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Distribution of Receptor Glycolipids for *Lactobacilli* in Murine Digestive Tract and Production of Antibodies Cross-reactive with them by Immunization of Rabbits with *Lactobacilli**

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In the digestive tract of mice (HR-1 strain), glycolipids belonging to the ganglio-series were revealed to be expressed in region-specific manners, *i.e.* FGA1 and FGM1 in the stomach, GA1 in the small intestine, and FGA1 and sulphatides in the cecum. The amount of GA1 as a receptor glycolipid for *Lactobacilli* was especially higher in the small intestine than in the other regions, it comprising 1.6–2.8 μg/mg dry weight. On immunization of rabbits with *Lactobacillus johnsonii* and *Lactobacillus intestinalis*, both of which are murine intestinal bacteria, antibodies toward bacterial glycolipids, *i.e.* Galα1–2Glcα1–3DG, and tri- and tetrahexaosyl DGs, were effectively generated and, in addition, they were found to cross-react with GA1 and GalCer, but not with structurally related glycolipids such as Lc_4Cer, nLc_4Cer and $IV^3Gal\alpha\text{-}nLc_4Cer$, indicating that GA1 is a preferable antigen for anti-lactobacillus antisera and suggesting the presence of epitopes common to both *Lactobacilli* and the host. In fact, molecules reacting with anti-GA1 antibodies were detected among bacterial proteins on Western blotting, indicating a possible occurrence of the carbohydrate structure mimicking GA1 in bacterial proteins.

Key words: asialo GM1, bacterial receptor, digestive tract, glycoglycerolipids, TLC-immunostaining.

Abbreviations: CL, cardiolipin; CMH, ceramide monohexoside; CS, cholesterol sulphate; DG, diacyl glycerol; Hep, L-glycero-D-mannoheptose; FGA1, fucosyl asialo GM1; FGM1, fucosyl GM1; GA1, asialo GM1; PG, phosphatidyl glycerol; Sul, sulphatide.

Glycolipids are ubiquitous membrane components of mammalian tissues and cells, and their carbohydrate moieties are included in antigens concerning blood group, species specificity and cellular differentiation and transformation, and receptors for bacteria, bacterial toxins and viruses (*1*). In the murine digestive tract, glycolipids belonging to the globo- and ganglio-series are separately distributed in the mesenchymal and epithelial tissues, respectively, and GA1, as the backbone structure of ganglio-series glycolipids, is expressed in association with cellular differentiation from the crypt to the villus in the intestinal microvilli (*2*), and provides receptors for several bacteria, such as *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus johnsonii* (*L. acidophilus*), *Bifidobacterium bifidum*, *Pseudomonas aeruginosa*, *Actinomyces maeslundii* and *Neisseria gonorrhoeae* (*1*). However, the carbohydrate moiety of GA1 has been revealed to be modified through transcriptional regulation of the sugar transferase gene on bacterial infection (*3–6*). Under conventional breeding conditions, expression of FGA1 from the fucosyltransferase gene in the small intestine occurs during the postnatal period from early suckling to weaning (*7–9*), and is enhanced by food containing dietary fibre at the time of weaning (*10*). Fucosylation does not occur under germ-free conditions, but infection by indigenous filamentous bacteria and wild-type *Bacteroides thetaiotaomicron* in germ-free mice triggers expression of the FUT2 gene, a counterpart of the human secretor (Se) gene, for synthesis of FGA1 only in the small intestine, *i.e.* not in other regions of the digestive tract (*5*, *6*). In fact, differences in the regulation of gene expression among different regions have been revealed by targeted deletion of the FUT1 and FUT2 genes (*6*, *11*). Although fucosylated glycolipids completely disappear from the antrum, cecum and colon of FUT2-null mice, those in the small intestine of FUT2-null mice are maintained at similar levels to those in the wild-type (*6*). In contrast, the amounts of fucosylated glycolipids in the stomach, cecum and colon are not affected by targeted deletion of the FUT1 gene, but those in the small intestine of FUT1-null mice are rather increased compared to in wild-type and

*The nomenclature for glycolipids and gangliosides is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [IUPAC-IUB Commission on Biochemical Nomenclature. (1977) The nomenclature of lipids. *Eur. J. Biochem.* **179**, 11–21] and Svennerholm [Svennerholm, L. (1963) Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**, 613–623], respectively.

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FUT2-null mice (*6*). These findings indicate that fucosylation in the antrum, cecum and colon is preferentially due to the FUT2 gene, but that in the small intestine to either the FUT1 or FUT2 gene. Accordingly, fucosylation of glycolipids in the stomach, cecum and colon due to the FUT2 gene occurs in a development-dependent manner, but that in the small intestine is regulated by the surrounding circumstances in mice, *i.e*. suppression of FUT2-gene expression under germ-free conditions, and activation of FUT2-gene expression under conventional breeding conditions and targeted deletion of the FUT1 gene. Thus, expression of GA1 in the epithelial cells of the digestive tract is actively regulated through modification of the carbohydrate structures, and is thought to influence the colonization of bacteria having the ability to bind with GA1 as a receptor, particularly of symbiotic bacteria, *i.e*. *Lactobacillus* and *Bifidobacterium* (*12–16*), which play a role in protection from infection by harmful bacteria passing into the digestive tract together with food.

However, the profile of glycolipid expression under region-specific regulation in the digestive tract is not clearly understood yet, and therefore we determined the glycolipid compositions in different regions of the digestive tract of conventional breeding mice, with special reference to the receptor distribution for intestinal *Lactobacilli*, the receptor glycolipids for *L. johnsonii* having been characterized as GlcCer, Gb_3Cer, GA1, nLc_4Cer and Lc_4Cer (*13*). In addition, we prepared antisera by immunization of rabbits with *L. johnsonii* and *L. intestinalis*, both of which are murine intestinal bacteria, and found that the antisera cross-reacted with GalCer and GA1, indicating the occurrence of an intestinal glycolipid-like structure in *Lactobacilli*, which might be related with a mechanism for evading immunological surveillance by the host for symbiosis with *Lactobacilli* in the digestive tract of mice.

MATERIALS AND METHODS

Lactobacilli—Lactobacillus johnsonii (*L. acidophilus*) (JCM No. 1022), *Lactobacillus casei* (JCM No. 1134) and *Lactobacillus intestinalis* (JCM No. 7548) were purchased from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center (Wako, Saitama, Japan), and cultured in *Lactobacilli* MRS broth (Difco, Beckton-Dickinson, Sparks, MD, USA).

Glycolipids and phospholipids—The glycolipids used in this experiment were purified from various sources in our laboratory: Galα1–2Glcα1–3DG from *L. casei* (*17*), GlcCer, LacCer, Gb_3Cer, Gb_4Cer, GM3 and IV^3NeuAc-nLc_4Cer from human erythrocytes, $IV^3GalNAc\alpha$-Gb_4Cer (Forssman antigen) from equine kidney, Gg_3Cer from guinea pig erythrocytes, GalCer containing non-hydroxy fatty acids (GalCer NFA) and 2-hydroxy fatty acids (GalCer HFA), sulphatides and GM1 from bovine brain, FGM1 from bovine thyroid, Lc_4Cer from human meconium and $IV^3Gal\alpha$-nLc_4Cer from rabbit erythrocytes (*18*). GA1, FGA1 and nLc_4Cer were prepared from GM1, FGM1 and IV^3NeuAc-nLc_4Cer, respectively, by treatment with *Arthrobacter ureafaciens* sialidase (*19*). *N*-Stearoyl glycolipids, as standards, for TLC-densitometry were prepared by deacylation with sphingolipid ceramide *N*-deacylase (*Pseudomonas sp*. TK4), followed by reacylation with stearoyl chloride. Dioleoyl derivatives of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl glycerol (PG), phosphatidic acid (PA) and sphingomyelin (SM) from human erythrocytes were kindly provided by Alfresa Pharma Co. (Osaka), and cardiolipin (CL) and phosphatidyl inositol (PI) were purchased from Sigma (St Louis, MO, USA). The concentrations of standard phospholipids in chloroform/methanol (1:1, v/v) were determined by the phosphomolybdate procedure after decomposition of the lipids with 70% $HClO_4$ and H_2O_2 (*20*).

Antisera—Rabbit polyclonal antibodies toward *L. johnsonii* and *L. intestinalis* were generated by immunizing rabbits (Japanese White; Japanese Biological Materials, Tokyo) intradermally with a water-in-oil emulsion prepared by mixing 15 mg of each bacterium in 1 ml of phosphate-buffered saline (PBS) with 1 ml of Freund's complete adjuvant (Sigma), and the antibody titres were subsequently monitored by enzyme-linked immunosorbent assaying (ELISA) with the respective bacterium (2 μg/well) as the antigen. In a similar way, polyclonal antibodies toward Forssman antigen and GA1 were generated by immunizing rabbits with 1 mg of glycolipids together with Freund's complete adjuvant, and also monoclonal antibodies toward FGM1 and FGA1 were prepared by immunizing mice with 20 μg of glycolipids together with *Salmonella minnesota* as the adjuvant, followed by hybridization of lymphocytes with murine myeloma P3X63Ag8. Anti-Forssman, anti-GA1, anti-FGM1 and anti-FGA1 antibodies generated characteristically reacted with the respective glycolipid antigens and no cross-reaction to structurally related glycolipids were observed (*5*, *21*).

Quantitation of lipids in murine tissues and bacteria—Mice (HR-1, 10 weeks of age) were kept under conventional breeding conditions at a room temperature of $24 \pm 1°C$ and a humidity level of $55 \pm 10\%$ with food and water *ad lib*. Animal treatment followed the animal care guidelines of Kinki University.

Tissues, *i.e*. stomach, duodenum, jejunum, ileum, cecum and colon, were rinsed with PBS and then lyophilized. Total lipids were extracted from the lyophilized tissues with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1:0, v/v/v), and then fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; GE Healthcare Bioscience, Piscatway, NJ, USA). Then, the neutral glycolipids were separated from the unabsorbed neutral lipid fraction by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the absorbed acidic lipid fraction containing gangliosides and cholesterol sulphate (CS) was saponified with 0.5 M NaOH in methanol to remove the ester-containing lipids, followed by dialysis (*22*, *23*).

The total lipids, and acidic and neutral lipids thus obtained were examined by TLC. The following solvent mixtures were used as the developing solvents for TLC, chloroform/methanol/water (65:35:8, v/v/v) for phospholipids and neutral glycolipids, chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v/v) for cholesterol sulphate and sulphatides, and chloroform/methanol/0.5%

$CaCl_2$ (55:45:10, v/v/v) for gangliosides, and the detection reagents were as follows, cupric acetate–phosphoric acid reagent for neutral lipids, phospholipids and CS, Dittmer's reagent for phospholipids, orcinol–sulphuric acid reagent for glycolipids and resorcinol–hydrochloric acid reagent for gangliosides. The density of spots on TLC plates was determined by image analysis (NIH image). Standard lipids, *i.e*. *N*-stearoyl derivatives of GalCer, LacCer, Gb_3Cer, GM3, Gb_4Cer, Forssman glycolipid, GA1, GM1, FGM1 and GD1a, and dioleoyl derivatives of PE, PS, PC, PG and PA, and CS (0.1–1.5 μg), were developed on the same TLC plates for the preparation of standard curves (*24*). Analysis of lipids from *Lactobacilli* was carried out by the same procedure, but quantitation of mono- to tetrahexaosyl DGs in *Lactobacilli* was performed using the standard curves for mono- to tetrahexaosyl ceramides as described above.

TLC-immunostaining—Lipids were developed on plastic-coated TLC plates (Macherey-Nagel, Düren, Germany), which were then blocked with blocking buffer (PBS containing 1% polyvinylpyrrolidone and 1% ovalbumin), and the spots were visualized by immunostaining with the above anti-glycolipid and anti-lactobacilli antibodies (1:500) diluted with dilution buffer (PBS containing 3% polyvinylpyrrolidone), followed by immunostaining with peroxidase-conjugated anti-rabbit IgG and IgM (1:1,000; Jackson Immunoresearch Lab., PA, USA), and anti-murine IgG and IgM antibodies (1:1,000; Sigma), and peroxidase substrates, 4-chloro-1-naphthol and H_2O_2, according to the procedure reported previously (*25*). Control staining with normal rabbit serum was performed simultaneously under the same conditions.

SDS–PAGE and Western blotting—*Lactobacilli* were suspended in PBS by ultrasonication, and the protein concentration of the resulting solution was measured by Bradford's procedure with bovine serum albumin as the standard protein (*26*). Then the solution, corresponding to 8 μg of protein, was denatured with 15 μl of sample buffer [1% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.125% bromophenol blue in 0.06 M Tris–HCl (pH 6.8)] at 95°C for 4 min, and then electrophoresed on an acrylamide gel (12%), which was stained with Coomassie brilliant blue (CBB) for proteins (*27*). Also, proteins on a SDS–PAGE gel were electrically transferred to a nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Lab., Hercules, CA, USA), and the membrane was immunostained with anti-lactobacilli and anti-GA1 antibodies according to the procedure for TLC-immunostaining described above.

RESULTS AND DISCUSSION

Glycolipid receptors for Lactobacilli in the murine digestive tract—As shown in Figs 1 and 2, the glycolipids, particularly ganglio-series ones, were revealed to be distinct in different regions of the murine digestive tract. Although Forssman glycolipid was present in all regions of the tract, amounting to 0.12–1.11 μg/mg dry weight, GA1 as a receptor for intestinal *Lactobacilli* was predominant in the duodenum, jejunum and ileum, in comparison to in the stomach, cecum and colon, where it was not detectable or was only present in a trace amount, as judged on TLC-immunostaining with anti-GA1 antibodies (Fig. 2). The amounts of GA1 in the duodenum, jejunum and ileum were 1.6, 2.8 and 2.2 μg/mg dry weight, respectively, corresponding to ~36–50% of the total neutral glycolipids. In contrast, FGA1 was abundant in the stomach and cecum, amounting to 0.9 and 1.6 μg/mg dry weight, respectively, corresponding to ~20% of the total neutral glycolipids. On comparison of the ratio of FGA1 to GA1 plus FGA1 as the rate of fucosylation of GA1, GA1 in the stomach, cecum and colon was revealed to be almost completely converted to FGA1, but the fucosylation rates in the duodenum, jejunum and ileum were only 8, 0.3 and 10%, respectively (Table 1). On the other

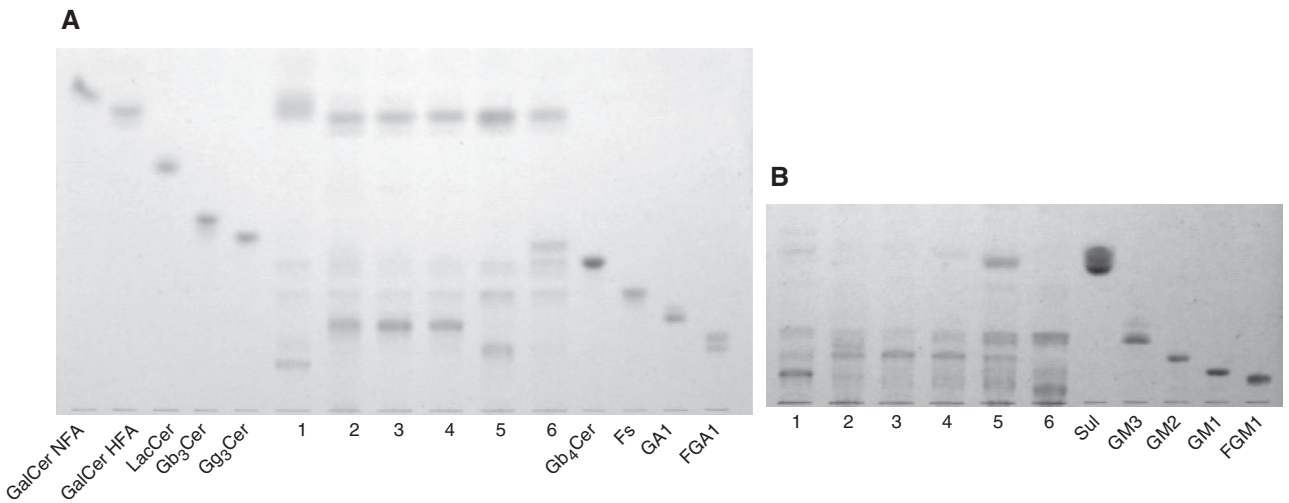

Fig. 1. **TLC of neutral (A) and acidic (B) glycolipids from different regions of the murine digestive tract.** Neutral glycolipids and gangliosides, corresponding to 0.5 mg dry weight, were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v) for A and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) for B, and were detected with orcinol–sulphuric acid reagent. 1, Stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon.

hand, FGM1 was abundant in the stomach, in which the concentration of FGM1 was similar with that of FGA1, and fucosylation of GM1 was almost complete like that of GA1, suggesting that the fucosyltransferase equally acts on GA1 and GM1 in the stomach (Fig. 1) (*5*).

Ceramide monohexosides (CMH) were the most abundant neutral glycolipids in all regions, amounting to 1.7–4.1 μg/mg dry weight. As shown in Fig. 1, gastric CMH migrated to a similar position on a TLC plate to GalCer containing 2-hydroxy fatty acyl sphingosine, but to a higher position than intestinal CMH, which is composed of 2-hydroxy fatty acyl phytosphingosine, as reported in the literature (*2*).

As to the acidic lipids, the distribution of sulphatides was restricted to the stomach and cecum, and the amount in the cecum was significantly higher than those in the other regions. Also, CS and GM3 were uniformly distributed in the tract, their highest amounts being observed in the ileum and colon, respectively.

Then the molar ratios of individual lipids to CMH were calculated using their mean molecular weights [behenic acid (22:0)-containing ones], that is, 783 for CMH, 1,310 for Gb_4Cer, 1,513 for Forssman glycolipid, 1,310 for GA1, 1,456 for FGA1, 466 for CS and 1,236 for GM3, the CMH:Gb_4Cer:Forssman glycolipid:GA1:FGA1:CS:GM3 ratio in the jejunum being found to be 1.00:0.05:0.04:0.73:0.002:0.17:0.05, which resembled the reported ratio (*2*), showing that the jejunum as well as the duodenum and ileum contain GA1 in relatively high molar proportions.

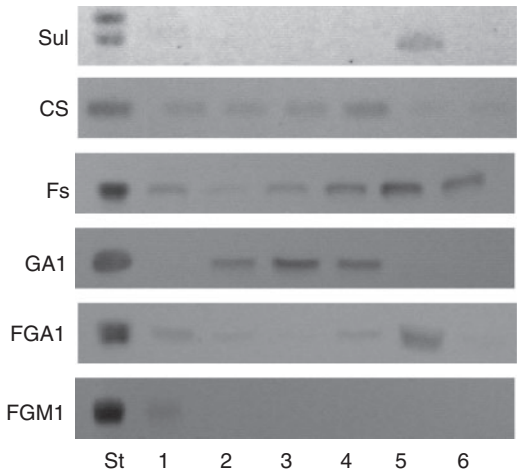

Fig. 2. **TLC- and TLC-immunostaining of lipids from different regions of the murine digestive tract.** For CS and sulphatides (Sul), acidic lipids, corresponding to 0.5 mg dry weight, were developed on TLC plates with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v/v), and were detected with cupric acetate–phosphoric and orcinol–sulphuric acid reagents, respectively. For TLC-immunostaining of glycolipids, total lipid extracts, corresponding to 0.1 mg dry weight, were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v), followed by detection with anti-Forssman (Fs), anti-GA1, anti-FGA1 and anti-FGM1 antibodies. 1, Stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon.

Thus, the following glycolipids were abundant in the murine digestive tract in region-specific manners, FGA1 and FGM1 in the stomach, GA1 in the small intestine, FGA1 and sulphatides in the cecum. Accordingly, the small intestine seemed to be the site for colonization by *Lactobacilli*, because GA1 was characterized as the receptor with strongest affinity toward *L. johnsonni, L. casei* and *L. reuteri* (*13*).

Bacterial lipids—Extensive studies on the structures of bacterial lipids including those of *Lactobacillus* species have appeared in the literature (*28*, *29–33*). In accord with previous reports, CL and PG were the major phospholipids in *L. johnsonii* and *L. intestinalis*, amounting to 0.12–0.65 μg of dry weight (Table 2) (*33*). Also, the major glycolipid, whose mobility on a TLC plate was similar with that of Galα1–2Glcα1–3DG from *L. casei*, was present in both bacteria, amounting to 1.03 μg/mg in *L. johnsonii* and 0.41 μg/mg in *L. intestinalis*, as determined by TLC-densitometry with *N*-stearoyl LacCer as the standard for quantitation. The other glycolipids in *L. johnsonii* were supposed to be Glcα1–3DG, Glcβ1–6Galα1–2Glcα1–3DG and Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG on the bases of their mobilities on a TLC plate,

Table 2. **Amounts of lipids in *Lactobacilli*.**

	L. johnsonii (μg/mg dry weight)	*L. intestinalis* (μg/mg dry weight)
CL	0.13	0.65
PG	0.12	0.21
Monohexaosyl DG (Glcα1–3DG)	0.15	tr
Dihexaosyl DG (Galα1–2Glcα1–3DG)	1.03	0.41
Trihexaosyl DG (Glcβ1–6Galα1–2Glcα1–3DG)	0.39	0.03
Tetrahexaosyl DG (Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG)	0.61	–

Quantitative determination of lipids was performed by TLC-densitometry with the following standards, CL from bovine heart, dioleoyl PG and N-stearoyl derivatives of GalCer, LacCer, Gb_3Cer and Gb_4Cer. tr, trace amount. Values are the means for three different experiments.

Table 1. **Amounts of lipids in the murine digestive tract (microgram/milligram dry weight).**

	Cho	CL	PE	PG	PC/PS	SM	CMH	Gb_4Cer	Fs	GA1	FGA1	FGM1	Sul	CS	GM3
Stomach	6.5	0.5	5.9	1.5	7.3	1.6	3.5	0.37	0.25	–	0.87	0.79	0.20	0.19	0.40
Duodenum	7.4	0.7	6.1	1.6	11.1	1.3	1.7	0.21	0.12	1.6	0.17	–	–	0.16	0.25
Jejunum	5.5	0.8	4.7	1.2	8.1	1.1	2.3	0.21	0.16	2.8	0.01	–	–	0.23	0.20
Ileum	6.1	0.2	4.0	1.0	6.6	0.9	2.4	0.69	0.33	2.2	0.28	–	tr	0.42	0.39
Cecum	6.3	0.3	5.3	1.2	7.5	1.2	4.1	0.53	1.11	tr	1.56	–	0.88	0.11	0.90
Colon	7.1	0.3	5.3	0.8	7.1	1.2	2.4	0.82	0.57	–	0.08	–	–	0.11	1.20

Cho, cholesterol; Fs, Forssman glycolipid; Sul, sulphatides; tr, trace amount. Values are the means for three different experiments.

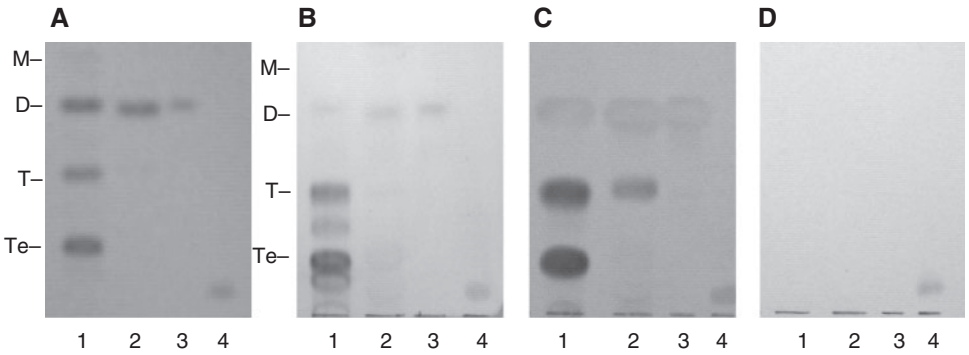

Fig. 3. **TLC-immunostaining of lipids from *Lactobacilli*.** Lipid extracts of *L. johnsonii* (*1*) and *L. intestinalis* (*2*), corresponding to 1 mg dry weight for A, and 0.1 mg dry weight for B–D, were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v), and the spots were visualized with cupric acetate–phosphoric acid (A), anti-*L. johnsonii* (B), anti-*L. intestinalis* (C), and anti-GA1 (D) antisera. 3, Galα1–2Glcα1–3DG from *L. casei*; 4, GA1. M, D, T and Te indicate the positions of mono-, di-, tri- and tetrahexaosyl DGs, respectively.

as described in the literature (*28*, *29–32*). Tri- and tetraglycolipids were present in *L. johnsonii* at significantly high concentrations, but were only present in trace amounts in *L. intestinalis*.

Bacterial antigens reacting with anti-L. johnsonii and anti-L. intestinalis antisera—Immunization of rabbits with *L. johnsonii* and *L. intestinalis* yielded antisera with ELISA titres of more than 1:12,800. Both the anti-*L. johnsonii* and anti-*L. intestinalis* antisera reacted with Galα1–2Glcα1–3DG in *L. casei*, as well as that in *L. johnsonii* and *L. intestinalis*, but not with monohexaosyl DG (Glcα1–3DG). Although tri- and tetrahexaosyl DGs were not detectable in *L. intestinalis*, even on spotting of lipids corresponding to 5 mg dried bacteria, those in *L. johnsonii* were intensively stained with anti-*L. intestinalis* antisera to a similar levels to in the case of anti-*L. johnsonni* antisera, indicating their strong antigenicities (Fig. 3). In fact, the relative densities of spots/μg of Galα1–2Glcα1–3DG were significantly lower than those of tri and tetrahexaosyl DGs, *i.e.* Glcβ1–6Galα1–2Glcα1–3DG and Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG, respectively (*28*,*32*). However, since Galα1–2Glcα1–3DG was widely distributed in *Lactobacillus* species in relatively higher amounts than those of tri- and tetrahexaosyl DGs, it was revealed to contribute to a *Lactobacillus* antigen, as already reported by others (*30*, *34*).

Similarly, the strong antigenicity of diglucosyl DGs, *i.e.* kojibiosyl (Glcα1–2Glc) DG in *Acholeplasma laidlawi* and gentibiosyl (Glcβ1–6Glc) DG in *Mycoplasma neurolyticum*, has been well studied (*34*), and antibodies towards digalactosyl DG have been reported not to cross-react with GalCer or gangliosides (*35*, *36*), but to be involved in the production of natural antibodies in patients suffering from multiple sclerosis (*37*). Accordingly, one can suggest that *Lactobacillus* antigens including glycolipids are also involved in the production of natural antibodies.

Lipid antigens reacting with anti-lactobacillus antisera in murine tissues—Anti-*L. johnsonii* and anti-*L. intestinalis* antisera contained antibodies that reacted with GA1, but glycolipids that reacted with anti-GA1 antibodies were not present in the lipids from either bacterium (Fig. 3). Therefore, antigens reactive with antisera were explored by TLC-immunostaining with lipids from the murine digestive tract and several standard glycolipids. Among the glycolipids examined, GalCer and GA1 were reactive with antisera, but structurally related glycolipids such as GlcCer, LacCer, Gb_3Cer, Gg_3Cer, Gb_4Cer, Forssman glycolipid, Lc_4Cer, nLc_4Cer and $IV^3Gal\alpha$-nLc_4Cer were not reactive, indicating that terminal galactose moieties are not always included in the epitope and that gangliotetraose is a preferable antigen for anti-lactobacillus antisera (Fig. 4). On comparison of the densities of spots per microgram of glycolipids stained with antisera, the intensities of Galα1–2Glcα1–3DG and GA1 were found to be similar, but that of GalCer was lower than those of Galα1–2Glcα1–3DG and GA1 (Fig. 4). Thus, symbiotic *Lactobacilli* were shown to generate antibodies toward receptor glycolipid GA1.

To determine why anti-GA1 antibodies were yielded on immunization with *Lactobacilli*, the antigens reacting with anti-GA1 antibodies in *Lactobacilli* were explored by Western blotting. As shown in Fig. 5, although the protein-profiles on SDS–PAGE differed between the two bacteria, anti-lactobacillus antisera reacted with the same antigenic proteins with molecular weights of 43, 50, 55 and 75 kDa. In addition, a protein with a molecular weight of 26 kDa in both bacteria exhibited a positive reaction with anti-GA1 antibodies, indicating the presence of gangliotetraose-like glycans in their protein fractions. If the molecules mimicking GA1 in *Lactobacilli* to generate anti-GA1 antibodies exhibit receptor activity for a ligand, binding of bacteria to GA1 in the small intestine and bacterial aggregation through the ligand might allow effective colonization on the surface of the small intestine. In this connection, modification of GA1 through fucosylation might regulate the number of colonies of *Lactobacilli*.

The production of antibodies to GA1 and gangliosides in human autoimmune diseases such as Guilain–Barré syndrome has been reported to be due to infection by *Campylobacter jejuni*, which causes gastroenteritis (*38*). In fact, an oligosaccharide mimicking GA1, that is, Gal-GalNAc-Gal-(Glc)HepII-(Glc)HepI, was detected in the lipooligosaccharides (LOS) of a gram-negative bacterium, *C. Jejuni* (*39*), and an immune response to the bacterial LOS was thought to result in autoimmune diseases through a reaction with gangliosides in neural tissues,

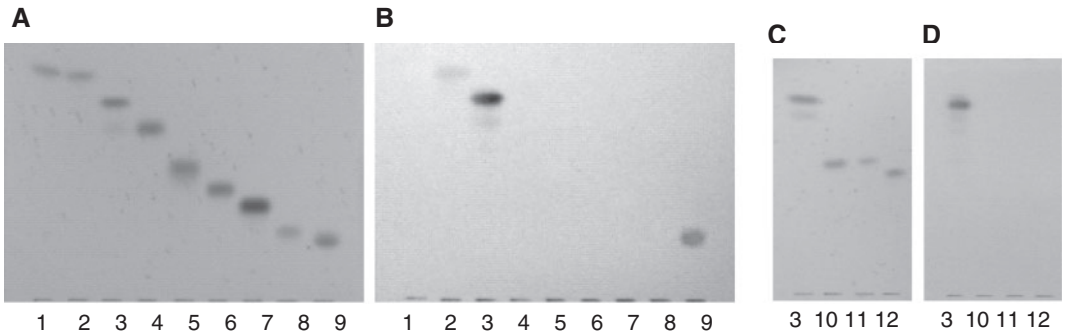

Fig. 4. **TLC and TLC-immunostaining of standard glycolipids.** Glycolipids (0.5–1.5 μg) were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v) for A and B, and with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) for C and D, and the spots were visualized with orcinol–sulphuric acid for A and C, and with anti-*L. johnsonii* antisera for B and D. 1, GlcCer; 2, GalCer NFA; 3, Galα1–2Glcα1–3DG from *L. casei*; 4, LacCer; 5, Gb_3Cer; 6, Gg_3Cer; 7, Gb_4Cer; 8, Forssman antigen; 9, GA1; 10, Lc_4Cer; 11, nLc_4Cer; 12, $IV^3Gal\alpha$-nLc_4Cer.

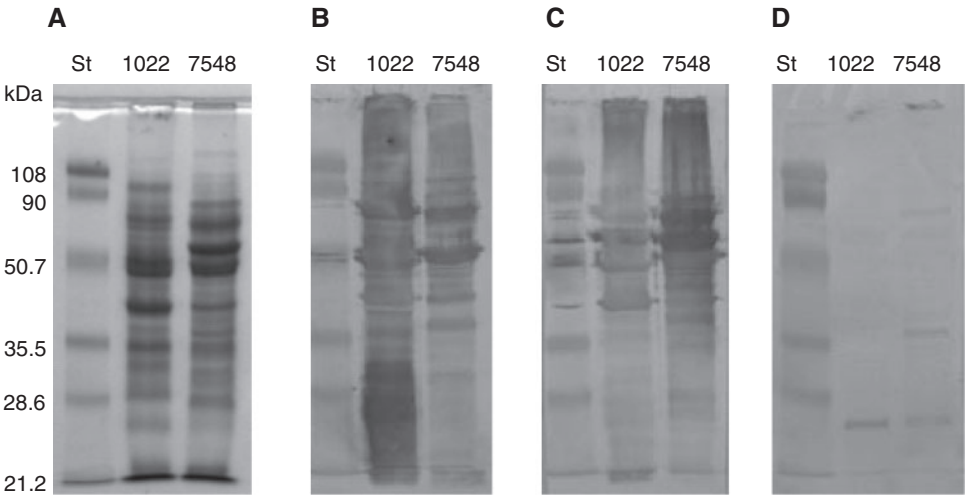

Fig. 5. **SDS–PAGE and Western blotting of proteins from *Lactobacilli*.** *L. johnsonii* (1,022) and *L. intestinalis* (7,548) suspended in PBS by sonication were denatured by heating with the sample buffer, and the resultant solutions were analysed by SDS–PAGE with CBB-staining (A), and by Western blotting with anti-*L. johnsonii* (B), anti-*L. intestinalis* (C), and anti-GA1 (D) antibodies. No band was obtained by staining with normal rabbit serum (1:500). St, protein molecular markers.

playing a crucial role in the pathogeneses of the diseases. In this connection, *Lactobacilli*, gram-positive bacteria, also carry glycans mimicking GA1 in the digestive tract of mice, and the resemblance in the epitope structure between bacteria and the host might be essentially related with a mechanism for evading immune responses to establish symbiosis with *Lactobacilli* in the digestive tract.

CONFLICT OF INTEREST

None declared.

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