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Binding Specificity of *Lactobacillus* to Glycolipids

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Lactobacillus, a representative useful bacterium, in the intestinal tract was found to bind to some specific glycosphingolipids, like the pathogenic intestinal bacteria. Thin layer chromatography overlay assays using rabbit antiserum against *Lactobacillus casei* revealed that the bacteria bound to G_{A1} and trihexosylceramide strongly, but not to any gangliosides. The bacteria generally bound to glycosphingolipids having short sugar chains and galactosyl moiety in the non-reducing terminal. *L.casei* did not bind to G_{M1}, but bound to the product after sialidase treatment, G_{A1}. This indicated that sialic acid inhibited the adhesion of *L.casei* to tissues. *L.casei* actually bound nonacid glycosphingolipids but not acid glycosphingolipids extracted from the small intestinal mucosa of rats. © 1996 Academic Press, Inc.

Many viruses, pathogenic bacteria, and bacterial toxins specifically recognize and bind to carbohydrate moieties of the eukaryotic cell surface (1). Their binding to the cells is essential to establish an infection or produce the toxin effect. Especially, the binding of viruses and pathogenic bacteria to cells is considered as the primitive form of cell-to-cell interaction. For intestinal bacteria, adherence to cell surfaces for colonizing epithelial tissues is an important initial event. They must colonize on the cell surface of the intestine in resistance against peristalic movement and flow of foods. Among such intestinal bacteria, the useful bacteria on the gut wall is associated with beneficial health effects for the host (2). Therefore, the adhesion of useful bacteria to the cell surface has been gathering attention (3).

We speculated that useful intestinal bacteria should be bound to glycosphingolipids of the eukaryotic cell surface because some of the pathogenic intestinal bacteria have been reported to bind particular glycosphingolipids for infection (4 - 7). We paid attention to *Lactobacillus* because the bacterium is the most representative useful one in the intestinal tract. Urinary tract infections caused by uropathogenic *Escherichia coli* are prevented by implantation of *Lactobacillus casei* (8). Then, we investigated the binding specificity of *Lactobacillus* and found that the bacteria specifically bind to some glycosphingolipids. This is the first report to show that *Lactobacillus* binds to specific glycosphingolipids.

MATERIALS AND METHODS

Bacterial strain and cultivation. Lactobacillus casei strain (IFO 3425) was obtained from the Institute of Fermentation, Osaka. The bacteria were cultivated in a liquid culture medium containing 1% glucose, 0.5% peptone, 0.3% yeast extract, 0.6% sodium acetate, 0.03% KH_2PO_4, 0.03% K_2HPO_4 and 0.01% $MgSO_4$, pH 7.0. The culture was carried out for 24 h with stationary cultivation. The bacteria were harvested by centrifugation, washed 5 times with phosphate-buffered saline (PBS, pH 7.4), and suspended with a small volume of the same buffer.

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Abbreviations used are: G_{M1}, II^3NeuAcGgOse$_4$Ceramide; G_{M2}, II^3NeuAcGgOse$_3$Ceramide; G_{M3}, II^3NeuAcLacCeramide; G_{D1a}, IV^3NeuAc, II^3NeuAcGgOse$_4$Ceramide; G_{D3}, II^3(NeuAc)$_2$LacCeramide; G_{T1b}, IV^3NeuAc, II^3(NeuAc)$_2$-GgOse$_4$Ceramide; G_{Q1b}, IV^3(NeuAc)$_2$, II^3(NeuAc)$_2$GgOse$_4$Ceramide; G_{A1}, GgOse$_4$Ceramide; CTH, GbOse$_3$Ceramide; SPG, II^3NeuAcLcOse$_4$Ceramide; Lc_4, LcOse$_4$Ceramide.

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Preparation of antibody to Lactobacillus casei. One milligram of *L.casei* cells in 0.5 ml of PBS were mixed with 0.5 ml of Freund's complete adjuvant and the cell emulsion was injected into the back of a rabbit. One week later, the rabbit was given a booster shot with 1 mg of cells. On the 7th day after the booster injection, the blood was drawn from an ear vein, and the serum was separated by centrifugation after incubating the blood at 37°C for 2 h. The serum was chromatographed on a Protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals) and the fraction of IgG, which was eluted with 100 mM sodium citrate buffer (pH 4.0), was obtained.

TLC-immunostaining (9, 10). Various glycosphingolipids were developed on a silica gel-coated plate (Polygram, Sil G, Macherey-Nagel Co.) with chloroform-methanol-0.2 % $CaCl_2$ (60 : 35 : 8, v/v) and the plate was dried. Then, the plate was treated with 0.4% polyisobutylmethacrylate (PIM) solution for 1 min and soaked in blocking buffer (1% polyvinylpyrrolidone (PVP) and 1% skim milk in PBS) for 2 h. After being washed 5 times with PBS, the plate was overlaid with intact cells (approximately 10^7 cells/ml) of *L.casei* in PBS containing 0.05% Tween 20 for 2 h, followed by washing 3 times with PBS containing 0.05% Tween 20 and PBS, respectively. Then, the plate was dipped with the blocking buffer for 1 h and washed 4 times with PBS, and it was allowed to react with rabbit anti-*L.casei* IgG solution diluted to 10 mg/ml with PBS containing 0.05% Tween 20 and 3% skim milk for 1.5 h. After being washed 3 times with PBS containing 0.05% Tween 20 and then PBS, the plate was incubated with a peroxidase-conjugated anti-rabbit IgG antibody (from goat) diluted to 1 : 500 for 1.5 h. The plate was washed 3 times with PBS containing 0.05% Tween 20 and then PBS. The amount of the enzyme remaining was visualized by incubation with 0.02% H_2O_2 and 0.6 mM nitrotetrazorium blue in phosphate buffer (100 mM, pH 7.0) containing 0.75 mM NADH for 5 min. After being washed with distilled water and dried, the plate profiles obtained by staining were read with a scanner (HP DeskScan II, Hewlett Packard Co.) and the color intensity of the spots was quantified with a computer using an NIH Image 1.54 Program.

Preparation of glycolipids from rats. Glycolipids of a rat (Wister, male) were extracted from the mucosa of the small intestine obtained by an operation. The mucosa was homogenized and suspended with a chloroform-methanol (2 : 1) solution, then, incubated at 37°C for 1 h. After filtrating the suspended solution, the filtration was concentrated and subjected to Forch partition (11) to obtain a crude preparation of glycolipids. Neutral (nonacid) and acid glycolipids were fractionated by a DEAE-Sephadex A-25 column (acetate form, Pharmacia Fine Chemicals) chromatography using a chloroform-methanol solution containing sodium acetate.

Analyses. For detection of glycosphingolipids, thin layer chromatography was carried out on a TLC plate (Merck Co.) using chloroform-methanol-0.2% $CaCl_2$ (60 : 35 : 8) as the developing solvent, and the plate was sprayed with orcinol-H_2SO_4 reagent for visualization. The protein concentration was measured by the method of Lowry et al.(12).

Materials. G_{A1}, lactosylceramide, glucosylceramide and G_{D3} were obtained from Wako Pure Chemical Co. Globoside, trihexosylceramide (CTH), galactosylceramide and G_{T1b} were obtained from Sigma Chemical Co. G_{Q1b} was purchased from Dia-Iatron Co. G_{M1}, G_{M2}, G_{M3}, G_{D1a}, sialylparagloboside (SPG), Forssman glycolipid, lactotetraose (Lc_4) and sulfatide were gifts from Dr. Hirabayashi, RIKEN. Peroxidase-conjugated anti-rabbit IgG antibody (from goat) and POD-Immunostaining Kit were purchased from Wako Pure Chemical Co. Skim milk was from Difco Laboratories Co. Sialidase (*Arthrobacter ureafaciens*) was kindly donated by Marukin Soysauce Co. β-*N*-Acetylhexosaminidase (Jack bean) was from Sigma Chemical Co. All other chemicals used were obtained from commercial sources.

RESULTS

Binding of L.casei to glycosphingolipids. To examine whether *Lactobacillus* binds to glycosphingolipids of cell membranes, we chose *L.casei* which commonly inhabits the animal intestine (13). Approximately 4 mg of various glycosphingolipids on silica gel thin layer chromatography plates were developed and visualized with resorcinol reagent. Another developed plate for the binding assay was subjected to TLC-immunostaining and the binding of the bacteria was quantitatively determined using a scanner and a computer. As shown in Table I, *L.casei* bound to some glycosphingolipids. *L.casei* bound strongly to trihexosylceramide (CTH) and also bound to G_{A1} and lactosylceramide similarly. *L.casei* bound to some of the glycosphingolipids having relatively short oligosaccharides, but not to any ganglioside glycolipids having sialic acids in the oligosaccharides.

Effect of the amount of glycosphingolipids on the binding of L.casei. Next, to certify the binding of *L.casei* to glycosphingolipids, we examined the effect of their amount on the binding. As shown in Fig. 1, the binding of *L.casei* to G_{A1} and CTH increased with the amount of these glycosphingolipids. The bacteria had slightly higher affinity to CTH than G_{A1}. On the other hand, no bacteria bound to the ganglioside glycolipids tested (G_{M1} and G_{D1a}) even in large amounts.

TABLE I
Binding Activity of *Lactobacillus casei* to Various Glycosphingolipids

Glycolipid	Structure	Relative Binding Intensity
Glucosylceramide	Glc β1-1′Cer	72
Galactosylceramide	Gal β1-1′Cer	46
Lactosylceramide	Gal β1-4Glc β1-1′Cer	89
CTH	Gal α1-4Gal β1-4Glc β1-1′Cer	100
Globoside	GalNAc β1-3Gal α1-4Gal β1-4Glc β1-1′Cer	0
Forssman hapten	GalNAc α1-3GalNAc β1-3Gal α1-4Gal β1-4Glc β1-1′Cer	0
GM3	NeuAc α2-3Gal β1-4Glc β1-1′Cer	0
GM2	GalNAc β1-4(NeuAc α2-3)Gal β1-4Glc β1-1′Cer	0
GM1	Gal β1-3GalNAc β1-4(NeuAc α2-3)Gal β1-4Glc β1-1′Cer	0
GD3	NeuAc α2-8NeuAc α2-3Gal β1-4Glc β1-1′Cer	0
GD1a	NeuAc α2-3Gal β1-3GalNAc β1-4(NeuAc α2-3)Gal β1-4Glc β1-1′Cer	0
GT1b	NeuAc α2-3Gal β1-3GalNAc β1-4Gal β1-4Glc β1-1′Cer NeuAc α2-8NeuAc α2$^{/3}$	0
GQ1b	NeuAc α2-8NeuAc α2-3Gal β1-3GalNAc β1-4Gal β1-4Gal β104Glc β1-1′Cer NeuAc α2-8NeuAc α2$^{/3}$	0
GA1	Gal β1-3GalNAc β1-4Gal β1-4Glc β1-1′Cer	94
LC4	Gal β1-4GlcNAc β1-3Gal β1-4Glc β1-1′Cer	35
SPG	NeuAc α2-3Gal β1-4GlcNAc β1-3Gal β1-4Glc β1-1′Cer	0
Sulfatide	HSO_3-3Gal β1-1′Cer	27

Abbreviations used: Gal, D-galactose; GalNAc, *N*-acetylgalactosamine; Glc, D-glucose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; Cer, ceramide.

Binding intensity of *L. casei* to CTH was taken as 100.

Binding of L.casei to G_{A1} derived from G_{M1}. To ascertain that *L.casei* specifically bound to G_{A1}, we incubated 1 mM G_{M1} with 1 unit *Arthrobacter* sialidase in 10 mM sodium acetate buffer (pH 6.0) at 37°C for various periods, and developed each reaction mixture on thin layer chromatography. As shown in Fig.2a, the orcinol-H_2SO_4 spray revealed that G_{M1} was converted to G_{A1} with incubation. However, TLC-immunostaining revealed only the staining spot corresponding with G_{A1}, and its intensity increased with the incubation time (Fig.2b). No staining was found at the position of G_{M1}. This indicated that *L.casei* specifically binds to G_{A1} not G_{M1}. On the other hand, a similar result was obtained by the treatment of globoside with Jack bean β-*N*-acetylhexosaminidase. Immunostaining on TLC revealed only CTH which was derived from globoside by β-*N*-acetylhexosaminidase treatment, but not on globoside (data not shown).

Binding of L.casei to glycosphingolipids from the small intestine mucosa of rat. *L.casei* is found in various animal small intestines (13). Then, the binding of *L.casei* to glycosphingolipids

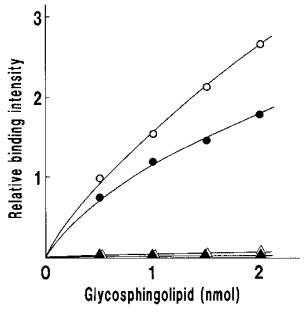

FIG. 1. Binding reactivity of *Lactobacillus casei* to various amounts of glycosphingolipids. The binding assay was performed as described under ''Materials and Methods''. Binding intensity of *L.casei* to 0.5 nmol CTH indicates as 1. ●: G_{A1}, ○: CTH, △: G_{M1}, ▲: G_{D1a}.

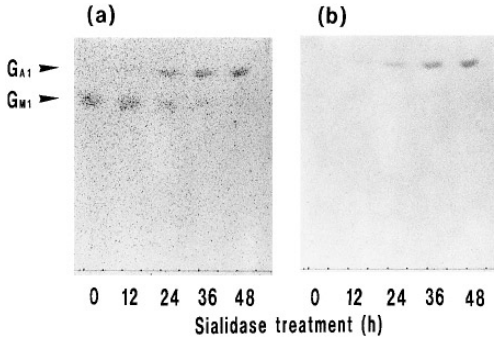

FIG. 2. Binding of *L.casei* to G_{M1} and G_{A1} derived from G_{M1} by sialidase treatment. Thin layer plates with the reaction mixtures of sialidase and G_{M1} were treated with orcinol-H_2SO_4 (a) and for *L.casei* binding and immunostaining (b), as described under ''Materials and Methods''. In this thin layer chromatography, solvent system of chloroform-methanol-0.2% $CaCl_2$ (50 : 50 : 12, v/v) was used.

extracted from the small intestine mucosa of Wister rat was examined by TLC-immunostaining because the presence of a large amount of CTH has been reported in this tissue (14). As shown in Fig.3b, *L.casei* bound to some nonacid glycosphingolipids, but not any acid glycosphingolipids. Although not certified, the nonacid glycosphingolipid fraction may contain G_{A1}, CTH and lactosylceramide judging from their developed positions on TLC (Fig.3a). Then, staining was found at the positions corresponding to their developed positions on TLC-immunostaining, although other unknown glycosphingolipids were also stained (Fig.3b).

DISCUSSION

Studies on the adhesion of *Lactobacillus* to epithelial cells have been focused on the absorption receptor of *Lactobacillus* cells (15 - 18). There are no reports about the adhesive epitope of epithelial cells for binding this bacteria. *Lactobacillus* associates with the gastrointestinal tract and is useful for maintenance of the host's health, but the binding specificity of *Lactobacillus* is only recognized as the fact that the bacteria attach to the mucosa on the gut wall and are colonized on it (2).

Herein, we showed that cells of a strain of *L.casei* attached to specific glycosphingolipids. The bacteria bound to some glycosphingolipids having a relatively short sugar chain, but did not bind gangliosides having sialic acids. The glycosphingolipids for adhesion of *L.casei* have

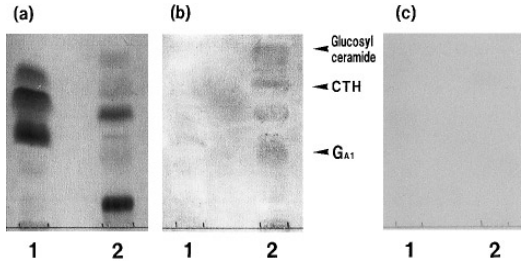

FIG. 3. Detection of glycosphingolipid mixture of rat small intestine mucosa interacting with *L.casei.* Thin layer plates with glycolipid mixtures extracted from rat small intestine mucosa were treated with orcinol-H_2SO_4 (a) and for *L.casei* binding and immunostaining (b), as described under ''Materials and Methods''. (c) is control plate for TLC-immunostaining without addition of *L.casei.* In lanes 1 and 2, an acid glycolipid mixture and a nonacid glycolipid mixture, respectively, were applied. Arrow indicates the developed position of each glycosphingolipid described.

a common structure that their non-reducing terminal saccharide in the sugar chain is a galactose moiety, except for glucosylceramide. Even though it has a different configuration of α and β, it seems to be in agreement with the proposal that the galactosyl residue on the non-reducing terminal of the sugar chain is the minimal requirement for bacterial adhesion. On the other hand, the presence of sialic acid inhibited the binding. Karlson proposed the common oligosaccharide sequence of the internal part in glycolipids responsible for the bacterial adhesion, and named it ''isoreceptor'' (1). However, we could not find such an isoreceptor in the glycosphingolipids *L.casei* bound to.

As shown in Table I, *L.casei* bound to some glycosphingolipids, and then, we attempted to determine the possibility that this bacteria actually binds to glycosphingolipids of the animal intestine. *Lactobacillus* inhabits the small intestine of rats (19). In fact, as shown in Fig.3, the bacteria bound to some nonacid glycosphingolipids of the tissue extract including CTH. CTH is also bound by uropathogenic *E.coli* (5), and it is possible that *Escherichia* and *Lactobacillus* compete with each other for the binding to CTH. The usefulness of therapy for urinary tract infection using *Lactobacillus* seems to be due to competition at the receptor level (8). Bacterial toxins are also bound to some glycosphingolipids (1). *Lactobacillus* may prevent their binding by competition with the receptor of glycosphingolipids.

We also found that *L.acidophilus* which is a predominant bacteria in the human intestine and some isolated strains of *Lactobacillus* from human feces also bound to some glycosphingolipids (data not shown). Further studies are in progress to isolate the receptor of the bacteria for binding.

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