective invasion, could be a response from the host to circumvent the infection, or it could be a general infectious response with no function in one specific infection. Lectin-binding assays have indicated the induction of glycosylation alterations in the small-intestinal mucus of rats upon infection with the intestine-dwelling parasite *Nippostrongylus brasiliensis*. The alterations coincide with the expulsion of the parasite from its host [13,14]. In particular, the lectin from *Helix pomatia* binding to terminal GalNAc residues

Abbreviations used: gpA, mucin glycopeptide A; Hex, hexose; HexNAc, *N*-acetylhexosamine; HexNAcol, *N*-acetylhexosaminitol; GalNAcol, *N*-acetylgalactosaminitol; Me, methyl; NeuGc, *N*-glycolyl neuraminic acid; 1D, one-dimensional; ROE, rotating-frame Overhauser effect; ROESY, ROE-correlated spectroscopy; HPAEC-PAD, high-performance anion-exchange chromatography-pulsed amperometric detection.

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has been shown to react strongly. Additional experiments have been done to explore the mechanism of this alteration using hypothymic rats [15].

In this report the alterations of sialylated mucin oligosaccharides from the *N. brasiliensis* infection of rats is explored. Evidence presented suggests that the alterations found are due to an altered glycosylation of the rat intestinal Muc2 mucin. Furthermore, these alterations are suggested to be due to changes in the glycosylation machinery by the regulation of specific enzymes.

MATERIALS AND METHODS

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

Inbred GOT-W, GOT-BW (from the Department of Medical Biochemistry, Göteborg University, Gothenburg, Sweden), WFU and WKY rats (Möllegaard, Skensved, Denmark), or outbred Sprague–Dawley rats (300–350 g) were infected subcutaneously with *N. brasiliensis* third-stage larvae [16]. Rats were killed by decapitation after ether anaesthesia and the mucosa from the small intestine were collected and stored at −80 °C or immediately homogenized in 6.0 M guanidinium chloride as described in [8]. The pellets collected after centrifugation were extracted repeatedly (2–4 times) in the above buffer and solubilized by reduction and alkylation [8]. These samples, referred to as 'insoluble' mucins, were dialysed against water and lyophilized.

Isolation of the rat Muc2 mucin domains

Mucin domains from rat Muc2 were isolated from the insoluble mucins (see above and [8]). Briefly, the insoluble mucins were digested with RNase and DNase, trypsin digested and the void-volume fractions were collected from Sephacryl S-200 gel filtration followed by separation into two mucin domains, gpA (mucin glycopeptide A, larger Muc2 mucin domain) and gpB (smaller Muc2 mucin domain), on Sephacryl S-500. The eluate was detected by PAS (periodic acid–Schiff) staining of glycopeptides absorbed on nitrocellulose membranes [17].

Immunochemical detection of oligosaccharide epitopes and apomucin

The isolated gpA (50 ng) was coated on to microtitre plates and washed and blocked as described in [18]. The primary antibodies used (100 μl) were the monoclonal antibodies anti-blood group H and anti-blood group A (both diluted 1:50; both from Dako, Copenhagen, Denmark). Lectin-binding assay was performed using 1 μg/ml biotinylated *Helix pomatia* lectin (Sigma). For immunochemical detection of Muc2, gpA (0.4 μg) or insoluble mucins (1–5 μg) were coated on to microtitre plates by slow evaporation of a water solution at 37 °C for 12 h. The plates were dried further in a dessicator for 2 h, and treated with gaseous hydrogen fluoride in a hydrogen fluoride apparatus (Peptide Institute, Tokyo, Japan) for 18 h at room temperature. The samples were recoated in their original wells by adding 100 μl of PBS and incubating for 24 h at 37 °C. Washing and blocking was performed as described above, followed by incubation for 2 h with α-gpA rabbit polyclonal antiserum (diluted 1:100) [8]. Fluoroimmunoassays and lectin assay were performed as described in [18] using secondary goat anti-rabbit antiserum and goat anti-mouse antiserum (Jackson Immunoresearch, West Grove, PA, U.S.A.), or streptavidin, all labelled with europium.

Release and fractionation of mucin oligosaccharides

Oligosaccharides were released from the insoluble mucins (5 mg) or from mucin glycopeptides (1–60 mg), using 0.05 M KOH containing 1.0 M $NaBH_4$ [8]. Collected oligosaccharides were separated into three fractions containing the neutral, sialylated and sulphated species as described in [19]. In short, oligosaccharides were applied to DEAE-Sephadex A-25 and the neutral oligosaccharide alditols eluted with methanol. The sialylated oligosaccharide alditols, either from a total mixture of mucin oligosaccharides, or from an acidic oligosaccharide pool, were derivatized to methyl esters by the repeated addition of DMSO/iodomethane, 5:1, and subsequently eluted by adding methanol. The sialylated species were converted into *N*-methyl amides using methylamine in methanol [19].

Isolation of individual sialylated oligosaccharides

The reduced and alkylated insoluble mucin complexes of six GOT-W rats from day 12 of the infection were digested with trypsin and subjected to Sephacryl S-200 chromatography in 4.0 M guanidinium chloride [8], and the material in the void volume was pooled and freeze-dried. Oligosaccharides were released and the sialylated fraction was isolated as their *N*-methyl amides as described above. The oligosaccharides were separated on a Lichrosorb-NH 5-μm column (25 cm × 4.6 mm; Hichrom, Reading, U.K.) using a linear gradient from 80% acetonitrile (Lab-Scan, Dublin, Ireland)/20% water (Fisons, Loughborough, U.K.) to 60% acetonitrile/40% water for 80 min (1.0 ml/min). Eluted oligosaccharides were detected by UV absorption (206 nm) and fractions were collected manually. Further purification was performed using a Kromasil KR 100-5 C_{18} column (25 cm × 4.6 mm; Hichrom) eluted isocratically with water (1 ml/min).

GC and GC-MS of permethylated oligosaccharides

Permethylation of the oligosaccharides was done using methyl iodide and NaOH in DMSO [19,20]. GC and GC-MS were performed using columns of 11–12 m (0.25 mm inner diameter) with 0.02–0.04 μm of cross-linked PS264 (Fluka, Buchs, Switzerland) and the temperature programme and parameters as described previously [19].

Linkage analysis using partially methylated alditol acetates

After permethylation the isolated oligosaccharides were subjected to acetolysis/hydrolysis, $NaBH_4$ reduction and acetylation [21], and analysed by GC-MS using a Hewlett-Packard 5890 Series II gas chromatograph connected to a Hewlett-Packard 5972 mass spectrometer. The samples were dissolved in 10 μl of ethyl acetate and 1 μl was introduced by splitless injection at 250 °C on to a HP-5MS column (30 m × 0.25 mm, 0.25 μm stationary film; Hewlett-Packard). The temperature was raised linearly from 70 °C (kept for 2 min) at 50 °C/min up to 180 °C followed by 8 °C/min up to 270 °C (kept for 8 min). Helium was used as the carrier gas (1.2 bar, constant flow of 50 cm/s at 70 °C). Retention times and mass spectra were compared with partially methylated alditol acetates made from purchased standards or oligosaccharides isolated in our department.

Monosaccharide composition analysis

Neutral monosaccharides from mucin oligosaccharide alditols were analysed as described in [22]. Sialic acids were determined after acidic hydrolysis in 100 μl of 0.1 M HCl at 80 °C by high-performance anion-exchange chromatography-pulsed ampero-

metric detection (HPAEC-PAD), using muramic acid as an internal standard. The column [CarboPac PA1 (4 × 250 mm; Dionex Corp., Sunnyvale, CA, U.S.A.) with a guard column CarboPac PA1 (4 × 50 mm)] was eluted isocratically at 1.0 ml/min using 0.05 M NaOH (from 50 % solution; J. T. Baker, Phillipsburg, NJ, U.S.A.) containing 0.1 M sodium acetate. Analysis of sialic acids from sialylated oligosaccharide methyl esters was preceded by saponification in 0.1 M NaOH (50 °C for 2 h) followed by the acidic hydrolysis and HPAEC-PAD as described above.

Proton-NMR spectroscopy

Deuterium-exchanged *N*-methylamide derivatives of isolated sialylated structures were dissolved in ^2H_2O (99.98 %; Glaser) and analysed at 25 °C by proton-NMR spectroscopy on an 11.7 T (500 MHz) JEOL Alpha spectrometer (JEOL, Tokyo, Japan) equipped with a pulsed-field gradient unit. Chemical shifts were given relative to sodium 4,4-dimethyl-4-silopentane-1-sulphonate, using internal acetone ($\delta = 2.225$ p.p.m.) as a reference. Assignments were performed using standard single-pulse one-dimensional (1D) experiments and two-dimensional homonuclear scalar-correlated spectroscopy (COSY) and nuclear Overhauser effect-correlated spectroscopy (NOESY) experiments with and without pulsed-field gradient support for coherence selection. The positional sequence of monosaccharides was determined from reference samples and nuclear Overhauser enhancements in the rotating frame (ROE) measured with a selective (Gaussian-shaped) 1D ROE-correlated spectroscopy (ROESY) experiment.

Northern-blot analysis

Preparation of mRNA was performed as described previously [9], from a 20-cm section of the proximal part of the small intestine from infected Sprague–Dawley rats. The mRNA was electrophoresed, blotted and probed with mouse CMP-NeuAc hydroxylase DNA [23]. This 1.7-kb DNA probe was ^{32}P-labelled using the Megaprime DNA-labelling system from Amersham Pharmacia Biotech. Hybridizing bands were visualized using autoradiography. After removal of the CMP-NeuAc hydroxylase probe, the membrane was also probed with β-actin DNA.

RESULTS

The effect of the *N. brasiliensis* infection on the expression of intestinal mucins

To examine what effect a mucosal infection has on the expression of the major intestinal mucin, rats were infected with the intestine-dwelling parasite *N. brasiliensis*. The parasite was injected subcutaneously and migrated via the bloodstream to the lungs and airways to the small intestine. These parasites remain in the small intestines before being expelled after 12–13 days of infection [16]. Mucosal scrapings were collected at different stages of the infection, and the 'insoluble' mucin complex was obtained by disulphide-bond reduction after repeated extractions with guanidinium chloride. The amount of collected insoluble mucins always increased up to days 12–13, measured as both dry weight and total carbohydrate (Figure 1). The same tendency was also observed when analysing the Muc2 content within the collected insoluble mucins immunochemically. As the Muc2 mucin is the major small-intestinal mucin [8] and is recovered in the insoluble mucin fraction as used here, it is likely that the Muc2 was one of the major mucins analysed.

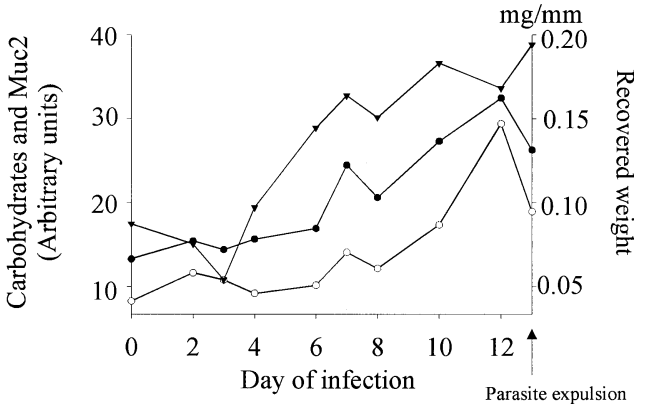

Figure 1 Amount of insoluble mucins and Muc2 isolated from the small intestine of Sprague–Dawley rats infected with *N. brasiliensis*

Rats were infected (infection 2) with an estimated 6000 third-stage larvae. The amount of collected insoluble mucins per length of the small intestine was measured as weight (mg/mm; ○) or as the amount of carbohydrate measured by PAS (periodic acid–Schiff) staining (mean of three experiments) of insoluble mucin absorbed on nitrocellulose membranes (●). The amount of rat Muc2 (▼) was measured on HF-deglycosylated insoluble mucins by the α-gpA antiserum against the Muc2 mucin large domain.

Analysis of released sialylated oligosaccharides

To analyse the potential glycosylation alterations, the oligosaccharides from the insoluble mucins collected from Sprague–Dawley rats were released. The sialylated oligosaccharides were isolated as their methyl esters eluted from an anion-exchange column after removing the neutral oligosaccharides as described before [19]. The mixture of sialylated oligosaccharides was converted into their *N*-methyl amides and permethylated. When these oligosaccharides were analysed by GC-MS, two glycosylation alterations were recognized in the sialylated oligosaccharides from the infected animals (Figure 2 and Table 1). The first, and most prominent, was a decreased amount of NeuGc-containing oligosaccharides (where NeuGc is *N*-glycolyl neuraminic acid) in favour of NeuAc-containing oligosaccharides, as revealed by the lower second peak in each pair (Figure 2). The second feature was the appearance of small amounts of four additional oligosaccharides (s4.1, s4.2, s5.1 and s5.2).

Transient suppression of the CMP-NeuAc hydroxylase

The gradual shift from NeuGc as the major sialic acid towards NeuAc as found by GC-MS was verified by sialic acid analysis using HPAEC-PAD after acidic hydrolysis of the insoluble mucins (Figure 3). NeuGc is predominant in uninfected animals, but during the infection a gradual increase in the amount of NeuAc and a decrease in the amount of NeuGc was observed. The day with the highest amount of NeuAc varied between the two infection experiments performed with Sprague–Dawley rats, but the dominance of NeuGc was in both experiments regained after parasite expulsion.

Using the probe from the mouse CMP-NeuAc hydroxylase [23] in Northern-blot experiments, it could be shown that the increased level of NeuAc was preceded by decreased expression of CMP-NeuAc hydroxylase during the infection (Figure 4). Thus a lowered amount of the sugar donor CMP-NeuGc accounted for the observed decreased levels of NeuGc-containing oligosaccharides. Towards the end of the infection cycle, the

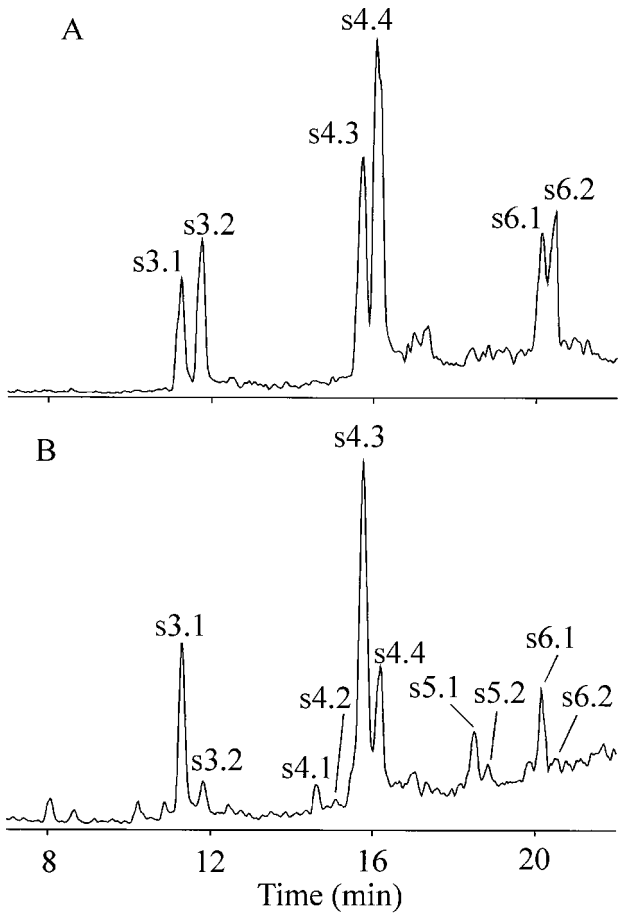

Figure 2 Mass chromatograms from GC-MS of permethylated *N,N*-dimethyl amide derivatives of sialylated oligosaccharides from Sprague–Dawley rats uninfected and infected by *N. brasiliensis*

Chromatograms of the relative intensity (*y* axis) of the B-type oxonium ions (sum of *m/z* 389 and *m/z* 419) from terminal NeuAc and NeuGc (*N*-glycolyl neuraminic acid), respectively. An uninfected animal (day 0) is shown in (**A**) and an infected animal in (**B**; infection 1, day 12 after infection). The samples were injected on-column at 70 °C (1 min) into a 10 m × 0.25 mm column with 0.02-μm stationary film. The temperature was programmed to rise from 80 to 200 °C at 50 °C/min and then by 10 °C/min to 390 °C (5 min). Helium was used as carrier gas (0.2 bar, linear velocity 65 cm/s at 80 °C). The numbers refer to structures interpreted by GC-MS, shown in Table 1.

Table 1 Structures of small-intestinal sialylated oligosaccharides derived from the insoluble mucin complex of Sprague–Dawley rats uninfected and infected with *N. brasiliensis*

Structures are based on GC-MS of permethylated *N,N*-dimethylamide derivatives of sialylated oligosaccharides. Hexose (Hex) residues are assumed to be Gal and deoxyhexoses to be Fuc-2, similar to the linkage in A- and H-determinants. *N*-Acetylhexosamine (HexNAc) residues are usually assumed to be GlcNAc based on the monosaccharide composition. GalNAc is assumed to be present only as NeuAc/NeuGc(GalNAc-)Gal- (where NeuGc is *N*-glycolyl neuraminic acid), and *N*-acetylhexosaminitol (HexNAcol) residues are assumed to be *N*-acetylgalactosaminitol (GalNAcol). Substituents on the C-6 branch of GalNAcol are marked in bold. Results are from infection 1.

No.	Sialylated oligosaccharide	Structures found in uninfected animals (day 0)	Additional structures induced by infection (day 11–12)
s3.1	NeuAcα2-3Galβ1-3GalNAcol*	x	
s3.2	NeuGc-Gal-3GalNAcol	x	
s4.1	NeuAc-(GalNAc-)Gal-3GalNAcol		x
s4.2	NeuGc-(GalNAc-)Gal-3GalNAcol		x
s4.3	NeuAcα2-3Galβ1-3**(GlcNAcβ**1-6)GalNAcol*	x	
s4.4	NeuGc-Gal-3**(GlcNAc-6)**GalNAcol	x	
s5.1	NeuAcα2-3(GalNAcβ1-4)Galβ1-3**(GlcNAcβ**1-6)GalNAcol*		x
s5.2	NeuGc-(GalNAc-)Gal-3**(GlcNAc-6)**GalNAcol		x
s6.1	NeuAc-Gal-3**(Fuc-2Gal-GlcNAc-6)**GalNAcol	x	
s6.2	NeuGc-Gal-3**(Fuc-2Gal-GlcNAc-6)**GalNAcol	x	

* Also isolated by HPLC from infected animals and structurally characterized by ^{1}H-NMR, monosaccharide analysis and linkage analysis by GC-MS of partially methylated alditol acetates.

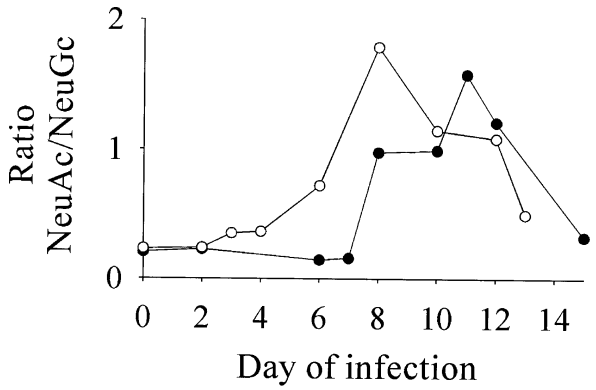

Figure 3 Analysis of the ratio NeuAc/NeuGc from insoluble small-intestinal mucins from Sprague–Dawley rats during the progress of *N. brasiliensis* infection

The ratio NeuAc/NeuGc in two infection series, infection 1 (●) and infection 2 (○), analysed by HPAEC-PAD after hydrolysis in 0.1 M HCl at 80 °C for 1 h. Each point represents the mean of two analyses.

CMP-NeuAc hydroxylase was expressed again and reached day-0 levels (results not shown), preceding the normalization of NeuGc levels found after the expulsion of the parasite.

The *N. brasiliensis* infection was also studied in three additional rat strains and the same infection course was seen in all cases (N. G. Karlsson and G. C. Hansson, unpublished work). The amount of NeuAc was increased late in the infection, before the parasite expulsion, in all the rat strains studied (Figure 5).

Isolation and characterization of sialylated oligosaccharides

Four induced sialylated oligosaccharides were detected late in the infected animals, as shown in the mass spectra from the GC-MS analysis (Figure 2 and Table 1). These components (s4.1, s4.2, s5.1 and s5.2) all contained the saccharide sequence NeuAc/NeuGc-(HexNAc-)Hex- (where Hex is hexose and HexNAc is *N*-acetylhexosamine) bound to the reduced GalNAc. In order to fully assign the structure of the induced sialylated epitope the oligosaccharide s5.1 was isolated by HPLC (Figure 6). Infected GOT-W rats were used as source because their s5.1 oligosaccharide level was slightly increased (approx. 50%) compared with the Sprague–Dawley rats in the experiments described previously. The s5.1 oligosaccharide and two additional oligosaccharides that are normally present (s3.1 and s4.3) all showed single peaks after purification when investigated by GC-MS after permethylation (Figure 6, inserts). The sequences of these three compounds could be deduced from the mass spectra of the

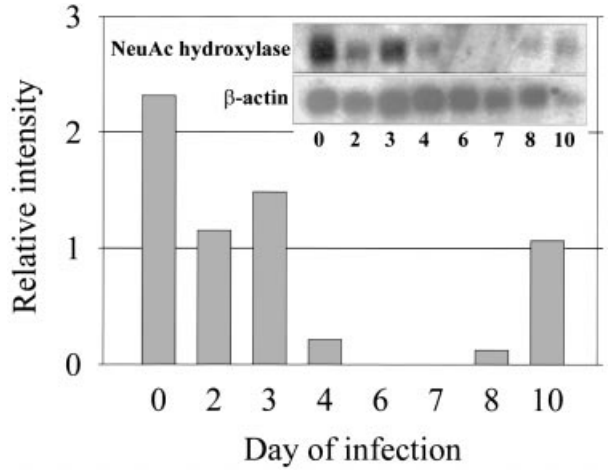

Figure 4 CMP-NeuAc hydroxylase mRNA levels in small intestine of Sprague–Dawley rats infected with *N. brasiliensis*

Northern-blot analysis was performed as described in the text (mRNA from infection 2). A single band of 2.3 kb was detected. The autoradiography film was scanned and the intensities of hybridizing bands are displayed as bars after correction against the β-actin levels for each lane, as determined on the same blot. The inserted pictures show the blot hybridized with CMP-NeuAc hydroxylase DNA and β-actin.

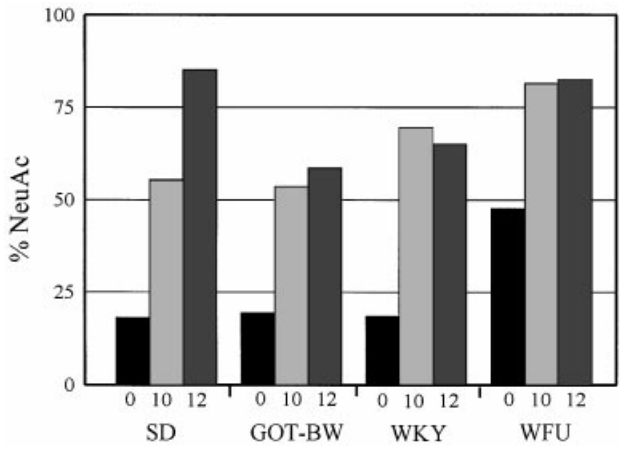

Figure 5 Relative amount of NeuAc in the insoluble mucin isolated from different rat strains after *N. brasiliensis* infection

The percentage of NeuAc in the total amount of sialic acid on insoluble mucin oligosaccharides from outbred Sprague–Dawley (SD) rats and inbred GOT-BW, WKY and WFU rats, uninfected (day 0) and infected with *N. brasiliensis* (days 10 and 12). Data are from sialic acid analysis as detected by HPAEC-PAD after hydrolysis of the insoluble mucins.

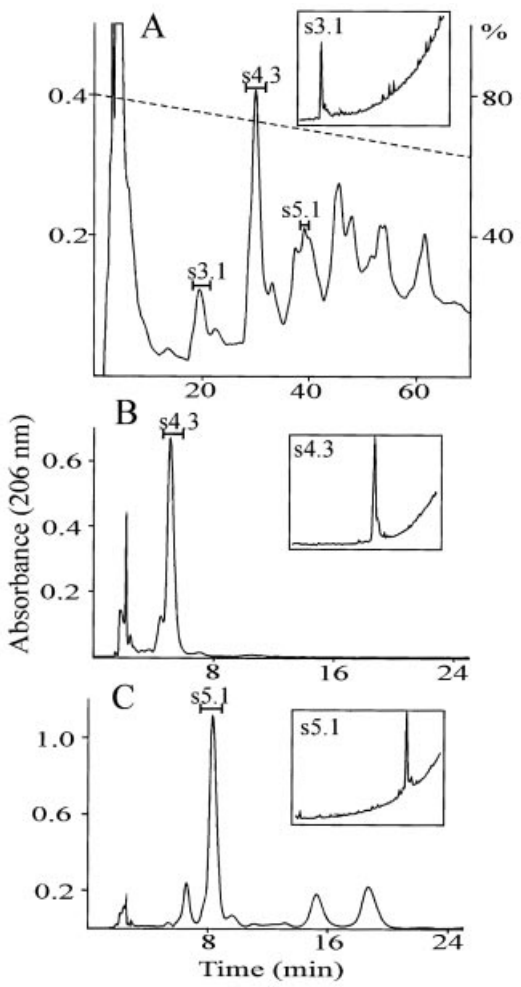

Figure 6 Isolation of released rat small-intestine sialylated oligosaccharides from day 12 of the *N. brasiliensis* infection by HPLC

GOT-W rats were infected subcutaneously with approx. 6000 third-stage larvae, and the insoluble-mucin tryptic glycopeptides were collected after Sephacryl S-200 gel filtration. Sialylated oligosaccharides were collected and derivatized, as described in the text, and subjected to HPLC on a Lichrosorb-NH column (**A**) eluted with a gradient of H_2O/acetonitrile (decreasing portion of acetonitrile). Collected fractions s4.3 (**B**) and s5.1 (**C**) were separated further on a Kromasil C_{18} column eluted isocratically with water. Inserts are from the total ion chromatogram (7–22 min) from the concomitant analyses by GC-MS of the permethylated fractions after HPLC purification.

subsequent GC-MS analysis (Figure 7). The simplest of these (s3.1), was shown by GC-MS to have the sequence NeuAc-Hex-3HexNAcol (where HexNAcol is *N*-acetylhexosaminitol; Figure 7A), and 3-linked *N*-acetylgalactosaminitol (GalNAcol) and Gal were concluded from monosaccharide analysis and linkage analysis (Table 2). The α2-3 linkage of the NeuAc(NHMe) (where Me is methyl) was confirmed by ^{1}H-NMR from the chemical shift of its $H3_{eq}$ and the low field shift of Gal H3 (Table 3 and Figure 8A). The values found were similar to previously published values for the un-derivatized species [24,25], except for a substantial (approx. 0.14 p.p.m.) downfield shift of NeuAc $H3_{ax}$ ($\delta = 1.94$ p.p.m.) and the extra methyl amide signal at 2.802 p.p.m.

The sequence of the s4.3 structure was found by GC-MS to be the same as s3.1 but with an extra HexNAc at C-6 of the HexNAcol (Figure 7B). This was consistent with the presence of terminal GlcNAc and GalNAcol substituted on both C-3 and C-6 in the linkage analysis (Table 2). Again ^{1}H-NMR (Table 3 and Figure 8B) showed a downfield shift of the NeuAc $H3_{ax}$ for the *N*-methyl derivative compared with the shift reported for the corresponding underivatized oligosaccharide [24].

The mass spectrum of the five-sugar compound s5.1 from GC-MS unambiguously revealed the sequence NeuAc-(HexNAc-) Hex-3(HexNAc-6)HexNAcol (Figure 7C). In the 1D spectrum two extra signals were identified easily: a β-anomeric signal around 4.55 p.p.m. and a methyl signal in the 2.05 p.p.m. region (in total, four methyl groups present). The individual mono-

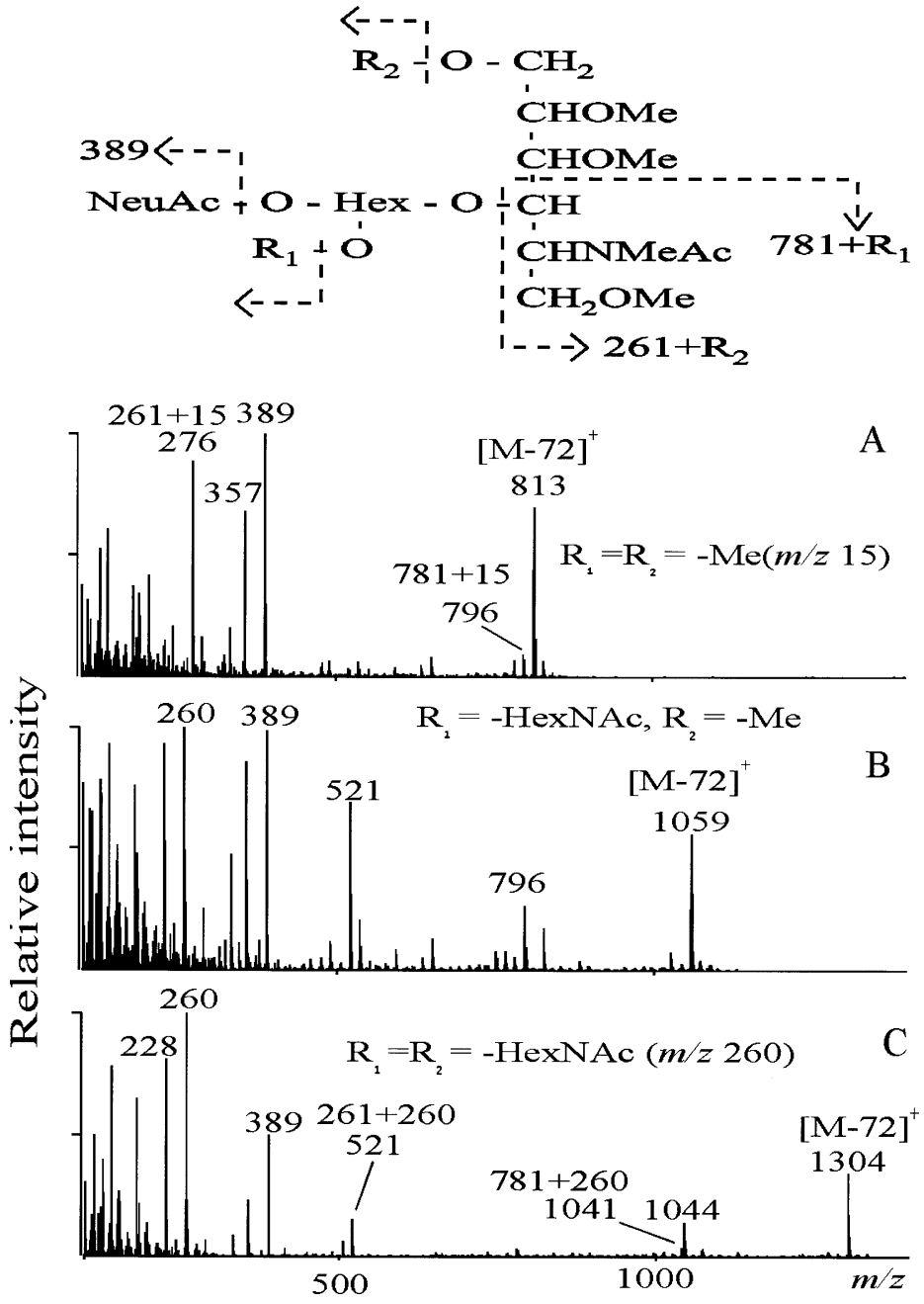

Figure 7 Mass spectra from GC-MS of the permethylated *N,N*-dimethyl amide derivatives of sialylated oligosaccharides from GOT-W rats on day 12 of infection

(**A**) Mass spectrum (mean of 12 scans, retention time 19.3 min) of the derivatized component s3.1. (**B**) Mass spectrum (mean of 17 scans, retention time 16.8 min) of the derivatized component s4.3. (**C**) Mass spectrum (mean of 9 scans, retention time 11.8 min) of the derivatized component s5.1. Spectra were background subtracted. The numbers refer to structures in Table 1.

saccharides were identified from their chemical shifts (Table 3 and Figure 8C) and couplings (only estimated as large or small from two-dimensional cross peaks and resolved 1D peaks), and were conclusive with the facts that GlcNAc, Gal, GalNAc and GalNAcol were the only moieties detected in the monosaccharide analysis (Table 2). The NeuAc $H3_{eq}$ signal was shifted downfield approx. 0.09 p.p.m. and a smaller change was also found on the $H3_{ax}$ signal, in agreement with a GalNAcβ added on the C-4 of Galβ. The Gal also showed expected small shifts on H2 and H3, while the normal downfield shift of H4 was not detected. Shift changes were also found for the GlcNAcβ H1 and on the H5 and one of the C-6 protons of the GalNAcol. The H5 of GalNAcol changed from 4.261 to 4.177 p.p.m. upon addition of GalNAcβ1-4 to Gal (s4.3 to s5.1), a shift that is similar to the one observed by Blanchard et al. [26] for the addition of a GalNAcβ1-4 to the Gal of NeuAcα2-3Galβ1-3(NeuAcα2-6) GalNAcol.

To better identify and confirm the saccharide sequence and linkage positions, selective 1D ROESY experiments were performed. An ROE is normally found between the anomeric

Table 2 Monosaccharide composition and linkage analysis of sialylated oligosaccharides isolated from GOT-W rats after 12 days of infection

The values represent the relative amount of each monosaccharide as analysed by HPAEC-PAD after acidic hydrolysis of purified compounds. The structural elements in parentheses are partially methylated alditol acetates detected by GC-MS after methylation analysis. Man or Fuc were not found in the analyses.

	Relative abundance (mol)		
Monosaccharide	s3.1	s4.3	s5.1
Gal	0.9 (–3Gal1-)	0.7 (–3Gal1-)	0.6 [–3(–4)Gal1-]
GlcNAc		1.4 (GlcNAc1-)	1.6 (GlcNAc1-)
GalNAc			1.6 (GalNAc1-)
GalNAcol	1.0 (–3GalNAcol)	1.0 [–3(–6)GalNAcol]	1.0 [–3(–6)GalNAcol]

Table 3 Chemical shifts of assigned protons in the three sialylated structures s3.1, s4.3 and s5.1 isolated from GOT-W rats after 12 days of infection

Spectra were recorded in ^2H_2O at 25 °C and the oligosaccharides were analysed as methyl amides.

		Chemical shift (p.p.m.)		
Saccharide	Proton	s3.1	s4.3	s5.1
NeuAcα2-3 (methyl amide derivative)	$H3_{eq}$	2.768	2.77	2.863
	$H3_{ax}$	1.939	1.939	1.956
	H4	3.74	–	3.68
	Methyl	2.038	2.038	2.04*
	Methylamide	2.802	2.803	2.843
GalNAcβ1-4	H1			4.52
	H2			3.901
	H3			3.72
	Methyl			2.088*
Galβ1-3	H1	4.559	4.547	4.548
	H2	3.602	3.597	3.484
	H3	4.078	4.07	4.101
	H4	3.844	3.845	3.87
GlcNAcβ1-6	H1		4.532	4.56
	H2		3.71	3.704
	H3		3.521	3.529
	H4		3.446	3.439
	H5		3.73	3.751
	Methyl		2.06	2.067*
GalNAcol	H1	3.78	–	3.781
	H2	4.387	4.385	4.381
	H3	4.082	4.074	4.058
	H4	3.508	3.458	3.47
	H5	4.181	4.261	4.177
	H6a	–	3.925	3.905
	H6b	3.670	3.68	3.683
	Methyl	2.043	2.06	2.054*

* These signals have been tentatively assigned based on reference substances.

proton and the proton on the other side of the glycosidic linkage. Irradiation of the Gal H4 (3.87 p.p.m.) gave rise to two strong ROEs at 4.52 and 4.11 p.p.m. The former was the anomeric proton of the GalNAcβ, further strengthening the conclusion of a glycosidic linkage GalNAcβ1-4Gal, and the latter was the H3 signal within the Gal. Together with the chemical shifts of the other isolated substances, and the results from GC-MS, methylation analysis and monosaccharide composition analysis, the oligosaccharide s5.1 was concluded to be NeuAcα2-3(GalNAcβ1-4)Galβ1-3(GlcNAcβ1-6)GalNAcol.

The induction of the Sd^a/Cad-type epitope during the infection

The epitope NeuAcα2-3(GalNAcβ1-4)Galβ1- found in structure s5.1 has previously been described in the Sd^a- and Cad-blood-group antigens, and is believed to be the consequence of a GalNAc transferase acting on terminating NeuAc/NeuGcα2-3Galβ1- sequences. All the induced sialylated structures found during the *N. brasiliensis* infection could be explained by the induction of such a GalNAc transferase. To estimate the level of oligosaccharides containing the Sd^a/Cad-like epitope throughout the infection course the intensities of their $[M-72]^+$-fragment ions (loss of -$CONMe_2$ from the molecular ion) was compared with the intensities of their assumed biosynthetic precursors recorded by GC-MS. The Sd^a/Cad-like oligosaccharides all increased from day 8 up to a maximum at the time of parasite expulsion (Figure 9).

Analysis of the glycosylation of glycopeptide A

To verify that the induced Sd^a/Cad-like epitopes were present on the large mucin domain (gpA) of the insoluble mucins from the small intestines of GOT-W-strain rats (day 12 of the infection), these were isolated after tryptic digestion and gel chromatography on Sephacryl S-200 and S-500. gpA is probably mostly made up of the large mucin domain of Muc2. The relative higher increase in the recovered Muc2 reactivity as compared with total amount of gpA might indicate a larger increase of Muc2 relative to other potential mucin domains present in the gpA fraction (Table 4). The monosaccharide composition of this Muc2-derived glycopeptide indicated that there was indeed an increased amount of GalNAc-containing compounds and also that the amount of NeuGc was lowered (Table 5). The GC-MS of released oligosaccharides showed the presence of the compounds s4.1 and s5.1 (see Table 1 for structures), whereas their NeuGc-containing analogues were not detected. The absence of NeuGc-containing Sd^a/Cad-like species was probably due to the generally lower NeuGc content in the GOT-W rats. This is indicated by the fact that the ratio NeuGc/NeuAc was 3:4 in the GOT-W strain compared with 4:1 in the Sprague–Dawley rats (Table 5 and Figure 3). The increased amount of sialylated oligosaccharides relative to GalNAcol and the concomitant decreased acidic oligosaccharides suggest a lower sulphation of gpA prior to expulsion of the parasite. The high amount of the Sd^a/Cad-like structures recovered from the gpA fraction of infected rats and the high reactivity of the antibody α-gpA with the deglycosylated glycopeptide suggested that the Sd^a/Cad-like oligosaccharides could be present on rat Muc2 (Table 4).

DISCUSSION

This study of the sialylated oligosaccharides during an infection by the *N. brasiliensis* parasite shows not only that the quantity of mucins changes throughout the infection, but also that there is a qualitative modification of the epitopes expressed. Both the level of NeuAc and the level of the Sd^a/Cad epitope were elevated at the end of the infection cycle, suggesting an involvement in the recovery from this infection. Additional alterations, including transient expression of a blood-group-A GalNAc transferase, not reported here, suggest that there could be a pre-programmed sequence of events occurring during this infection. The precise

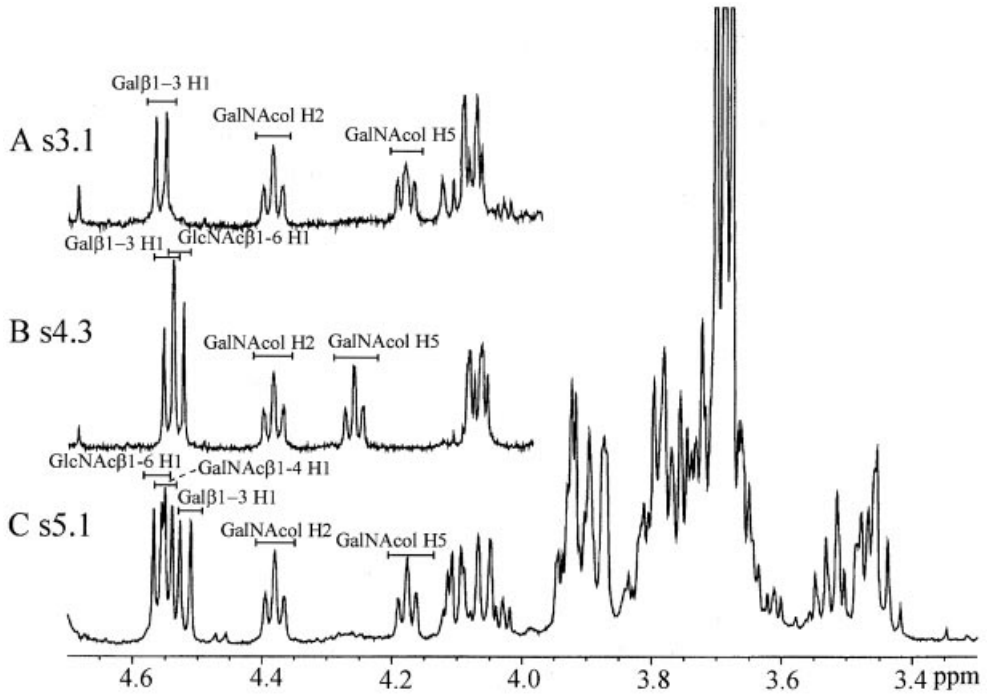

Figure 8 Proton-NMR spectra of the isolated sialylated oligosaccharide *N*-methyl amide derivatives

Ring proton signal region for the component s3.1 (**A**), component s4.3 (**B**) and component s5.1 (**C**) recorded in ^2H_2O at 25 °C isolated from GOT-W rats on day 12 of the infection.

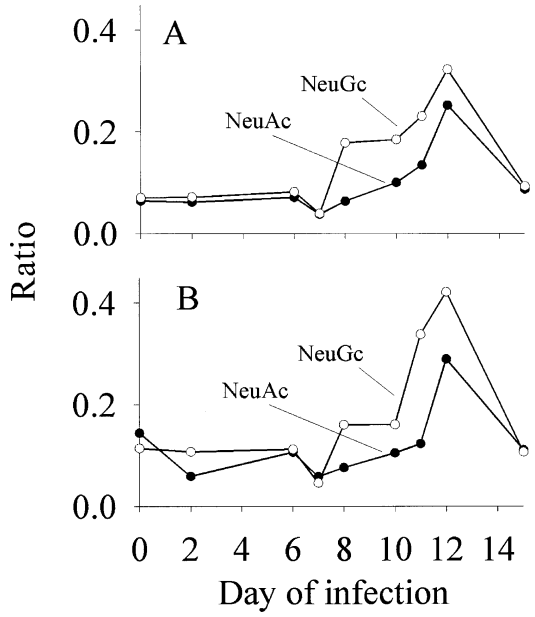

Figure 9 Expression of Sd^a/Cad-like epitope, NeuAc/NeuGc-(GalNAc-)Gal-, on the insoluble small-intestinal mucins during the parasite infection of Sprague–Dawley rats

The product/substrate ratio of a presumed GalNAcβ1-4 transferase, during infection 1, measured from GC-MS of permethylated *N,N*-dimethyl derivatives measured by the $[M\text{-}72]^+$-fragment ions. (**A**) Ratio of NeuAc/NeuGc-(GalNAc-)Gal-3GalNAcol to NeuAc/NeuGc-Gal-3GalNAcol. (**B**) Ratio of NeuAc/NeuGc-(GalNAc-)Gal-3(**GlcNAc-6**)GalNAcol to NeuAc/NeuGc-Gal-3(**GlcNAc-6**)GalNAcol. NeuAc-containing (●) and NeuGc-containing (○), respectively.

Table 4 Characterization of gpA from GOT-W rats uninfected and infected (day 12) with *N. brasiliensis*

	Uninfected	Infection day 12
Recovered amount (mg/rat)	6.2	17.8
Relative amount of Muc2*	1	4.6
Neutral oligosaccharides (mol%)†	51	69
Acidic oligosaccharides (mol%)†	49	31
No. residues/GalNAcol (neutral)†	5.1	6.0
No. residues/GalNAcol (acidic)†	13.0	10.2
No. residues/GalNAcol (sialylated)†	7.6	10.3
Amount of Sd^a/Cad-epitopes (nmol/mg of gpA)‡	0	2.0
H-Activity§	100	116
A-Activity§	0	0
Helix pomatia lectin binding§	0	0

* The amount of Muc2 was measured as reactivity of HF-deglycosylated glycopeptides with the α-gpA antiserum.

† Released carbohydrates measured by monosaccharide compositional analysis in each fraction eluted from the anion-exchange column.

‡ The amount of Sd^a/Cad-containing oligosaccharides was estimated by GC-MS.

§ Relative antibody- and lectin-binding against gpA of uninfected rat.

day for these transient phenomena varied, as illustrated in Figure 3 (infections 1 and 2), but their order remained constant. The reason for this variation in onset is not known, but could probably be attributed to differing amounts or infectability of the worm preparations used. This is the first observation of transient glycosylation alterations of mucins during an infectious process suggesting a significant biological role for mucins. Such dynamic

Table 5 Monosaccharide composition of the sialylated mucin oligosaccharides

The total fraction of sialylated oligosaccharides released from gpA of GOT-W rats uninfected and infected with *N. brasiliensis*. Values are the means of two analyses by HPAEC-PAD, and are given relative to the amount of GalNAcol.

	Relative abundance (mol)	
Monosaccharide	Uninfected animals	Infection day 12
GalNAcol	1.0	1.0
Fuc	0.5	0.9
GlcNAc	1.6	2.4
GalNAc	0.6	1.8
Gal	1.7	2.3
NeuAc	0.8	1.1
NeuGc	0.6	0.2

glycosylation modifications are in contrast to previous views of mucins as only acting as passive protection barriers.

A gradual shift from a predominance of NeuGc to a predominance of NeuAc was found among the sialylated oligosaccharides in the later part of the infection. After the parasite expulsion, the levels returned to normal. During the biosynthesis of sialylated oligosaccharides, the sialic acids are transferred by specific sialyltransferases to the precursor oligosaccharides as their CMP derivatives (CMP-NeuAc or CMP-NeuGc). The conversion of the acetyl form to the glycolyl form occurs at this sugar nucleotide level, in a reaction catalysed by the CMP-NeuAc hydroxylase using CMP-NeuAc as a substrate [23]. Any changes in the final relative amounts between NeuAc and NeuGc will affect all sialylated glycoconjugates and are likely to be due to regulation of this enzyme. The expression levels of CMP-NeuAc hydroxylase were thus analysed in Northern-blot experiments (Figure 4), where expression declined from day 0 to day 4 of the infection and started to increase again on day 10. This correlated well with the change of the NeuAc/NeuGc ratio detected by monosaccharide composition analysis, although there is a discrepancy of a few days. The delay is most probably an effect of the rather slow turnover rate of mucins, accumulating 'older' mucins in the small intestine. That mucins have a slow turnover has been shown previously for MUC2, where estimates of 48 h have been suggested [27]. We suggest that the regulation of CMP-NeuAc hydroxylase is the major underlying reason for the shift from NeuAc to NeuGc seen during the infection.

Mucin oligosaccharide expression patterns have been shown to differ markedly between different tissues as well as between individuals within one species [28,29]. One can thus question the generality of glycosylation events studied in single inbred animals. With this in mind, we also performed *N. brasiliensis* infection experiments on three additional rat strains. The shift from NeuGc towards NeuAc during the infection was observed in all the strains studied, strengthening this as a general effect of *N. brasiliensis* infection in rats. In this context, it should also be mentioned that the course of the infection was independent of the rat strain.

The oligosaccharide characterization also revealed a second glycosylation alteration during the *N. brasiliensis* infection. A terminal epitope characterized as an Sd^a/Cad-like antigen not normally expressed in the small intestine of Sprague–Dawley rats was found in small amounts. The appearance of this epitope may be traced to the regulation of a single enzyme, in this case an up-regulation of a GalNAc transferase using the continuously expressed NeuAc/NeuGcα2-3Galβ1- epitope as substrate. Cloned transferases acting on a similar epitope of the G_{M2}-glycosphingolipid from human, mouse and rat [30–32] and the murine T-lymphocyte CT-1 antigen found on glycoproteins [33] use a similar biosynthetic pathway. Also, biosynthesis of the Sd^a and the Cad blood-group antigens [34] is believed to be due to transferases adding GalNAc to the sialylated precursors. This has been shown, at least for the Sd^a transferase, by enzymic studies [35–37]. Our preliminary results indicate that the transferase responsible for making the Sd^a/Cad-related epitope in the parasite-infected rats is different from the one found in the murine T-lymphocytes. A Northern blot using the CT-1 cDNA probe encoding the full-length murine transferase [33] was negative throughout the infection (results not shown). As the GalNAc transferase induced by the parasite has not been purified or cloned, its specificity and potential activity on substrates other than mucins cannot be evaluated.

At this stage it is too premature to judge whether the observed alterations are a prerequisite for expelling the parasite and for the host to recover from this particular infection. We can also only speculate as to what mechanism causes these alterations to occur. Somehow the host must sense the presence of parasites in the small intestine regardless of the parasite being incapable of physically penetrating the intestinal wall. Thus the effect is most likely triggered by substances secreted from the parasite or expressed on the parasite surface.

That microbes can up-regulate glycosyltransferases has been suggested by exposing germ-free mice to specific intestinal bacteria causing an altered fucosylation of intestinal glycoconjugates [12]. The transient and dynamic control of the glycosylation in the intestinal rat Muc2 as shown in this report illustrates that the terminal monosaccharide residues can be susceptible to further modifications under the influence of infections. The understanding of how intestinal microbes affect glycosylation is important for the understanding of how mucosal surfaces are involved in the defence against an infection. One possibility is that the pathogen induces the glycosylation alterations for its own benefit, as suggested by Bry et al. [12]. However, another scenario is that this is part of a general defence strategy utilized by the host and affecting the mucin Muc2, but maybe also other glycoconjugates. Support for a general mechanism comes from the observed binding of pathogens to glycans [11] and the diversity of glycans between species, individuals and different organs and cells within one organism [38]. The evolution and biological role of this diversity for protecting species from infections has recently been discussed by Gagneux and Varki [29]. The present observation with transient glycosylation alterations within one organ further develops this idea. A possible pathway for a gastrointestinal response to a microbial infection could be a repertoire of inductive glycosylation alterations launched sequentially by the host until one combination eventually affects the infection or potential binding sites for the infectious microbe. A process like this with a pre-programmed response is in line with the current concept of the innate immune system where pre-programmed responses effectively protect an organism also lacking a T- and B-cell-based immune system [39]. Mucins and their glycan parts can be suggested to belong to the innate immune system.

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