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Sialic acid-dependent attachment of mucins from three mouse strains to *Entamoeba histolytica*

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ABSTRACT

Mouse strain-specific differences in the carbohydrate composition of intestinal mucins were hypothesized to account for strain-dependent susceptibility to *Entamoeba histolytica*. To test this hypothesis, intestinal mucins from susceptible and resistant inbred strains of mice were analyzed for their *O*-glycan content and for their ability to inhibit amoebic adherence to $(GalNAc)_{12-27}$-HSA neo-glycoproteins. The results showed that the colorectal mucin *O*-glycan of susceptible CBA mice was lower in sialic acid content than that of resistant C57BL/6 and BALB/c mice. Mucins from CBA mice were more potent inhibitors of *E. histolytica* adherence to neo-glycoproteins than were mucins from C57BL/6 or BALB/c mice. Consistent with the role of terminal Gal/GalNAc as a receptor for amoebic adherence, sialidase treatment of C57BL/6 and BALB/c colorectal mucins increased their ability to inhibit *E. histolytica* adherence to the neo-glycoproteins. These results provide evidence of mouse strain-specific differences in the sialic acids content of mucin *O*-glycans. These dissimilarities likely contribute to the differential susceptibility of the three mouse strains to *E. histolytica* infection.

1. Introduction

The anaerobic parasitic protozoan *Entamoeba histolytica* (*E. histolytica*) is estimated to infect about 1% of the world's population (about 50 million people), resulting in 40,000–100,000 deaths annually. Transmission of the parasite is primarily through the fecal-oral ingestion of cysts, which pass through the stomach to reach the small intestine. There, excystment results in the release of trophozoites, migration to the large intestine, and colonization by adherence to the mucous layer. Attachment of the parasite to the intestinal wall requires galactose (Gal)/*N*-acetylgalactosamine (GalNAc) inhibitable lectins, specifically, their heavy subunits (Hgls), which have been shown to bind the Gal and GalNAc residues exposed on the luminal surface of host mucins [1,2].

Studies on mice have described strain-specific differences in their susceptibility to *E. histolytica*, with resistance to infection shown to be H-2 haplotype and lymphocyte independent [3,4]. Moreover, resistance to the parasite was observed even in IL-12, IFN-γ or iNOS deficient mice of resistant mouse strains, thus ruling out the involvement of the immunological compartment in resistance, at least in the initial stage of *E. histolytica* infection. Since *E. histolytica* parasitizes the large intestine and parasite Hgls are vital for host attachment and infection [2], we hypothesized that strain-specific differences in mucus glycosylation in the large intestine mediated susceptibility vs. resistance.

Mucin is a family of large glycoproteins whose function includes the protection of intestinal epithelial cells from physical and physiological damage [5–9]. *O*-glycans attached to mucin protein backbones play an important role in mucin function. However, despite their importance in disease resistance [10], differences in the *O*-glycan structures of mucins among mouse strains have not been investigated. Thus, in the present study, we compared the *O*-glycomic profiles of large intestinal mucus from C57BL/6, CBA, and BALB/c mice, known to differ in their susceptibility to *E. histolytica* infection. The results showed that the affinity of *E. histolytica* toward mouse mucins is determined by the relative abundance of sialic acids on their *O*-glycans and that differences in sialic acid content may contribute to host susceptibility to the parasite.

Abbreviations: ABC, ammonium bicarbonate; aoWR, N^{α}-((aminooxy)acetyl)tryptophanylarginine methyl ester; DHB, 2,5-dihydroxybenzoic acid; MTT, 3-methyl-1-p-tolyltriazene; PAS, periodic acid-schiff.

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2. Materials and methods

2.1. Ethics statement

Animal experiments conducted in the present study were approved by the Animal Care and Use Committee, Nagasaki University, and the experimental procedures were performed in accordance with the Guidelines for Animal Experimentation established by Nagasaki University (project number 1104190916).

2.2. Isolation and semi-purification of colorectal mucins from mice

C57BL/6J (B6) and CBA/J (CBA) mice (6- to 7-weeks-old, male) were purchased from KBT Oriental Co., Ltd. (Saga, Japan) and BALB/c (BALB) mice (6- to 6.5-weeks-old, male) were purchased from Japan SLC, Inc. (Fukuoka, Japan). All animals were fed under specific-pathogen-free (SPF) condition. Materials containing mucins were prepared as described [11]. For each experiment, B6, CBA and BALB mice ($n = 5$ for each strain) were sacrificed by diethyl ether anesthesia and the colorectum was collected. The luminal contents were removed and the tissues were rinsed with ice-cold saline. Mucosal samples were obtained by scraping and were immediately frozen at −80 °C. The frozen mucosal samples (150 mg, wet weight) were homogenized on ice in 1 ml of ice-cold 5 mM EDTA solution (pH 7) for 1 min using a Potter–Elvehjem homogenizer. The homogenized samples were then treated with 50 μl of protease (P6110, Sigma) and incubated at 37 °C for 80 min. The digested samples were left on ice for 10 min, after which 3.5 μl of protease inhibitor cocktail (P8340, Sigma) was added to each one. The samples were centrifuged at 10,000 rpm for 60 min at 4 °C. The mucin-containing supernatants were purified on a Sepharose CL-4B (GE Healthcare) column equilibrated with 0.2 M NaCl, applying 1 ml of each sample on a column containing 8 ml of gel slurry and then eluting 0.6 ml fractions with 0.2 M NaCl. After removing aggregates by centrifuging at 10,000 rpm for 10 min at 4 °C, aliquots (9 μl) of each fraction were subjected to 4–12% Bis-Tris NuPAGE gels electrophoresis (Invitrogen). The mucin-containing fractions confirmed by PAS staining (Pierce glycoprotein staining kit, Thermo) were concentrated 20-fold using an Amicon Ultracel-100 K (Millipore) and directly used in binding inhibition assays or exchanged with 50 mM ammonium bicarbonate (ABC) for glycoblotting. The samples obtained using this protocol would contain the mixture of mucin species as described previously [12].

2.3. Glycoblotting-assisted glycomics of colorectal mucin from mice

A high-throughput method for glycan analyses of mucins was performed as described previously [13]. This method releases both *O*- and *N*-glycans from glycoproteins, although in this study only *O*-glycans were analyzed. Briefly, 80 μl of H_2O, 5 μl of 1 M ABC, and 5 μl of PNGase F (1 U/μl, Roche, IN) were added to 10 μl of concentrated samples, which were then incubated at 37 °C for 24 h. After the addition of 200 μl of 50 mM ABC, the samples were applied to YM-10 columns. The column was washed twice with 280 μl of 50 mM ABC by centrifugation at 12,000×*g* for 25 min at 25 °C. Flow-through fractions contained *N*-glycans, while the portion of the supernatant remaining on the column consisted of *O*-glycosylated proteins. The latter were concentrated to 25 μl, treated with 20–30 mg of ammonium carbamate (Tokyo Chemical Industry Co., Ltd.), and incubated at 60 °C for 20 h followed by the addition of 500 μl of H_2O and SpeedVac processing at high temperature to remove ammonium carbamate. The residual material was reconstituted in 25 μl of H_2O, with 1 pmol of chitotetraose (GN4, Seikagaku Biobusiness, Tokyo) added as an internal standard. An aliquot of this solution was subject to glycoblotting [13] using BlotGlyco H beads (50 μl) (10 mg/ml suspension, Sumitomo Bakelite Co., Tokyo). On-bead methyl esterification of sialic acid carboxyl groups was carried out by incubating the samples with 100 μl of 100 mM 3-methyl-1-p-tolyltriazene (MTT) in 1,4-dioxane at 60 °C to dryness, followed by three serial washes with 1,4-dioxane, MeOH, and H_2O. The glycans bound to the beads were subjected to transiminization by treating them with a mixture of 180 μl 2% acetic acid in MeCN and 20 μl of 5 mM N^{α}-((aminooxy)acetyl)tryptophanylarginine methyl ester (aoWR) at 80 °C to dryness. Tagged glycans were eluted by the addition of 100 μl of H_2O and an aliquot was purified using a MassPREP HILIC plate (Waters). Purified samples were directly mixed with DHB matrix solution (10 mg/ml in 30% MeCN) and dried to afford crystals. These were subjected to MALDI-TOF MS analysis on an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a reflector and controlled by the FlexControl 3.3 software. The masses of the precursor ions and fragment ions obtained in reflector mode were annotated using the FlexAnalysis 3.3 software. The relative amounts of the *O*-glycans were quantified against 1 pmol of internal standard. The glycans were structurally identified by mass spectrometric analysis as well as by using the GlycoSuite database (http://glycosuitedb.expasy.org/glycosuite/glycodb). All experiments were done at least twice and the data obtained were consistent and reproducible among the experiments.

2.4. Preparation of E. histolytica membranes

E. histolytica HM1:IMSS trophozoites were cultured in TYI-S-33 medium supplemented with 15% adult bovine serum (GemCell), 2.2% vitamin mix (Sigma), and penicillin/streptomycin (PenStrep, Gibco). Sub-confluent cultures of the parasites were harvested and the trophozoites were washed twice with wash buffer (75 mM Tris–HCl, pH 7.2, with 65 mM NaCl) at 200×*g* for 5 min at 4 °C, after which a 9-fold volume of osmotic lysis buffer (10 mM Na-phosphate (pH 8.0) with protease inhibitor (EDTA-free) and 5 mM EDTA) was added to the pellet. The mixture was incubate for 5 min at 37 °C followed by resuspension of the pellet (0.5 ml) in 4.5 ml of osmotic lysis buffer and a second incubation at 37 °C for 5 min. The lysed samples were sonicated on ice using a BRANSON Sonifier sonicator until the parasite particles could no longer be detected by light microscopy. The sample was centrifuged at 50,000×*g* for 1 h at 4 °C and the resulting pellet was resuspended in 5 ml of osmotic lysis buffer, followed by centrifugation at 100,000×*g* for 1 h at 4 °C. The pellet was suspended again in 1 ml of osmotic lysis buffer and used as *E. histolytica* membranes in subsequent experiments. The presence of Hgls in the isolated membranes was confirmed by Western blotting using an anti-Hgl antibody (7F4).

2.5. Binding inhibition assay using neo-glycoproteins, E. histolytica membranes, and mouse-derived mucins

Sialidase from *Vibrio cholerae* (Roche) was used as an active or heat-inactivated (at 80 °C for 30 min) enzyme. Mucins from the colorectum of B6, CBA, and BALB mice were incubated at 37 °C overnight with 0.33 μl of the enzyme (active or inactivated) per reaction in a total reaction mixture volume of 2 μl (adjusted with 0.2 M NaCl). Reactions without mucins were prepared as control samples. All samples were incubated at 80 °C for 30 min, mixed with 100 μl of 50-fold diluted *E. histolytica* membranes, and incubated at 4 °C overnight with rotation. $(GalNAc)_{12-27}$-HSA (Tn Antigen-HSA) was purchased (DEXTRA Laboratories, UK) and coated on 96-well plates (NUNC, Denmark) at 10 pmol/100 μl/well at 4 °C overnight. The plate was washed three times with TBST and the incubated samples were loaded onto the plate, which was kept

at 4 °C overnight. On the following day, the plate was washed three times with TBST followed by the addition of 100 μl of 1:5000 diluted HRP-7F4 (2.56 mg/ml, Techlab, USA) to each well and incubation at 4 °C overnight. Finally, the plate was washed three times with TBST and incubated with 100 μl of 1-Step™ Ultra TMB-ELISA solution (Thermo)/well at room temperature for 15 min. The reactions were stopped by the addition of 50 μl of 1 M sulfuric acid/well and absorbance at 450 nm (A_{450}) was subsequently detected using a Labsystems Multiskan MS plate reader. The percentages of binding inhibition were calculated as: binding inhibition (%) = [(A_{450} of sample without mucin) − (A_{450} of sample with mucin)]/(A_{450} of sample without mucin) × 100. Mucins treated with active or inactivated sialidase were compared, with the average of six independent results per group expressed as scatter plots.

2.6. Statistical analysis

In binding inhibition studies, six independent experiments per group were conducted. The Mann–Whitney *U*-test was applied in comparisons of the active-sialidase-treated and inactivated-sialidase-treated groups.

3. Results

3.1. O-glycomics of mucin derived from B6, CBA, and BALB mice as determined by the ammonium carbamate method

After obtaining the mucin samples from B6, CBA, and BALB mice, the *O*-glycomic profiles of the three mouse strains were compared. *O*-glycans were liberated from the mucin samples using ammonium carbamate, and then purified and labeled with aoWR tag using the glycoblotting technique [13–15]. After the removal of excess reagent, the labeled glycans were analyzed by MALDI-TOF MS. As shown in Fig. 1, *N*-glycans not cleaved completely by PNGase F were also released (signals shown with asterisks) by this method, but they were not studied further. These *N*-glycan derived peaks were also observed when the flow-through fractions of PNGase F-treated samples were analyzed by MALDI-TOF MS (data not shown). Fairly similar *O*-glycan profiles were observed among the three mice strains, with the greatest similarity between B6 and BALB samples (Fig. 1). At least 30 types of *O*-glycans were detected (Table 1), 17 of which were neutral while the rest were acidic. *O*-glycans that differed in their expression profile between the *E. histolytica* susceptible strain (CBA) and the resistant strains (B6 and BALB) include *O*-glycans which possess sialic acid residues (Table 1 and Fig. 2). There were no significant differences in the relative intensities of the deduced neutral *O*-glycans among the samples (Fig. 2A), whereas, in general, the expression of sialylated *O*-glycans tended to be lower in CBA mice than in B6 and BALB mice. Specifically, the expression levels of all identified sialylated *O*-glycans, except for Nos. 15 and 29, were 2- to 5-fold higher in B6 and BALB mice than in CBA mice (Fig. 2B). To confirm that each peak obtained by MALDI-TOF MS analyses truly reflected the deduced composition of acidic *O*-glycans, each of the glycans was further fragmented using LIFT TOF/TOF MS mode (Ultraflex III, Bruker) and the resulting fragments were then annotated with the FlexAnalysis 3.3 program. Among the 13 types of deduced acidic *O*-glycans, nine were confirmed to have NeuAc, as determined by MS/MS analyses. A typical spectrum obtained in MS/MS analyses of NeuAc-containing *O*-glycans involves the −305 Da peak (corresponding to a loss of the terminal NeuAc(OMe) from the precursor ion peak), which can be easily detected (Supplemental Fig. 1). The MS/MS spectra for the other four types of deduced sialylated *O*-glycans were obtained but could not be analyzed because of excess noise.

3.2. Binding inhibition assay with sialidase-treated mucins

To assess the role of sialic acids of mucin *O*-glycans in the attachment of *E. histolytica* to mouse intestinal mucins, binding inhibition assays using $(GalNAc)_{12-27}$-HSA coated microtiter plates were conducted, as shown in Fig. 3A. The assay is based on the binding of Hgls on the *E. histolytica* membrane to GalNAc of $(GalNAc)_{12-27}$-HSA in the absence of mucins or in the presence of untreated mucin in which the sugars have been masked by sialic acids. However, if the Gal and GalNAc sugars on these mucins are exposed by sialidase treatment, they compete with Hgl binding and interfere with this association. If the mucins contain low levels of sialic acids, there will be no difference between the addition of sialidase-treated mucin and the control mucin treated with heat-inactivated sialidase. Thus, to confirm the role of sialic acids in parasite attachment, the samples were treated with sialidase before their inclusion in the inhibition assay (Fig. 3). The amounts of mucin used in this study were converted to and expressed as "Starting mucus amount (mg, wet weight)" because quantification of mucins is difficult using traditional protocols for quantifying protein concentrations due to the high density of *O*-glycans attached to mucin molecules. For B6 mucins, the results showed slight, but not significant, differences in sialidase (+SA)- vs. inactivated sialidase (−SA)-treated mucins (Fig. 3B), indicating that sialic acid of B6 mucin inhibits *E. histolytica* membrane binding to some extent. For CBA mucins, there was no difference in the ability of sialidase- and inactivated-sialidase-treated CBA mucins to compete with Hgl binding to $(GalNAc)_{12-27}$-HSA (Fig. 3C). This observation suggested that CBA mucins contain relatively few sialic acids, in agreement with the results obtained by MALDI-TOF MS/MS analysis (Figs. 1 and 2). As predicted, but nonetheless surprising, sialidase-treated BALB mucins exhibited significantly higher binding inhibition than inactivated-sialidase-treated BALB mucins, both at lower (0.8 mg of mucus) and higher (1.7 mg of mucus) amount of mucin. Strikingly, in some experiments, inactivated-sialidase-treated BALB mucins enhanced *E. histolytica* membrane binding to $(GalNAc)_{12-27}$-HSA, suggesting their strong inhibitory potential (Fig. 3D). These observations are in good agreement with the above-described results of the glycomic analysis.

4. Discussion

The most important finding of this work was the differences in the sialic acids content of large intestinal mucins among the three strains of mice tested, as it can, at least in part, explain the observed differential susceptibilities to *E. histolytica* infection. Our results are consistent with those reported by Asgharpour et al. [3], who found that *E. histolytica* persisted in the large intestine of CBA mice for more than 30 days after infection while the parasite was cleared 4 days after the infection of B6 mice. Houpt et al. also reported that BALB mice were resistant to *E. histolytica* infection [4] and that their resistance was H-2 haplotype and lymphocyte independent. Since Hgls are crucial for *E. histolytica* infection, these earlier findings led us to examine the role of host mucin *O*-glycans as the basis for the different murine susceptibilities.

Indeed, the sialic acids content of mucins isolated from the large intestines of B6 and BALB mice were higher than those isolated from CBA mice (Fig. 2). This observation suggested that the greater attachment of *E. histolytica* to the large intestinal mucus of CBA mice is due to the higher affinity of Hgl for Gal/GalNAc (neutral sugars) than for acidic sugars [16]. It would also explain the parasite's ability to remain for more than 30 days post-infection in the large intestine of CBA mice but not in the intestines of B6 or BALB mice [3,4]. To confirm this hypothesis, we conducted a binding inhibition assay using a neo-glycoprotein, *E. histolytica* membrane,

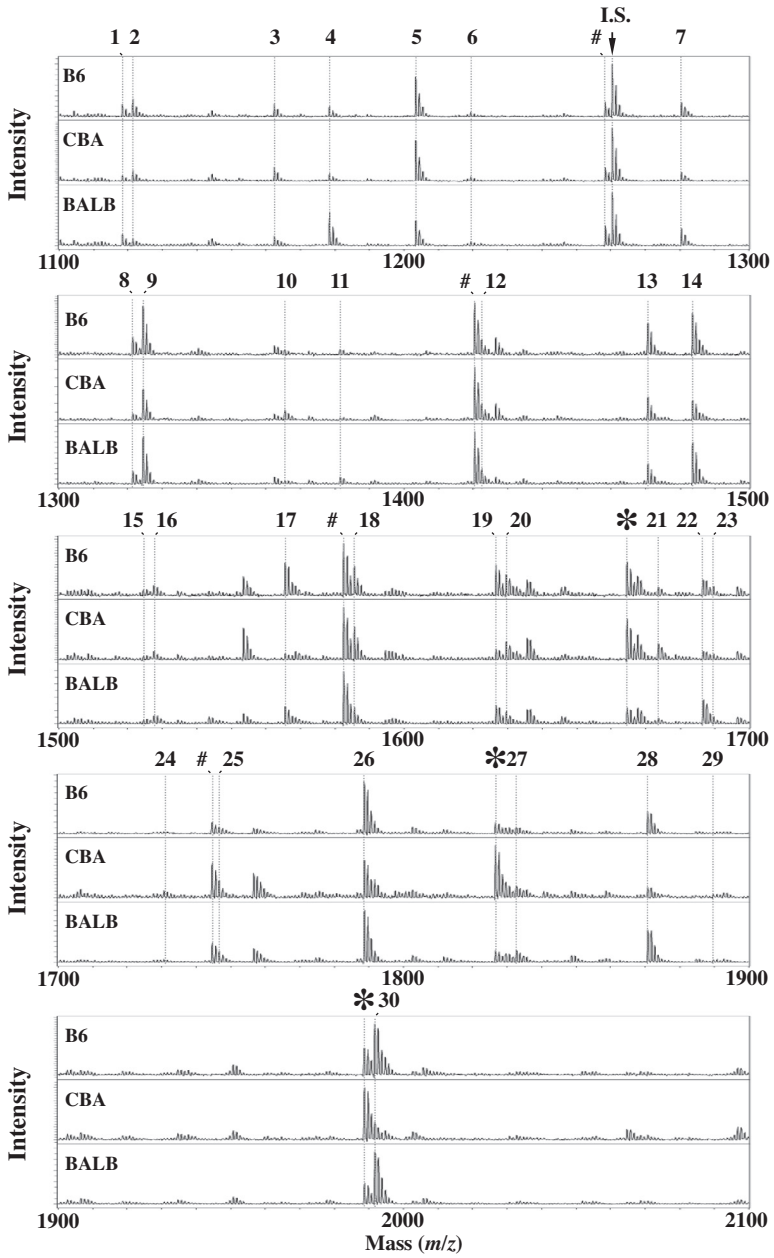

Fig. 1. *O*-glycan analyses of colorectal glycoproteins from B6, CBA, and BALB mice. MALDI-TOF MS chart (m/z = 1100–2100) of ammonium–carbamate-released *O*-glycans. The numbers on the top of each chart correlate with the *O*-glycans shown in Table 1. Asterisks indicate *N*-glycans remaining on mucins even after PNGase F treatment but released after ammonium carbamate treatment, and hash signs indicate the contaminated hexose oligomers. I.S. indicates internal standard (GN4).

and mucins derived from mouse large intestines. The results showed that mucin derived from resistant BALB mice could not inhibit *E. histolytica* membrane binding to a neo-glycoprotein, whereas inhibition was increased when the mucin was first treated with sialidase (Fig. 3D). On the other hand, regardless of sialidase treatment, CBA mucin was able to inhibit *E. histolytica* membrane binding to $(GalNAc)_{12-27}$-HSA, suggesting that it did not contain a large amount of sialic acids (Fig. 3C). However, glycans from BALB and B6 while having similar sialic acid content/structures (Fig. 2) behave differently in the binding assay (Fig. 3B and D). This might be because the sites and density of *O*-glycans having sialic acids on the mucins differ between these two mouse strains and the reason why the parasites persisted in B6 mice for 4 days but BALB mice were completely resistant to this infection. Further study needs to be done to clarify this possibility in the future. The presence of sulfation on colonic mucin *O*-glycans might be also important for the resistance, even though we could not detect sulfated *O*-glycans in this study probably because the amount of those glycans were very few, as previously observed for human colonic MUC2 [17].

The expression patterns of colorectal mucus *O*-glycans were mouse strain-specific (Figs. 1 and 2), with an abundance of certain acidic *O*-glycans detected on B6 and BALB colorectal mucins but not on CBA mucins. Thus, seven sialylated *O*-glycans were more highly expressed in the colorectal mucins of B6 and BALB mice than in those of CBA mice (Fig. 2B). All the deduced structures in Table 1, except HexNAc4NeuAc2, have been published previously or are available in the GlycoSuite database [18,19] (http://glycosuitedb.expasy.org/glycosuite/glycodb). The differential *O*-glycans expression observed among the three investigated mouse strains might reflect differences in the expression levels and patterns of glycosyltransferases in the organs responsible for generating these compounds, including sialyltransferases in

Table 1
Deduced composition of *O*-glycans.

Number	Mass (*m*/*z*)	Deduced composition of *O*-glycan
1	1118	Hex1HexNAc1NeuAc1
2	1121	Hex2HexNAc1DeoxyHex1
3	1162	Hex1HexNAc2DeoxyHex1
4	1178	Hex2HexNAc2
5	1203	HexNAc3DeoxyHex1
6	1219	Hex1HexNAc3
7	1280	Hex2HexNAc1NeuAc1/ Hex1HexNAc1DeoxyHex1NeuGc1
8	1321	Hex1HexNAc2NeuAc1
9	1324	Hex2HexNAc2DeoxyHex1
10	1365	Hex1HexNAc3DeoxyHex1
11	1381	Hex2HexNAc3
12	1422	Hex1HexNAc4
13	1470	Hex2HexNAc2DeoxyHex2
14	1483	Hex2HexNAc2NeuAc1
15	1524	Hex1HexNAc3NeuAc1
16	1527	Hex2HexNAc3DeoxyHex1
17	1565	HexNAc4NeuAc1
18	1585	Hex2HexNAc4
19	1626	Hex1HexNAc2NeuAc2
20	1629	Hex2HexNAc2DeoxyHex1NeuAc1
21	1673	Hex2HexNAc3DeoxyHex2
22	1686	Hex2HexNAc3NeuAc1
23	1689	Hex3HexNAc3DeoxyHex1
24	1730	Hex2HexNAc4DeoxyHex1
25	1746	Hex3HexNAc4
26	1788	Hex2HexNAc2NeuAc2
27	1832	Hex3HexNAc3DeoxyHex2
28	1870	HexNAc4NeuAc2
29	1889	Hex2HexNAc4NeuAc1
30	1991	Hex2HexNAc3NeuAc2

the mouse large intestine. Support for this possibility comes from a study of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases, one of the glycosyltransferase families responsible for mucin *O*-glycosylation, in which different expression patterns were determined in the kidneys of the examined mouse strains [20]. So far, 20 isoforms of mouse sialyltransferases have been reported [21]. It will be of interest in the future to test which sialyltranseferase is responsible for determining the susceptibility of the mice to *E. histolytica*.

In conclusion, in colorectal mucin samples, the profiles of *O*-glycans, and especially of those containing sialic acid, differed among B6, CBA, and BALB mice. The susceptibilities of those mice to *E. histolytica* infection may thus be determined by the expression levels of sialylated *O*-glycans, as measured in the *E. histolytica* lectin binding inhibition assay conducted in this study. Our results contribute to explaining some of the differences in the outcomes of *E. histolytica* infection studies in mice and to a better understanding of the basis of infectious diseases.

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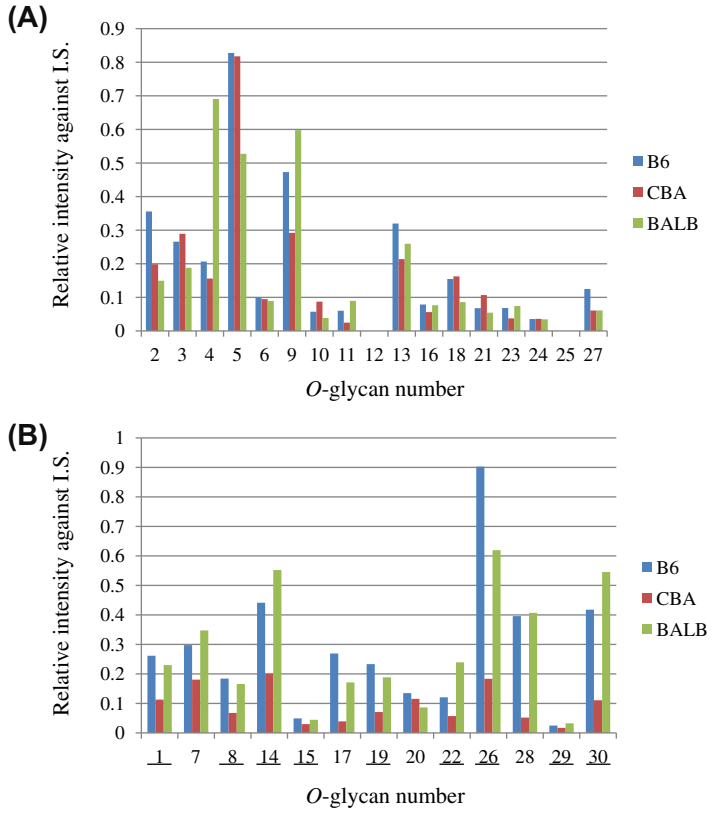

Fig. 2. Comparison of neutral and acidic *O*-glycans derived from the colorectal mucins of three mouse strains. Relative intensities of colorectum-derived neutral (A) and acidic (B) *O*-glycans calculated with respect to the intensity of the internal standard (GN4). Acidic *O*-glycans from the colorectum of B6, CBA, and BALB mice are shown with blue, red, and green bars, respectively. Underlined *O*-glycan numbers indicate *O*-glycans containing sialic acids, as confirmed by MALDI-TOF MS/MS (Supplemental Fig. 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

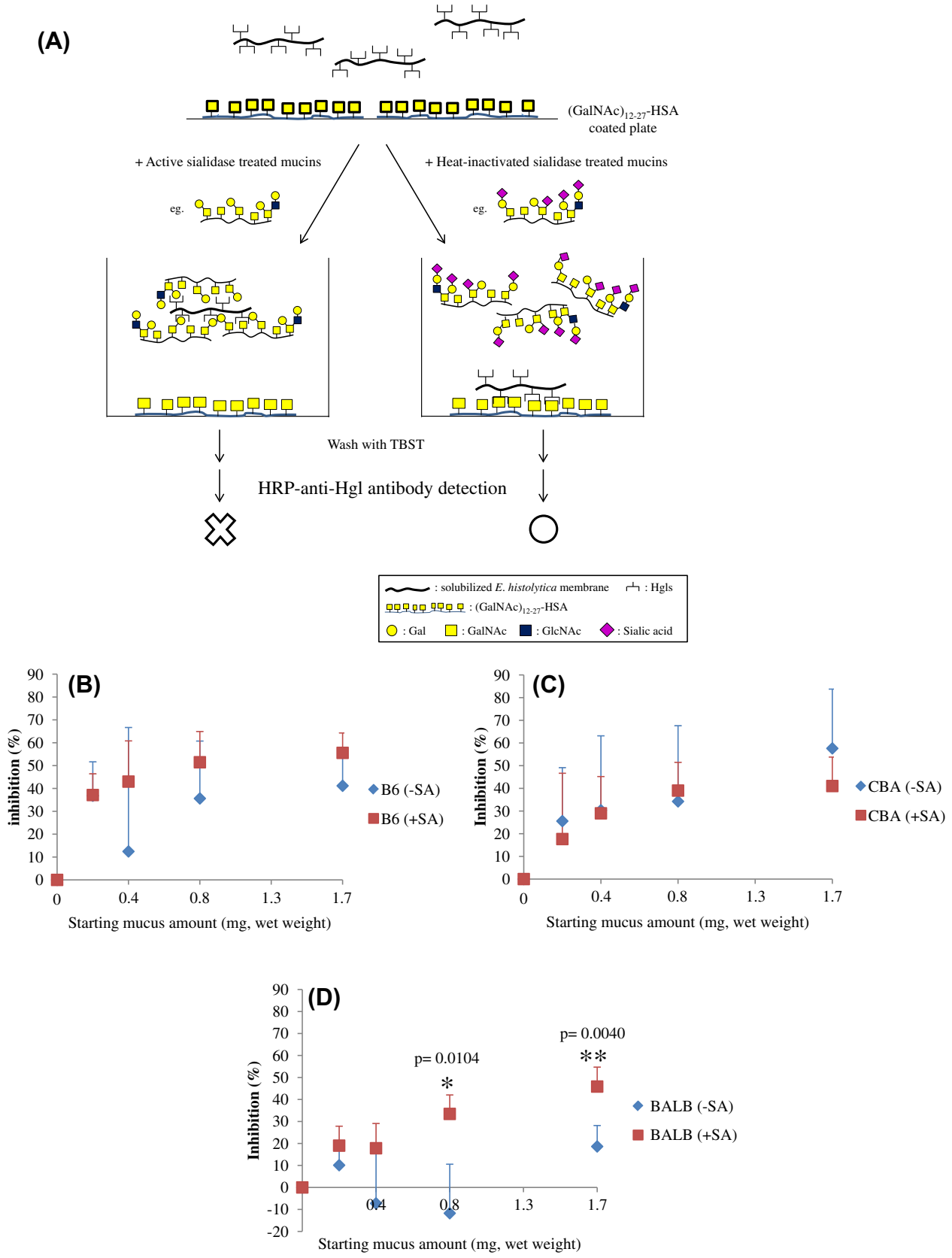

Fig. 3. *Entamoeba histolytica* membrane binding inhibition assay using a neo-glycoprotein. (A) Schema of the inhibition assay. (B) B6 mucins treated with sialidase [B6 (+SA)] or heat-inactivated sialidase [B6 (-SA)] were incubated with *E. histolytica* membranes and loaded onto a $(GalNAc)_{12-27}$-HSA-coated microtiter plate. Six independent experiments were conducted for each condition. The results are shown as a scatter plot with standard deviations. The results obtained with (C) CBA mucins and (D) BALB mucins. Asterisks indicate significant differences between sialidase- and inactivated-sialidase-treated BALB mucins, as determined in a Mann–Whitney's *U*-test ($^{*}p < 0.05$, $^{**}p < 0.01$).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.085.

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