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Changes in bacterial glycolipids as an index of intestinal lactobacilli and epithelial glycolipids in the digestive tracts of mice after administration of penicillin and streptomycin

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Abstract The major lipid constituent of symbiotic gram-positive bacteria in animals are phosphatidylglycerol, cardiolipin and dihexaosyl diglycerides (DH-DG), whose hydrophobic structures are characteristic of the environments, and the carbohydrate structures of DH-DGs are bacterial species-characteristic. Immunization of rabbits with intestinal lactobacilli generated antibodies against DH-DGs and their modified structures, among which Galα1-6-substituted DH-DG, *i.e.*, *Lactobacillus* tetrahexaosyl diglyceride (LacTetH-DG), reacted with antibodies more intensely than DH-DG. Whereas, from the 16S-rRNA sequence, the intestinal lactobacilli in murine digestive tracts were revealed to be *L. johnsonii,* in which LacTetH-DG is present at the concentration of 2.2 ng per 1×10^6 cells. To obtain more accurate estimates of intestinal lactobacilli in several regions of the digestive tract of mice, LacTetH-DG was detected by TLC-immunostaining with anti-Lactobacillus antisera, being found in the stomach, cecum and colon of normal breeding mice, 1.0×10^9, 3.5×10^9 and 7.4×10^9 cells, respectively. Administration of penicillin and streptomycin for 6 days resulted in a reduction in the number of intestinal lactobacilli, the levels being 0 %, 30 % and 4 % of the control ones in the stomach, cecum and colon, respectively, which was associated with the accumulation of the contents in the tracts from the stomach to the cecum and with diarrhea. In addition, a reduced amount of fucosyl GA1 (FGA1) and a compensatory increase in GA1 due to the reduced activity of α1,2-fucosyltransferase in the small intestine and the enhanced discharge of FGA1 into the contents occurred in mice, probably due to the altered population of bacteria caused by administration of penicillin and streptomycin.

Keywords Bacterial glycolipids · TLC-immunostaining · Asialo GM1 · Fucosyl asialo GM1 · Antibiotic-associated diarrhea · Digestive contents

Abbreviations

PS	Penicillin and streptomycin
GA1	Asialo GM1
FGA1	Fucosyl GA1
cy	Cyclopropane
ai	Anteiso
PG	Phosphatidylglycerol
CL	Cardiolipin
GL	Glycolipid
LJ	*Lactobacillus johnsonii*
LI	*Lactobacillus intestinalis*
SE	*Staphylococcus epidermidis*
LacDH-DG	*Lactobacillus* dihexaosyl diglycerides
LacTH-DG	*Lactobacillus* trihexaosyl diglycerides
LacTetH-DG	*Lactobacillus* tetrahexaosyl diglycerides.

Introduction

The plasma membrane of gram-positive bacteria is mainly composed of phosphatidylglycerol (PG), dihexaosyl diglycerides (DH-DG), and cardiolipin (CL), whose hydrophobic diglyceride (DG) structures are essentially identical in each bacterial species, but are bacterial species-characteristic, that is, two fatty acids in DG, as the major molecular species, are saturated (16:0 or 18:0) and cyclopropane ring-containing (cy19:0) ones for Lactobacillus species, two anteiso (ai15:0 and ai17:0) ones for Staphylococcus species, and saturated (16:0) and unsatutated (18:1) ones for Streptococcus species

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[1]. These unique hydrophobic structures seem to be required for the adaptation of bacteria to changes in growth circumstances, *i.e.*, lactic acid-producing bacteria with cy19:0, which retains similar chemical structure to that of unsaturated fatty acids, but prevents radical formation of unsaturated fatty acids by lactic acid, and epidermal bacteria with anteiso fatty acids, which form a solid membrane with strong water-holding activity, as shown in Fig. 1. In addition to the hydrophobic DG structures, the carbohydrate moieties of DH-DG are bacterial species-characteristic, that is, Glcα1-2Glcα1-3′DG for Streptococcus (StrDH-DG), Glcβ1-6Glcβ1-3′DG for Staphylococcus (StaDH-DG), and Galα1-2Glcα1-3′DG for Lactobacillus (LacDH-DG)(Fig. 1) [1, 2]. Also, structural modification of LacDH-DG with Gal linked by an α1-6 linkage and Glc linked by a β1-6 linkage have been reported in different Lactobacillus strains [1–3]. Since antisera elicited by direct immunization of a rabbit with individual bacteria, together with Freunds' complete adjuvant, reacted with these bacterial glycolipids, they have been revealed to possess strong antigenicity, although their configuration on the plasma membrane, as well as on the cell wall, is obscure.

In our previous study, we have attempted to detect *Lactobacillus* trihexaosyl DG (LacTH-DG) and tetrahexaosyl DG (LacTetH-DG) in murine feces by TLC-immunostaining with anti-*Lactobacillus johnsonii* antisera for estimation of intestinal lactobacilli in feces [4]. However, the relative antigenecities of several bacterial glycolipids, particularly Galα1-6- and Glcβ1-6-modified LacDH-DG, were not clearly disclosed in our previous study [4], further characterization of anti-Lactobacillus and anti-Staphylococcus antisera with glycolipids purified from several bacteria was undertaken to obtain information on the cross-reactivity among structurally related carbohydrate moieties, and subsequently a new method was developed for the determination of intestinal lactobacilli in the contents of different regions of the digestive tract of mice with and without administration of penicillin and streptomycin (PS).

In addition, although the ratio of FGA1 to GA1, that is, fucosylation of GA1 in the small intestine, has been demonstrated to change in association with changes in the intestinal bacteria [5, 6], it is not clearly understood yet whether an alteration of the bacterial population by antibiotics influences the fucosylation of GA1 in the digestive tract. Accordingly, the amounts of FGA1 and GA1 in the tissues and contents of different regions of the digestive tract, and the activities of α1,2-fucosyltransferase in the duodenum of mice with and without PS-administration were determined to clarify the possible involvement of intestinal bacteria in the modification of the carbohydrate structures of the host.

Materials and methods

Bacteria

The bacteria used in this study were purchased from the Japan Collection of Microorganisms (JCM; RIKEN, Wako, Saitama, Japan): *Lactobacillus johnsonii* (LJ)(JCM 1022), *L. intestinalis* (LI)(JCM7548), *L. reuteri* (JCM1112), *L. casei*

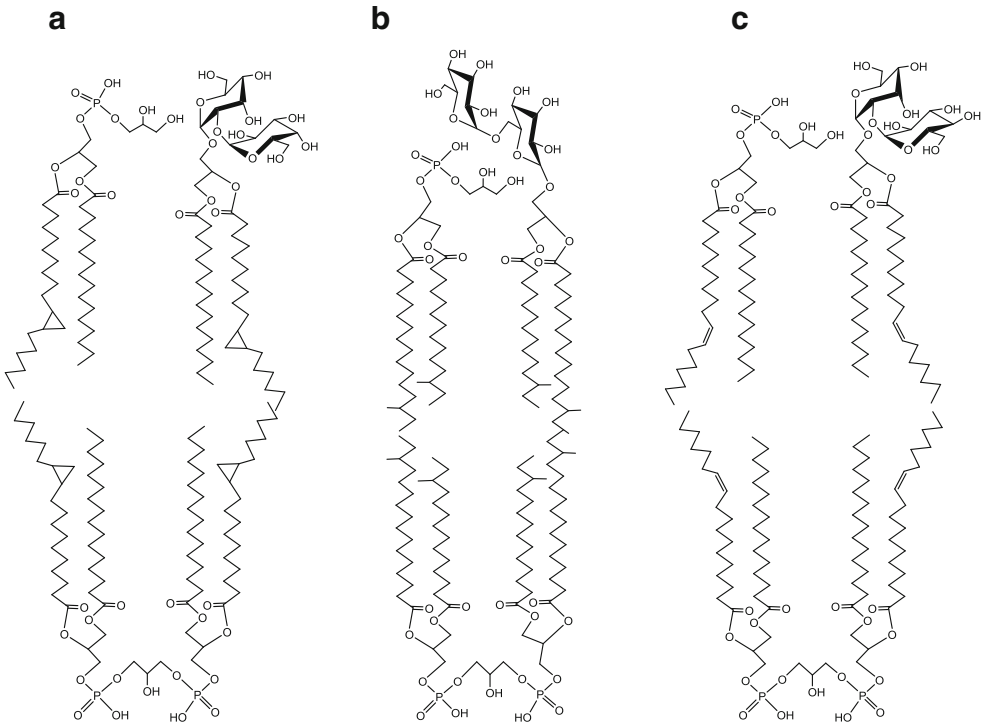

Fig. 1 Possible molecular organization of lipids in the plasma membranes of *L. reuteri* (**a**), *S. epidermidis* (**b**), and *S. salivarius* (**c**). The major lipids in these bacteria were PG, CL and GL, among which CL, and PG and GL were supposed to be distributed in the inner and outer leaflets of the lipid bilayer, respectively, from their space filling model. The DH-DGs characteristic of Lactobacillus, Staphylococcus and Streptococcus species were Galα1-2Glcα1-3′DG, Glcβ1-6Glcβ1-3′DG and Glcα1-2Glcα1-3′DG, respectively

(JCM 1134), *Staphylococcus epidermidis* (SE)(JCM2414), and *Streptococcus salivarius* (JCM5707). The culture media for bacteria were as follows: MRS broth (Beckton-Dickinson, Sparks, MD, USA) and GYP (1 g glucose, 1 g yeast extract, 0.5 g peptone, 0.2 g meat extract, 0.2 g sodium acetate, 20 mg $MgSO_4$, 1 mg $MnSO_4$, 1 mg $FeSO_4$, 1 mg NaCl and 50 mg Tween 80 in 100 ml water) for Lactobacillus, tryptic soy broth (Beckton-Dickinson) for Staphylococcus and heart infusion broth (Beckton-Dickinson) for Streptococcus. The numbers of Lactobacilli were determined from the colony formation on $CaCO_3$-containing GYP-agar plates (0.5 % $CaCO_3$ and 1.2 % agar in GYP medium) [7].

Mice

Mice (HR-1, female, 8–10 weeks old of age) were kept under conventional breeding conditions with lighting from 6:00 to 18:00 at a room temperature of 24±1 °C and a humidity level of 55±10 % with food (MF, Oriental Yeast, Tokyo) and water, with and without penicillin (1,000U/ml) and streptomycin (1 mg/ml)(Sigma, St. Louis, MO, USA), *ad lib*. Animal treatment followed the animal care guidelines of Kinki University. After anesthesia with pentobarbital (Abbott, Osaka), the tissues and contents of each of three mice were collected separately. The upper and lower halves of the tracts between the duodenum and cecum were used as the jejunum and ileum, respectively, whose contents were collected by injecting water with a syringe. The solid contents of the stomach, cecum and colon were collected with a spatula.

Materials

Standard glycolipids from various sources were purified in our laboratory: GM1 from bovine brain, Forssman glycolipids from equine kidney, and fucosyl GM1 (FGM1) from bovine thyroid. GA1 and FGA1 were prepared from GM1 and FGM1, respectively, by treatment with *Arthrobacter ureafaciens* sialidase [8, 9]. Polyclonal antibodies to LJ, LI, SE, GA1, and Forssman glycolipids, and monoclonal antibodies to FGA1 (LFA-1) and FGM1 (LFM-1) were generated in our laboratory by immunizing rabbits or mice with LJ, LI, SE or purified glycolipids together with *Mycobacterium tuberculosis* or *Salmonella minnesota* as the adjuvant [10, 11].

Analysis of lipids

Total lipids were extracted from the lyophilized bacteria, tissues and contents with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1:0, by vol.), and the combined extracts were used as the total lipids. A part of each extract was applied on a DEAE-Sephadex column (A-25, acetate form; GE Healthcare Bioscience, Piscataway, NJ, USA), and the unabsorbed and absorbed fractions were desalted by dialysis, and then used as the neutral and acidic lipids, respectively. Also, the neutral glycosphingolipids were separated from the neutral lipids by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the acidic glycosphingolipids were prepared from the acidic lipids by saponification with 0.5 M NaOH in methanol [3, 4].

The total, acidic and neutral lipids, and acidic and neutral glycosphingolipids thus obtained were applied on plastic-coated (Macherey-Nagel, Düren, Germany) and glass-coated (Merck, Darmstadt, Germany) TLC plates, which were then developed with chloroform/methanol/water (65:25:4, by vol.) for bacterial glycolipids, and (65:35:8, by vol.) for neutral glycosphingolipids, and chloroform/methanol/0.5 % $CaCl_2$ in water (55:45:10, by vol.) for acidic glycosphingolipids, and the spots were visualized with orcinol-sulfuric acid reagents for glycolipids. The densities of spots on TLC plates were determined by image analysis (NIH image).

Structural analysis of glycolipids

The individual glycolipids were purified using a silica gel (Iatrobeads 6RS8060; Iatron Laboratory, Tokyo) column by gradient elution with chloroform/isopropyl alcohol/water (85:15:0.2 and 40:60:2, by vol.) The purified bacterial glycolipids were analyzed by positive ion FABMS (JMS-700 TKM; JEOL, Tokyo) with triethanolamine as the matrix solvent [1, 2]. For analyses of the fatty acid and carbohydrate compositions, they were methanolized with 5 % HCl in methanol at 80 °C for 16 h. The resulting fatty acid methyl esters were extracted with n-hexane, and 1-*O*-methyl hexoses in the methanol phase were converted to trimethylsilyl derivatives with pyridine/hexamethyl disilazane/trimethylchlorosilane (10:2:1, by vol.) at 60 °C for 5 min, and then analyzed with a GLC-mass spectrometer equipped with a DB-1 column (0.25 mmø×30 m) (Shimadzu, Kyoto). Also, linkage analysis of carbohydrates was carried out by conversion of glycolipids to permethylated glycolipids, and then to partially methylated aldohexitol acetates, followed by analysis with a GLC-mass spectrometer [1, 2].

TLC-immunostaining

Lipids, corresponding to 0.05–0.1 mg dry weight, and standard glycolipids, 25–250 ng, were developed on plastic-coated TLC plates (Macherey-Nagel), 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-LJ, anti-LI, anti-SE and anti-glycolipid antibodies diluted with dilution buffer (PBS containing 3 % polyvinylpyrrolidone), followed by immunostaining with peroxidase-conjugated anti-rabbit IgG and IgM, and peroxidase-conjugated anti-mouse IgG and IgM antibodies (1:1000; Jackson Immunoresearch Lab., PA, USA), and peroxidase substrates, 4-chloro-1-naphthol and H_2O_2,

according to the procedure reported previously [4, 11]. The densities of spots on TLC plates were determined by image analysis (NIH image).

Assaying of α1,2-fucosyltransferase

The duodenal tissues were homogenized with a Polytron homogenizer (Kinematica Luzern, Switzerland) in four volumes of 0.25 M sucrose, and then centrifuged at 1000 xg for 10 min at 100,000 xg for 60 min to obtain the cytosol and microsomal fractions, and the microsomal fraction was suspended in 0.25 M sucrose with the aid of sonication. The protein concentrations were measured by the protein dye binding method with bovine serum albumin as the standard [12]. The activity of α1,2-fucosyltransferase was determined with GA1 and GDP-fucose as the substrates, and with the microsomal fraction as the enzyme source. The standard assay mixture comprised 38 nmol GA1, 20 mM $MnCl_2$, 1 % Triton X-100, 50 mM cacodylate-HCl buffer (pH5.8), 0.37 μM GDP-^{14}C-fucose (270.0 mCi/mmol), and enzyme (0.4 mg protein), in a final volume of 100 μl. After incubation at 37 °C for 1 h, the reaction was terminated with 300 μl of chloroform/methanol (2:1, by vol.), and then the products in the lower phase were separated by TLC with chloroform/methanol/0.5 % $CaCl_2$ (55:45:10, by vol.). The radioactivity incorporated into glycolipids was determined with an X-ray film (RX-U; Fuji Film, Tokyo) and with a liquid scintillation counter (Tri-Carb 1500; Packard) [13].

Sequence of bacterial 16S-rRNAs

Bacteria picked up with a toothpick from the colonies on a $CaCO_3$-GYP-agar plate were treated with proteinase K (10 mg/μl), EDTA (5 mM), NaCl (400 mM), and SDS (0.3 %) in 20 mM Tris–HCl buffer (pH 8.0) at 55 °C for 2 h, and the resultant DNAs were used for PCR with DNA polymerase (NovaTaq ™Hot start DNA polymerase; Novagen, San Diego, CA, USA) in Ampdirect Plus (Shimadzu, Kyoto) under the following conditions: 27f sense primer, AGAGTTTGATCCTGGCTCAG, 1544r antisense primer, AGAAAGGAGGTGATCCAGCC, 95 °C, 10 min, followed by 35 cycles of 94 °C for 30 s, 53 °C for 1 min and 72 °C for 1 min, and then 72 °C for 7 min [4]. The PCR products were purified with a MinElute PCR purification kit (Qiagen, MD, USA), and their sequences were determined with a DNA analysis system with BigDye® Terminator Cycle Sequencing Reagent (v. 3.1) according to the manufacturer's instructions (ABI Prism 310 genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

Results

Glycolipid antigens detected with anti-LJ, anti-LI and anti-SE antisera

The bacterial glycolipids used for the characterization of antisera were purified from the following bacteria: Glcα1-3′DG and Glcα1-2Glcα1-3′DG (StrDH-DG) from *S. salivarius*, Glcβ1-3′DG and Glcβ1-6Glcβ1-3′DG (StaDH-DG) from *S. epidermidis*, Galα1-2Glcα1-3′DG (LacDH-DG), Galα1-6Galα1-2Glcα1-3′DG (LacTH-DG), and Galα1-6Galα1-6Galα1-2Glcα1-3′DG (LacTetH-DG) from *L. johnsonii*, and Glcβ1-6Galα1-2Glcα1-3′DG (LacβTH-DG) and Glcβ1-6Galα1-2Glc(6FA)α1-3′DG (LacβTH-DG-FA) from *L. casei*. As shown in Fig. 2, the anti-SE antisera preferentially reacted with StaDH-DG. As to the anti-Lactobacillus antisera, although the anti-LI antisera reacted with intestinal Lactobacillus

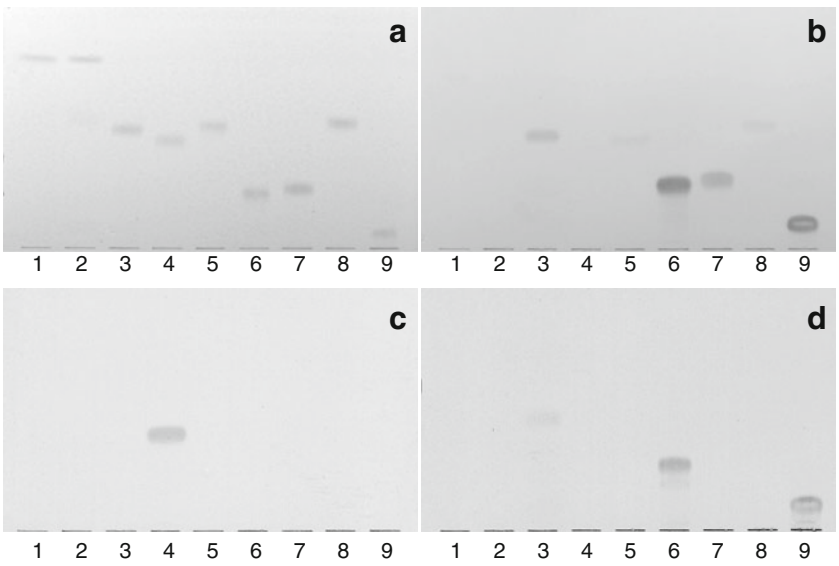

Fig. 2 TLC and TLC-immunostaining of bacterial glycolipids. Purified glycolipids, 0.2 μg, were developed on plates with chloroform/methanol/water (65:25:4, by vol.), and then detected with orcinol-sulfuric acid reagent (**a**), and with anti-LJ antisera (**b**), anti-SE antisera (**c**) and anti-LI antisera (**d**). 1, Glcα1-3′DG; 2, Glcβ1-3′DG; 3, LacDH-DG; 4, StaDH-DG; 5, StrDH-DG; 6, LacTH-DG, 7, LacβTH-DG; 8, LacβTH-DG-FA; 9, LacTetH-DG

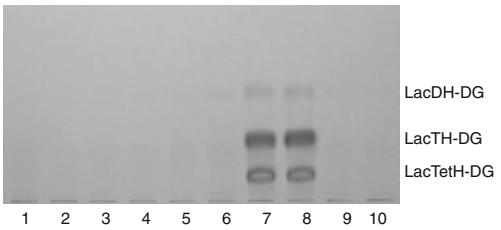

Fig. 3 TLC-immunostaining with anti-LJ-antisera of bacterial glycolipids. The cecal contents of mice without PS-administration were suspended in PBS and then cultured on a $CaCO_3$-GYP-agar plate. All colonies with a clear zone were picked up with a toothpick and then grown in 20 ml of GYP liquid medium. Then, bacterial glycolipids from individual colonies were examined by TLC-immunostaining with anti-LJ antisera. 1–10, individual colonies

glycolipids, LacTetH-DG, LacTH-DG and LacDH-DG, the relative binding intensities being 1.00, 0.47 and 0.24, respectively, LacβTH-DG, LacβTH-DG-FA and StrDH-DG, together with above Lactobacillus glycolipids were detected with the anti-LJ antisera, the order of reactivities being LacTH-DG (1.00), LacTetH-DG (0.66), LacβTH-DG (0.39), LacDH-DG (0.19), LacβTH-DG-FA (0.07), and StrDH-DG (0.06). Among the glycolipids that reacted with the anti-LJ and anti-LI antisera, the reactivities of LacTH-DG and LacTetH-DG were considerably higher than those of the others. However, as shown in Fig. 2a, although LacTH-DG migrated close to the position of LacβTH-DG, no structurally related glycolipids was observed at the position of LacTetH-DG, which was present in intestinal lactobacilli, *i.e.*, *L. johnsonii, L. intestinalis and L. reuteri*. Accordingly, the amount of LacTetH-DG, which was present in the amount of 0.22 μg in 10^8 cells of *L. johnsonii,* was employed to determine the numbers of intestinal lactobacilli in the contents of digestive tracts by TLC-immunostaining with either anti-LJ or anti-LI antisera.

Intestinal lactobacilli in the contents of murine digestive tracts

When the cecal contents suspended in PBS were cultured on $CaCO_3$-GYP-agar plates, intestinal lactobacilli producing lactic acid yielded colonies surrounded by a clear zone due to the dissolutions of $CaCO_3$, and their glycolipids were analyzed by TLC-immunostaining with anti-LJ antisera. As shown in Fig. 3, LacDH-DG, LacTH-DG and LacTetH-DG were detected in 20 % of the total colonies, and they were identified as *L. johnsonii* from the sequences of their 16S-rRNAs.

Glycolipids in the contents of digestive tracts of mice with and without PS-administration

The administration of PS resulted in increases of the contents in the digestive tract other than the colon, where the contents in PS-administered mice were wet compared with in the controls, indicating the enhanced secretion of water in the colon of PS-administered mice that leads to diarrhea. Also, the average size of the cecum of PS-administered mice became greater and the weight of the dried material was about 1.7 times that in the controls (Table 1). Although 45 % of the total dried materials in the whole digestive tract of control mice was distributed in the colon, 45 % was in the cecum of the PS-administered mice, indicating the diminished transit of cecal contents to the colon. As shown in Fig. 4, LacTetH-DG was present in the contents of the stomach, cecum and colon of the control mice at amounts of 0.12–0.2 μg per mg dry weight, but StaDH-DG was not detected, even on spotting of lipids corresponding to 5 mg dry weight. Thus, the habitable regions for intestinal lactobacilli were clearly demonstrated to be the stomach, cecum and colon, 62 % of the total intestinal lactobacilli in the whole digestive tract being in the colon (Table 1). However, PS-administration was revealed to affect the amount of LacTetH-DG, it being zero in the stomach, one-sixth of the control level in the cecum and one-tenth of the control level in the colon, demonstrating that an effect of PS on intestinal lactobacilli was clearly observed on TLC-immunostaining of LacTetH-DG (Table 1). The total amounts of LacTetH-DG in the whole digestive tract of the control and PS-administered mice were 26.1 μg and 2.9 μg, respectively, which corresponded to 1.2×10^{10} cells and 1.3×10^{9} cells, respectively, showing a 90 % reduction of intestinal lactobacilli on PS-administration for 6 days. On the contrary, the amounts

Table 1 Dry weights and concentrations of glycolipids in the contents of the digestive tracts of mice with and without PS-administration

			(μg/mg of dry weight)					
	Dry weight (mg/mouse)		GA1		FGA1		LacTetH-DG	
	PS	Control	PS	Control	PS	Control	PS	Control
Stomach	21.4±4.3	10.8±3.2	0.70±0.05	0.27±0.02	0.33±0.04	0.33±0.05	–	0.20±0.02
Jejunum	12.4±3.7	8.8±2.4	0.84±0.08	0.76±0.06	0.20±0.05	0.19±0.04	–	–
Ileum	28.1±4.4	20.1±6.3	0.88±0.11	1.15±0.09	0.27±0.05	0.30±0.06	–	–
Cecum	109.1±6.9	64.4±9.3	0.97±0.19	0.66±0.05	0.33±0.05	0.23±0.06	0.02±0.005	0.12±0.01
Colon	72.6±7.1	85.1±5.5	0.94±0.20	0.86±0.04	0.29±0.04	0.22±0.05	0.01±0.002	0.19±0.02

Values are the means for three samples. PS, PS-administered mice

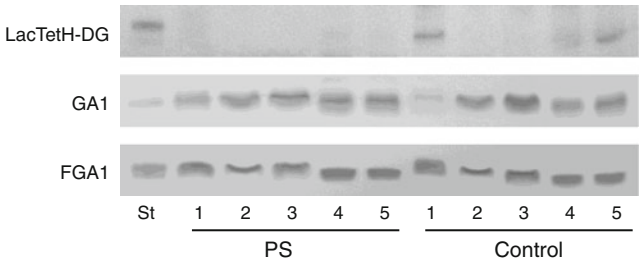

Fig. 4 TLC-immunostaining of LacTetH-DG, FGA1 and GA1 in the contents of the digestive tracts of mice with and without PS-administration. Lipid extracts, corresponding to 0.1 mg dried contents, were developed on plates with chloroform/methanol/0.5 % $CaCl_2$ in water (55:45:10, by vol.), and then detected with anti-LI antisera (LacTetH-DG), and anti-FGA1 and anti-GA1 antibodies. St, respective standard glycolipids; 1, stomach contents; 2, jejunal contents; 3, ileal contents; 4, cecal contents; 5, colonal contents; PS, PS-administered mice; Control, control mice

of GA1 and FGA1per mg of the cecal contents of PS-administered mice were higher than those of the controls, being 2.5-fold in the controls (Table 1). Also, the total amounts of GA1 and FGA1 in the whole digestive tract were 148.4 μg and 44.8 μg for the control, and 224.1 μg and 74.3 μg for the PS-administered mice, respectively, suggesting enhanced discharge of epithelial cells into the contents of mice on PS-administration (Table 2).

Glycolipids in the tissues of the digestive tracts of mice with and without PS-administration

Since the above results regarding the amounts of GA1 and FGA1 in the contents indicated the possibility that PS-administration affected the metabolism of GA1 and FGA1 in epithelial tissues, those in the tissues of various regions of the digestive tract were compared between PS-administered and control mice. As shown in Figs. 5 and 6, although Forssman glycolipid and FGA1 were ubiquitously distributed in murine digestive tracts, the distribution of GA1 was restricted to the duodenum, jejunum and ileum of the small intestine. Since FGA1 is synthesized through fucosylation of GA1, GA1 in the stomach, cecum and colon was completely fucosylated to FGA1, as reported in our previous papers [3, 4]. PS-administration resulted in an increase of GA1 and a decrease of FGA1 in the small intestine, indicating lowering of the fucosylation of GA1 in the small intestine of PS-administered mice as compared with in controls. As shown in Table 2, the total amount of GA1 in the small intestine of PS-administered mice was about 1.7 times that in the control mice, but that of FGA1 in PS-administered mice was reduced to one half of that in the control mice. The rates of fucosylation of GA1 in the duodenum of control and PS-administered mice were 12.6 % and 6.4 %, respectively, and those in the ileum were 14.9 % and 4.3 %, respectively (Fig. 6). They were closely correlated with the specific activities of GA1 α1,2-fucosyltransferase, *i.e.* 240±44 fmol/mg protein/hr and 87±11 fmol/mg protein/h, in the duodena of control and PS-administered mice, respectively, indicating that the reduced fucosylation in PS-administered mice is due to the retarded specific activity of α1,2-fucosyltransferase. On the contrary, the concentrations and total amounts of FGA1 in the stomach and cecum of PS-administered mice rather increased to 1.2–1.4 times those in the control mice (Table 2). Also, when the total amounts of GA1 and FGA1 in the whole contents to those in whole tissues were compared, the ratios of GA1 and FGA1 for the control mice were found both to be about 22 %, but those in the PS-administered mice were 20 % and 38 %, respectively, indicating that discharge of FGA1-bearing epithelial cells from the stomach and cecum with increased amounts of FGA1, as well as from the small intestine to the contents, is enhanced compared to that in GA1-bearing cells in the PS-administered mice (Table 3).

Table 2 Dry weights and total amounts of glycolipids in whole tissues of the digestive tracts of mice with and without PS-administration

Tissue	Dry weight (mg/mouse)		(μg/mouse) Fs		GA1		FGA1	
	PS	Control	PS	Control	PS	Control	PS	Control
Stomach	43.3±3.8	46.5±3.0	16.8±1.5	22.3±1.4	–	–	41.3±3.6	33.9±2.2
Duodenum	25.6±3.0	22.8±4.0	6.9±0.8	5.5±1.0	97.0±11.4	70.9±12.4	7.4±0.9	11.4±2.0
Jejunum	173.3±19.8	134.0±32.0	45.1±5.1	40.2±9.6	634.3±72.5	363.1±86.7	tr	tr
Ileum	125±20.8	119.5±28.5	50.2±8.3	40.6±9.7	418.2±69.3	253.3±60.2	21.4±3.5	49.0±11.6
Cecum	33.0±4.9	25.8±7.4	22.1±3.3	22.7±6.5	–	–	75.2±11.2	54.5±15.6
Colon	45.2±3.5	47.1±4.1	30.3±2.3	39.1±3.4	–	–	50.6±3.9	53.7±4.7
Total amount (μg/mouse)			188.7	165	1149.5	687.3	195.9	201.4

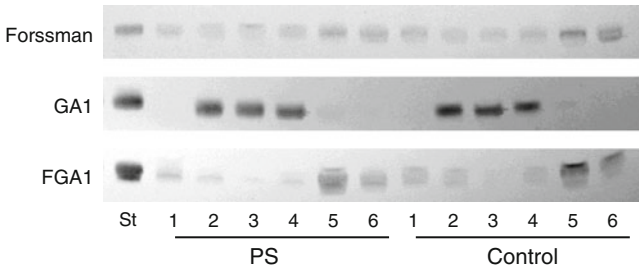

Fig. 5 TLC-immunostaining of Forssman glycolipid, FGA1 and GA1 in tissues of the digestive tracts of mice with and without PS-administration. Analysis was carried out as described in the legend to Fig. 3. St, respective standard glycolipids; 1, stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon; PS, PS-administered mice; Control, control mice

Discussion

As shown in this paper, glyceroglycolipids, whose carbohydrate structures were bacterial species-characteristic, exhibited strong antigenicity, and antibodies to them were revealed to be useful for the detection of individual bacterial species [1, 2]. However, careful examination of the modification of carbohydrate structures in individual bacterial species and cross-reactivities of antibodies toward structurally related glycolipids was required prior to their use as an index of intestinal lactobacilli. As reported previously [2], LacDH-DG was the major lipid constituent in several Lactobacillus strains as a Lactobacillus species-characteristic glycolipid, but its modified structures, LacTH-DG and LacTetH-DG, whose concentrations were lower than that of LacDH-DG, were revealed to react with anti-LJ and anti-LI antisera more intensely than LacDH-DG and LacβTH-DG, indicating that Galα1-6-substitution in LacTH-DG and LacTetH-DG maintains strong antigenicity. On a TLC-plate, LacTH-DG closely migrated with LacβTH-DG, and LacβTH-DG-FA with LacDH-DG, but no glycolipid from Lactobacilli co-migrated at the position of LacTetH-DG. Also, since Lactobacillus species in the murine digestive tract have been determined to be preferentially *L. johnsonii* by 16S-rRNA, the immunodetection of LacTetH-DG was judged to be a useful index of intestinal lactobacilli in the digestive tracts of mice. The lower limit of detection was 10 ng LacTetH-DG, corresponding to 4.5×10^6 cells of LJ, and the method was applicable to clarification of a change of intestinal lactobacilli in the digestive tracts of mice under PS administration.

Antibiotics including PS are well known to influence the intestinal bacteria and to cause gastrointestinal illness. For example, the major intestinal bacteria of infants after 1 week of age have been identified as to be Bifidobacterium, which assists the growth of intestinal lactobacilli, resulting in suppression of virulent bacteria, including Clostridium, Bacteoides and Enterococcus species, but a dosage of antibiotics *via* the mother's milk inhibited the growth of Bifidobacterium and Lactobacilli, which has been assumed to be a cause of allergy [14]. Also, antibiotic-associated diarrhea has been accounted for by abnormal growth of *Clostridium difficile* after a dosage of penicillin or cephalosporin, and can be cured by administration of Lactobacilli [15–17]. Accordingly, a more exact and reliable method for detecting intestinal lactobacilli is required to clarify habitable regions for them in the digestive tract and alterations in bacterial population caused by antibiotics. The TLC-immunostaining procedure reported in this paper was revealed to exhibit sufficient specificity and sensitivity, as described above for the characterization of intestinal lactobacilli. On application of the procedure, intestinal lactobacilli were found to inhabit the stomach, cecum and colon of control mice, 1×10^9, 3.5×10^9 and 7.4×10^9 cells, respectively, and to be reduced to 0, 1×10^9 and 3×10^8 cells, respectively, in mice after administration of PS for 6 days. In control mice, 62 % of the total intestinal lactobacilli in the whole digestive tract were in the colon, where intestinal lactobacilli were reduced to 4 % of the control level by PS-administration. The great reduction in the number of symbiotic intestinal lactobacilli, particularly in the colon, was assumed to cause a serious problem in the digestive process, *i.e.* retarded transit of the contents from the stomach to the cecum, accumulation of the cecal contents, and diarrhea. Since the amount of LacTetH-DG in the feces was identical with that in the colon, its analysis in the feces should become an index of intestinal lactobacilli in the colon of the digestive tract.

On the other hand, FGA1, which is synthesized from GA1 by α1,2-fucosyltransferase, has been revealed to be ubiquitously distributed in murine digestive tracts [3]. Because FGA1 disappeared from the stomach, cecum and colon of FUT2-null mice, fucosylation of GA1 was revealed to be preferentially

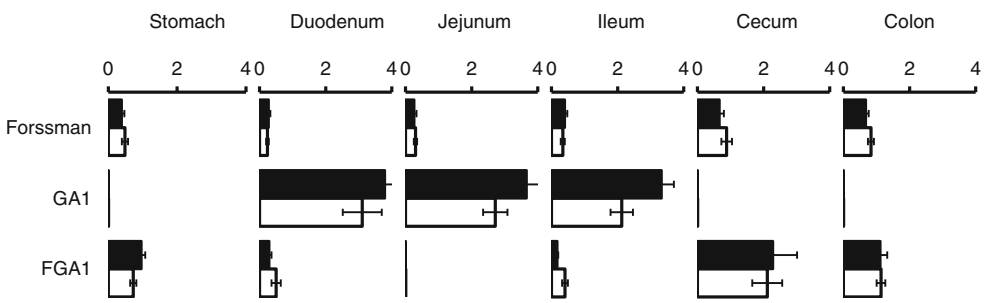

Fig. 6 Amounts of Forssman glycolipid, GA1 and FGA1 in several regions of the digestive tract of mice with (closed columns) and without (open columns) PS-administration (μg/mg dry weight). Values are the means for three samples

Table 3 Total amounts of GA1 and FGA1 in whole contents and their ratios to those in the tissues of the digestive tracts of mice with and without PS-administration

Contents	(μg/mouse)			
	GA1		FGA1	
	PS	Control	PS	Control
Stomach	15±3.0	2.9±0.9	7.1±1.4	3.6±1.1
Duodenum + Jejunum	10.4±3.1	6.7±1.8	2.5±0.7	1.7±0.5
Ileum	24.7±3.9	23.1±7.2	7.6±1.2	6.0±1.9
Cecum	105.8±6.7	42.5±6.1	36.0±2.3	14.8±2.1
Colon	68.2±6.7	73.2±4.7	21.1±2.0	18.7±1.2
Total amount (μg/mouse)	224.1	148.4	74.3	44.8
$\frac{\text{Total amounts in whole contents}}{\text{Total amounts in whole tissues}} \times 100\,(\%)$	19.5	21.6	37.9	22.1

carried out by a FUT2-encoded enzyme, which completely fucosylate GA1 in these tissues [6]. Either a FUT1 or FUT2-encoded enzyme has been shown to be involved in the synthesis of FGA1 in the small intestine [6], but the large contribution of a FUT2-encoded enzyme has been proven by the finding that the amount of FGA1 in FUT1-null mice was exceedingly higher than those in wild and FUT2-null mice. In addition, although the small intestine, particularly the duodenum and jejunum, of germ-free mice expresses GA1, but not FGA1, administration of bacteria in the feces of conventionally breeding mice has resulted in transcription of the FUT2 gene to synthesize FGA1 up to 20 % of the total GA1 [2, 3, 6]. Although the bacterial factor responsible for transcription of the FUT2 gene in the small intestine has not been completely identified, the reduced amount of FGA1 and the reduced activity of α1,2-fucosyltransferase caused by PS is considered to be due to the disappearance of bacteria, including intestinal lactobacilli. The rates of fucosylation of GA1 in the small intestine of control and PS-administered mice were 7.3 % and 2.2 %, respectively, and the lower fucosylation activity led to accumulation of the substrate GA1, amounting to 1.7-fold that in the controls. On the other hand, synthesis of FGA1 in the stomach and cecum of PS-administered mice was rather accelerated compared to that in control mice, giving 1.2 fold- and 1.4-fold amounts of FGA1 in the stomach and cecum compared to in the control mice, respectively. Although several bacteria, *i.e.* segmented filamentous bacteria and *Bacteroides thetaiotaomicron*, have been reported to be participated in the induction of α1,2-fucosyltransferase, a mechanism underlying the transcriptional induction of α1,2-fucosyltransferase by bacteria is not clearly understood yet [18, 19]. Characterization of the bacterial transcription factor and sequential analysis of up-stream promoter regions of α1,2-fucosyltransferase are required to clarify the mechanism underlying fucosylation of GA1 in murine digestive tracts, and are now in progress in our laboratory.

Also, epithelial glycolipids, GA1 and FGA1, were excreted into the feces without digestive degradation, and their amounts in the contents were 0.3–1.2 μg and 0.2–0.3 μg per mg dried content, respectively [4]. Since GA1 is known to constitute the receptor for several bacteria, *i.e. L. casei, L.reuteri, L. johnsonii, Bifidobacterium bifidum, Pseudomonas aeruginosa, Actinomyces maeslundii and Neisseria gonorrhoeae*, GA1 in the contents is presumed to bind bacteria for facilitation of their discharge and for prevention of irregular diffusion of intestinal bacteria. The total amounts of GA1 and FGA1 in the whole contents in PS-administered mice were higher than those in control mice, but their ratios in the contents to in the tissues of control mice were identical, indicating that the discharge of the GA1- and FGA1-bearing epithelial cells into the contents occurs at a similar rate. But, although the ratio of GA1 in the whole contents to in whole tissues of PS-administered mice was the same as that in the controls, the ratio of FGA1 in PS-administered mice was much higher than that in the controls, suggesting enhanced discharge of FGA1-bearing epithelial cells from the stomach, small intestine and cecum of PS-administered mice. Thus, glycolipids with receptor activity for bacteria in the digestive tract were demonstrated to be dynamically metabolized in response to an alteration in the intestinal bacterial population. On the basis of the data obtained, subsequent experiments are now carried out on mice given other antibiotics, *i.e.* cephalosporin, vancomycin, neomycin and metronidazole, for further characterization of the relationship between the enhanced discharge of FGA1 and diarrhea [20–22].

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