Intestinal Glycolipids and Their Possible Role in Microbial Colonization of Mice

Yoshinori UMESAKI

*Yakult Central Institute for Microbiological Research,
Yaho 1796, Kunitachi, Tokyo, 186*

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The alimentary tract is colonized by more than one hundred species of and more than one thousand billion microbes (per gram contents). It is well known that the microbes colonized characteristically according to the site of the alimentary tract and species and physiological conditions of the host animal such as age (16, 17, 19). Comparative studies between germfree and conventional animals clearly show that the intestinal microbes greatly affect physiology and morphology of the intestine of the host animal. However, we have not yet been able to obtain precise mechanisms of the difference between germfree and conventional animals in spite of great efforts thus far.

On the other hand, the sphingoglycolipids are now studied from the aspect of the host receptor-intestinal microbe interaction. The glycolipids are very abundant in the microvillus membrane of the small intestinal epithelial cells (11, 14). The composition of these glycolipids are different among species (3), developmental ages of the animals (12, 18), and between normal and pathological conditions of the host animals (6, 9). And also these glycolipids have, at least *in vitro*, high affinity for the microbes (10) as well as the enterotoxins produced by pathogenic microorganisms (27). Thus there is some reason to consider that the glycolipid might be a mediator between host animals and intestinal microbes.

In this review, we summarize what changes occur in the intestinal glycolipids by association of the intestinal microbes with germfree animals, discuss the mechanism of this glycolipid change, and finally consider what roles these glycolipids play in the colonization of the intestinal microbes.

THE GLYCOLIPIDS OF THE SMALL INTESTINAL EPITHELIAL CELLS IN MICE

The glycolipids present in the mammals are, generally, sphingoglycolipids. The sphingoglycolipids are usually divided into neutral glycolipids and acidic glycolipids such as gangliosides and sulfatides. Table 1 summarizes the structures of glycolipids present in the small and the large intestine of mice. Glucosyl ceram-

Table 1. Structures of intestinal glycolipids in mice

Glycolipids	Carbohydrate structure	Expression period	References
Neutral glycolipids			
Glucosyl ceramide	Glc-Cer	throughout life	(18, 21)
Asialo GM1	Gal β 1-3 β GalNAc β 1-4Gal β 1-4Glc-Cer	after weaning	(21)
Fucosyl asialo GM1	Fuc α 1-2Gal β 1-3 β GalNAc β 1-4Gal β 1-4Glc-Cer	at weaning ¹⁾	(23)
Gangliosides			
GM3	Neu α 2-3Gal β 1-4Glc-Cer	before weaning	(18)
GM1	Gal β 1-3GalNAc β 1-4(Neu α 2-3)Gal β 1-4Glc-Cer	before weaning	(18)
GD1a	Neu α 2-3Gal β 1-3GalNAc β 1-4(Neu α 2-3)Gal β 1-4Glc-Cer	before weaning	(18)
Sulfatide			
Sulfo-asialo GM1	HSO ₃ -2Gal β 1-3GalNAc β 1-4Gal β 1-4Glc-Cer	adult mice	(15)

¹⁾ Fucosyl asialo GM1 was also transiently induced when germfree mice were conventionalized.

ide and asialo GM1 were major components after birth and weaning, respectively (18, 21). Fucosyl asialo GM1 was expressed during the weaning stage, and thereafter present as a minor component (18, 22, 23). GM3, GM1, GD1a were major glycolipids before weaning (18). HSO₃-asialo GM1 was a major component in acidic glycolipids in adult mice (10). The amount of neutral glycolipids exceed the acidic glycolipids after the weaning stage. The glycolipid composition of other species are described in detail elsewhere (3). The composition of the glycolipids are very characteristic among species of animals. Strain-specificity in intestinal glycolipid composition of rat was reported (3), although its difference is not known in mice. In humans, the diversity of glycolipids are present among individuals according to the blood type and secretor phenotype (1).

The ceramides, the hydrophobic portion of glycolipids, in the intestine have α -hydroxy fatty acids in combination with phytosphingosine (21). Therefore, the glycolipids of the intestinal epithelial cells are very hydrophilic compared to the those of other organs. It is also shown that this hydrophilic structure affects binding ability of the microbes to glycolipids *in vitro* (7).

THE DISTRIBUTION OF GLYCOLIPIDS IN THE SMALL INTESTINAL EPITHELIAL CELLS

The distribution of the intestinal glycolipids of mice and rat are reported in detail by biochemical and histochemical methods. These glycolipids are distributed in epithelial cells from crypt bottom to the villus tip (4, 24). Glucosyl ceramide and GM3 are more abundant in villus than in crypt portion in rat (2). Immediately after conventionalization, fucosyl asialo GM1 was more strongly expressed in villous cells than in crypt in mice. Subcellular distribution of glycolipids show enrichment of glycolipids in microvillus membrane, facing the lumen of the

intestine (24).

There is clear difference in glycolipid composition between lamina propria cells and the epithelial cells, reflecting the ontogenic difference of the two tissues (8, 20, 22).

THE DIFFERENCE OF GLYCOLIPID COMPONENTS BETWEEN GERMFREE AND CONVENTIONAL ANIMALS

Comparative study of glycolipid composition between germfree and conventional mice has been done. A small amount of fucosyl asialo GM1 is present only in conventional adult mice (25). Incorporation of radiolabeled fucose into glycolipid fraction was not detected at all in germfree counterparts. Immediately after conventionalization of germfree mice by introduction of fecal suspension of conventional mice, an abrupt increase in fucosyl asialo GM1 content occurred corresponding to the induction of GDP-fucose: asialo GM1 α (1-2) fucosyltransferase (FT) activity as shown in Fig. 1 (26). About two weeks after conventionalization, fucosyl asialo GM1 content decreased and maintained a constant low level thereafter. There is a qualitative difference in the presence of fucosyl asialo GM1 between germfree and conventional mice. In rats, such a difference in the intestinal glycolipids has not been observed, although the time course of the conven-

Fig. 1. A thin-layer chromatogram of the lipid fractions containing asialo GM1 and fucosyl asialo GM1. At the days after conventionalization indicated in the figure, glycolipid fractions were prepared from the mucosal homogenate (7.0 mg protein) as described elsewhere (26). Thin-layer chromatogram was developed with chloroform-methanol-water (60/40/8, v/v) and glycolipids were detected by spraying with 50% sulfuric acid. Lane St indicates the standard glycolipids isolated from mouse small intestine.

tionalization process was not followed (5). In this case, the increase in H-active glycolipid was reported in the stomach.

MECHANISMS FOR ALTERATIONS OF THE GLYCOLIPID COMPOSITION BY BACTERIA

Microbial colonization changed the glycolipid composition by the following two mechanisms. One mechanism was *de novo* synthesis of glycolipids. This mechanism is due to induction or activation of glycosyltransferase. The other mechanism was hydrolysis of glycolipids by glycosidases produced by intestinal microbes.

FT activity appeared by conventionalization of germfree mice as described in the previous section. It is not certain that this induction reflects *de novo* synthesis of glycosyltransferase. FT induction also occurred in the weaning stage of conventional mice and in some injurious treatments on the small intestine in germfree as well as conventional mice (23). The proteinaceous fraction which could induce FT activity by intravenous injection into germfree mice was obtained from the homogenate of small intestine. This fraction, although not completely, lost FT-inducing activity by pronase and heat treatment. Its activity was separated into void volume fraction and M.W. 30,000–10,000 fraction on Sephadex G-200 column chromatography (unpublished data). All these facts suggest the presence of a proteinaceous factor which controls the expression of the fucoglycolipid in the mouse small intestine. This substance(s) is a candidate for a mediator of glycolipid change in the intestine.

The degradation of intestinal glycolipids was noted in humans (12, 13). The glycosidase involved in mucin degradation was shown to hydrolyze glycolipids. Some strains of *Ruminococcus* and *Bifidobacterium* have high glycosidase activities such as β -N-acetyl galactosaminidase, β -galactosidase, α -fucosidase, and α -neuraminidase. The degradation of glycolipids in the intestine is thought to be dependent on the transit time of intestinal contents. The degradation was not so clear in small animals such as mice and rats (5), probably due to fast transit of intestinal contents.

GLYCOLIPIDS ALTERATION IN GNOTOBIOTES

The intestinal glycolipids were converted from asilo GM1 into fucosyl asialo GM1 by conventionalization of germfree mice. To investigate what kind of bacteria induced this glycolipid change, FT activity was measured. As the first step, the interrelationship between the cecal bacterial composition and FT activities induced by antibiotic treatment was analyzed (Table 2). Generally, administration of antibiotics immediately after conventionalization repressed FT induction. Interrelation coefficient between the bacterial number of each genus and FT activity was estimated by analysis of variance based on the results of Table 2. *Enterobacteriaceae* ($r^2=0.63$), *Streptococci* ($r^2=0.547$), and *Lactobacilli* ($r^2=0.478$) showed

Table 2. Cecal flora and FT activity after oral administration of antibiotics

	Control	Gentamicin		Erythromycin
		0.5 mg/ml	1.0 mg/ml	1.0 mg/ml
Microbes				
<i>Enterobacteriaceae</i> ¹⁾	8.8±0.2 ¹⁰⁾	4.6±0.5	5.0–3.2	6.0±1.0
<i>Staphylococci</i> , <i>Bacilli</i> ²⁾	3.6	3.6	3.6	3.6
<i>Streptococci</i> ³⁾	9.2±0.1	3.6	4.1–1.4	3.6
<i>Lactobacilli</i> ⁴⁾	8.8	4.5±0.5	3.7–1.0	3.6
<i>Bacteroides</i> ⁵⁾	10.0±0.1	9.9±0.1	7.2±1.2	3.6
<i>Bifidobacterium</i> ⁶⁾	5.7±0.7	7.3±0.4	3.7±1.0	3.8–1.1
<i>Pseudomonas</i> ⁷⁾	3.6	3.6	3.6	3.7–1.0
Fungi, Yeast ⁸⁾	3.6	3.6	3.6	3.6
Total bacteria ⁹⁾	10.4±0.2	10.0±0.2	9.0±0.1	9.2±0.2
FT activity	251±170 ¹¹⁾	49±16	35±12	82±37

¹⁻⁹⁾ Each microbe was counted in the agar plate containing selective medium as follows: 1, MacConkey; 2, Mannit salt; 3, SF; 4, LBS; 5, modified NBGT; 6, YBS; 7, NAC; 8, PD; 9, GAM.

¹⁰⁾ The values are logarithmic number of bacteria (mean±SD, $n=4$) per gram cecal contents.

¹¹⁾ The values are expressed as pmol/mg protein/min ($n=4$).

Table 3. FT induction in conventionalized and gnotobiotic mice

Mice	Days after administration of bacteria	
	3 days	7 days
Germfree	0.65±0.06 ⁵⁾	—
Gnotobiot 1 ¹⁾	2.37±0.65	—
Gnotobiot 2 ²⁾	2.27±0.92	2.92±0.27
Gnotobiot 3 ³⁾	3.22±1.25	—
Conventionalized mice ⁴⁾	132±48	87.5±22

¹⁾ The bacterial suspension of *E. coli*, after washing with saline, was administered into the stomach.

²⁾ The mixture of suspension of *E. coli*, 2 strains (one strain was the same as in gnotobiot 1 group), and *Proteus* sp. was administered.

³⁾ The mixture of *L. acidophilus*, *L. fermentum*, *L. murini*, *S. faecalis*, and two species each of *Bifidobacterium* and *Bacteroides*, in addition to the same three stains of gnotobiot 2 group, was administered.

⁴⁾ The mice were transferred from isolator to open air, and the fecal suspension was administered.

⁵⁾ Enzyme activity was expressed as pmol/mg protein/min ($n=5$).

high coefficients. Coefficients of *Bifidobacterium* ($r^2=0.046$), *Bacteroides* ($r^2=0.033$), and other genera ($r^2=0$) were very low. Based on these results, three kinds of gnotobiotics were prepared (Table 3). A little elevation of FT activity was observed in these gnotobiotics. However, the values were extremely low compared to those of the conventional mice group. There was little difference in FT activity between *E. coli* mono-contaminated mice and multi-contaminated mice.

As *E. coli* showed some affinity to asialo GM1-coated plastic plate as described

Fig. 2. FT induction in the gnotobiotic mice contaminated with *E. coli*. *E. coli* was isolated from asialo GMI-adherent experiment as shown in Fig. 3 (GB 2). In comparison, *E. coli* was directly isolated from cecal contents of mouse (GB 1) and rat (GB 3) using MacConkey agar plate. GF shows the germfree mice.

in the next section, we tried to make some kinds of gnotobiotics associated with *E. coli* isolated from adhesion experiment. There is still great difference in FT activity induction between conventionalized mice and gnotobiotic mice associated with *E. coli* showing some affinity to asialo GM1-coated plastic plate, although some gnotobiotic induced FT activity to near the level of conventionalized mice (Fig. 2).

THE INTESTINAL GLYCOLIPIDS AS ATTACHMENT SITE FOR BACTERIA

The functions of intestinal glycolipids are obscure, except that they are known to be the receptors of enterotoxins. However, it has been clear that some neutral glycolipids show high affinity to bacterial cells in *in vitro* experiment using glycolipid-coated plastic plate or thin-layer plate. We investigated the extent to which alteration of glycolipids affects affinity of bacterial cells to the intestinal cell surface glycolipids *in vitro* using glycolipid-coated plastic plate. Figure 3 is a diagram of this method. An especially large number of bacteria in *Enterobacteriaceae* were recovered from asialo GM1-coated plastic plate at one dilution rate of bacterial suspension, as shown in Fig. 4. Recovery of bacteria of other genera were very low compared to

Fig. 3. Schematic presentation of bacterial adhesion test using a glycolipid-coated plastic plate. The cecal contents of conventionalized mice for 3 days were suspended, briefly spun, and the supernatant was obtained. This supernatant was centrifuged again at high speed (10,000 rpm, 30 min) and washed with saline. Bacterial suspension was prepared at 10 times concentration of original cecal supernatant. The bacterial suspensions serially diluted were overlaid in the wells of a microplate which was previously coated with glycolipid. After incubation at 37°C for 2 hr, the plate was gently rinsed with medium A (saline), and finally the microbes were scraped with medium B (0.05% Tween 80 in saline) using a plastic rod. The microbes of this suspension thus obtained were analyzed in various kinds of selective media as shown in Table 2.

Enterobacteriaceae. On the other hand, of all genera tested, no bacteria was recovered from fucosyl asialo GM1-coated plastic plate. It is not known why many bacteria were recovered from the ceramide (hydrophobic portion of glycolipids)-coated plate. The bacterial compositions recovered from asialo GM1- and fucosyl asialo GM1-coated plastic plate are shown as percentages in Fig. 5. This result suggests a possibility that fucosylation of asialo GM1 glycolipid represses the adhesion of *Enterobacteriaceae*, which is superior in number just after conventionalization of mice, to host cells. The possibility of a defense mechanism by alteration of sugar structures of the membrane needs further investigation.

Other glycolipids present in humans, rats, etc. have been tested for bacterial binding (10). In these studies, glycolipids were developed on thin-layer plate, radiolabeled bacterial cell suspensions were overlaid on the plate, and finally the binding was detected by radioautography. *Propionibacterium* and *E. coli* (patho-

Fig. 4. Bacterial adhesion to the glycolipid-coated plastic microplate. Microbe suspensions serially diluted (5 times) were overlaid in the glycolipid-coated plate. After washing with phosphate-buffered saline, the residual number of each bacteria was counted in each selective medium, respectively. $0.5 \mu\text{g}$ of asialo GMI glycolipid (crossed bar), fucosyl asialo GM1 (hatched bar), or ceramide (open bar) was previously coated in a 96-well plastic plate. The figures in the abscissa indicate the dilution rate of the microbe suspension. The method for bacterial adhesion is illustrated in Fig. 3. The viable number of each bacteria was counted as shown in Table 2.

genic) are shown to strongly bind to some glycolipids on thin-layer plate. Especially, lactosyl ceramide, present in human feces (13) and the rat (5), was recognized by many kinds of bacteria.

CONCLUDING REMARKS

The intestinal glycolipids are closely associated with the microbes as described in this review. In mice, the infection or colonization of microbes in the alimentary

Fig. 5. Composition of the bacteria recovered from the asialo GM1 (GA1)- and the fucosyl asialo GM1 (FGA1)-coated plastic plate, in comparison with the original cecal bacterial suspension (Control). The data at the bacterial concentration ($\times 25$ dilution of the original suspension) in Fig. 4 were recalculated. Bactero, Lacto, Entero, Bifido, and Strepto represent *Bacteroides*, *Lactobacilli*, *Enterobacteriaceae*, *Bifidobacterium*, and *Streptococci*, respectively.

tract greatly affect the glycolipid structure. This step might be mediated via a proteinaceous soluble factor present in the intestinal tissue. On the other hand, the glycolipids may play a role as attachment site for the microbes from the results of *in vitro* experiments concerning the binding of the microbes to the glycolipids. In mice, *Enterobacteriaceae* was shown to attach to asialo GM1 to some extent. However, *Enterobacteriaceae* lost its affinity to fucosyl asialo GM1. *Enterobacteriaceae* contamination of germfree mice induced fucosylation of asialo GM1 to a small extent. These results prompt a hypothesis that the host animal has a defense mechanism against the infection by alteration of cell-surface glycolipids. To test this hypothesis, further investigation is required.

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