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Inhibitory Effects of Milk Gangliosides on the Adhesion of *Escherichia coli* to Human Intestinal Carcinoma Cells

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The effects of milk gangliosides and their derivatives on the adhesion of enterotoxigenic and enteropathogenic *Escherichia coli* to Caco-2 cells, a human intestinal carcinoma cell line, were investigated. Human milk gangliosides inhibited the adhesion of enterotoxigenic *E. coli* to Caco-2 cells in the same proportion, regardless of the lactational stage, but bovine milk gangliosides were less effective. The most effective inhibitor was monosialoganglioside 1 (G_{M1}); the adhesion rate of enterotoxigenic *E. coli* in the presence of G_{M1} was less than 20% of the positive control. The adhesion of *E. coli* was also depressed to 31.4% by monosialoganglioside 3 (G_{M3}). However, the inhibitory effect of disialoganglioside 3 (G_{D3}) was less than that of G_{M3}. G_{D3} lactone, ceramide lactoside, and *N*-acetylneuraminic acid did not inhibit *E. coli* adhesion to Caco-2 cells. G_{M3} also inhibited the adhesion of enteropathogenic *E. coli* to Caco-2 cells. Thus, these results suggest that G_{M3} possibly behaves as a physiological component in the intestinal tract of infants to protect them against enteric infections.

Bacterial adhesion to the intestinal epithelium is the initial event in gastrointestinal infections caused by enteropathogenic bacteria.[1] Most commonly, adhesion is mediated by binding of bacterial surface proteins, colonization factor antigens, to receptors of host epithelial cells.[2] The receptors appeared to be carbohydrate residues of glycoprotein and glycolipid on the cell membrane.[3,4] Then, it is considered that exogenous substances containing the same carbohydrate residues might competitively inhibit bacterial adhesion to intestinal cells. On the other hand, numerous studies have indicated that breast milk protects against enteric infections.[5,6] Non-immunoglobulin components as well as the high content of secretory immunoglobulin A seem to be responsible for the protective effect of breast milk. Silva and Giampaglia[7] reported that human milk inhibited the adhesion of different serotypes of *Escherichia coli* to Hela cells, a human uterocervical carcinoma cell line, and suggested that structure analogs in human milk of cell membrane receptors for enteric pathogens might have been involved in the inhibition process. Holmgren *et al.*[8] demonstrated that the non-immunoglobulin fraction of human milk inhibits the binding of *E. coli* and *Vibrio cholerae* enterotoxin to the epithelial cells of the intestinal tract. Kolstø Otnæss *et al.*[9] suggested that human milk gangliosides might protect infants against enterotoxin-induced diarrhea. We have found that the predominant ganglioside in human colostrum is disialoganglioside (G_{D3}) and that in mature milk is monosialoganglioside (G_{M3}).[10] Thus, it would be interesting to know whether these gangliosides inhibit the adhesion of *E. coli* to human intestinal cells. Moreover, it was demonstrated that ceramide lactoside (CDH) and G_{D3} lactone were formed from milk gangliosides under the same acidic conditions found in an infant's stomach.[11] This suggests the possibility that these ganglioside derivatives may also protect infants against enteric infections. In this study, we investigated the effects of milk gangliosides and their derivatives on the adhesion of enterotoxigenic and enteropathogenic *E. coli* (ETEC and EPEC, respectively) to Caco-2 cells, a human intestinal cell line.

Materials and Methods

Gangliosides. G_{M3} and G_{D3}, purified from bovine milk were purchased from Wako Pure Chemicals (Osaka, Japan); monosialoganglioside 1 (G_{M1}), purified from bovine brain, and *N*-acetylneuraminic acid (NeuAc) were from Sigma (St. Louis, MO, U.S.A.). Ceramide lactoside was prepared from G_{M3} by treatment with neuraminidase (Sigma, N-6514). Briefly, the mixture (0.5 ml final volume) containing 50 μg of G_{M3}, 20 mU of neuraminidase, 10 mM calcium chloride, and 50 mM sodium acetate buffer (pH 5.5) was incubated at 37 °C for one hour. G_{D3} lactone was prepared by incubating G_{D3} at pH 2.2 as described previously.[11]

Milk specimens. Human milk specimens were obtained at 3–482 days postpartum from mothers living in various areas of Japan.[10] Colostral (3–5 days postpartum), mature (31–60 days) and terminal (241–482 days) composite milks were prepared by mixing specimens from 20 mothers. Bovine colostrum and terminal milk were obtained from Snow Brand Embryo Transplantation Laboratory (Tomakomai, Japan), and unprocessed bovine raw milk was purchased from Zenraku Milk Co. (Sayama, Japan). To extract the lipid fraction from milk, nine ml of milk was mixed with 24 ml of chloroform and 12 ml of methanol; the upper layer was collected and evaporated. The ganglioside-enriched fraction was purified from the extracted lipid fraction by chromatography using a DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (15 mm × 100 mm) equilibrated with chloroform–methanol–water (4 : 8 : 3) and an Iatrobeads 7RS 8060 (Iatron Labs, Tokyo, Japan) column (15 mm × 100 mm) equilibrated with chloroform–methanol (85 : 15) as described previously.[10] Before the Iatrobead chromatography, the eluate from the DEAE-Sephadex column was incubated under basic conditions (pH 12) at 37°C for 2 h to cleave the ester-containing lipids, and dialyzed against deionized water at 4°C overnight. After evaporation, the ganglioside-enriched fraction was suspended in deionized water and lyophilized.

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Abbreviations: G_{M1}, monosialoganglioside 1; G_{M3}, monosialoganglioside 3; G_{D3}, disialoganglioside 3; NeuAc, *N*-acetylneuraminic acid; CDH, ceramide lactoside; FITC, fluorescein 5-isothiocyanate; PBS, phosphate-buffered saline; *E. coli*, *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*.

Measurement of gangliosides. Gangliosides were measured by thin layer chromatography using silica gel 60 thin layer plates (Merck, Darmstadt, Germany) according to a method previously described.[10] Briefly, each plate was developed with chloroform–methanol–water containing 0.2% calcium chloride (55:45:10), sprayed with a resorcinol-copper sulfuric acid-hydrochloric acid reagent to color glycolipids containing sialic acid, and then incubated at 100°C for color development. The chromatogram was scanned at 580 nm with a Dual-Wavelength TLC scanner CS-930 (Shimadzu, Tokyo, Japan).

Bacterial strains. The strains used in this study were ETEC Pb-176 (serotype: O6, K15, H16) obtained from Dr. Y. Kudo (Tokyo Metropolitan Research Laboratory of Public Health, Japan) and EPEC ATCC29552 (serotype: O111ab, K58, H21), which was isolated from the stools of an infant with diarrhea, from American Type Culture Collection (Rockville, MD, U.S.A.). The bacteria was subcultured on agar containing bouillon broth (Eiken Chemical, Tokyo, Japan). Before the adhesion assay, the bacteria were incubated aerobically in bouillon broth for 18 h at 37°C and labeled with fluorescein 5-isothiocyanate (FITC; Sigma). Briefly, the bacteria were gently stirred in phosphate buffered saline (PBS, pH 7.9) containing 0.5% FITC for 3 h at 4°C. The pellet of bacteria was recovered by centrifugation at 3000 × *g* for 10 min and then washed three times with PBS. FITC-conjugated bacteria were suspended in PBS at a cell density of 2.5×10^9 cfu/ml.

Culture of intestinal cells. Caco-2 cells, a human enterocyte carcinoma cell line, were purchased from Dainihon Seiyaku (Osaka, Japan). Cells were routinely grown in Dulbecco's modified minimal essential medium (Gibco Laboratories, Grand Island, NY, U.S.A.) with 7% fetal calf serum (Flow Laboratories, North Ryde, Australia) and 1% nonessential amino acids (Flow Laboratories, McLeen, VA, U.S.A.), and maintained at 37°C in a 5% CO_2/95% air atmosphere. Caco-2 cells cultured in one ml of medium were used for the adhesion assay at late post confluence.

Adhesion assay. The adhesion assay was done as follows. Briefly, the Caco-2 monolayer, grown in conical tubes (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.), was washed twice with PBS. Then, 0.4 ml of FITC-conjugated bacteria suspension was mixed with 0.2 ml of PBS containing the ganglioside-enriched fraction or purified gangliosides. The ganglioside-enriched fraction was dissolved in PBS at a concentration of 2 mg/ml, and purified gangliosides at concentrations between 0.002 and 5 mg/ml. After one h at 37°C, the bacteria were added to the Caco-2 cell cultures and incubated at 37°C for 30 min; the supernatant was then removed by centrifugation at 170 × *g* for 5 min. The monolayers were washed three times with PBS, and detached by treatment with 500 U/ml of trypsin and 0.002% of EDTA. One ml of 0.1% SDS was added to the tubes to lyse *E. coli* and Caco-2, and stirred thoroughly. The fluorescence of the lysate was measured at an excitation wave length of 490 nm and an emission wave length of 520 nm on a fluorometer F-3000 (Hitachi, Tokyo, Japan). The adhesion rate of *E. coli* to intestinal cells was calculated by the following equation:

$$\text{Adhesion rate (\%)} = (\text{The fluorescence of the lysate of cells assayed in the presence of inhibitors}) / (\text{The fluorescence of the lysate of cells assayed in the absence of inhibitors}) \times 100$$

Observation of E. coli adhesion to intestinal cells. E. coli adhesion to Caco-2 cells was observed by the method of Scaletsky *et al.*[12] Caco-2 cell monolayers, grown on eight-well Lab-Tek chamber slides (Nunc Inc., Naperville, IL, U.S.A.), were washed twice with PBS. Apart, 0.4 ml of ETEC Pb-176 suspension was mixed with 0.2 ml of PBS containing G_{M1} and G_{M3} (1 mg/ml) and incubated for one h; the mixtures were then added to the Caco-2 cell cultures, and incubated at 37°C for 30 min. Finally, the monolayers were washed three times with PBS, fixed with methanol, stained with Giemsa (Sigma), and observed microscopically. As controls of positive adhesion, an *E. coli* suspension incubated in the absence of inhibitors was added to Caco-2 cell cultures.

Statistical analysis. Data were analyzed by Student's *t*-test.

Results

Ganglioside-enriched fractions from milk collected at different stages of lactation were investigated for their ability to inhibit *E. coli* adhesion to intestinal cells. Table I summarizes the adhesion rates of ETEC Pb-176 to Caco-2 cells in the presence of ganglioside-enriched fractions extracted from milk. Human milk gangliosides inhibited the adhesion of *E. coli* to intestinal cells in similar proportions, regardless of the lactational stage, but less inhibition was observed with bovine milk gangliosides. This finding suggests that human milk gangliosides are inhibitors of *E. coli* adhesion to intestinal cells and their ability is superior to that of bovine milk. Table II shows the content of

Table I. The Inhibitory Effects of Ganglioside-enriched Fraction fron Milk on the Adhesion of *E. coli* Pb-176 to Caco-2 Cells

Origin of the lipid extract	Adhesion rate (%)	Level (Student's *t*-test)
Human		
Colostrum (day 3–5)	27.2±14.8	<0.01
Mature milk (day 31–60)	19.8±8.7	<0.01
Terminal milk (day 241–482)	24.6±11.1	<0.01
Bovine		
Colostrum (day 3)	87.9±12.5	NS
Mature milk	92.5±9.6	NS
Terminal milk (day 432)	101.6±10.2	NS
PBS	100.0±8.9	

Values represent the means±SD of four determinations, each of whicl was assayed in duplicate.

p values were calculated as compared to the adhesion rate of the contro (PBS) group. NS means not significant.

Table II. The Content of Gangliosides in Milk Collected at Differen Stages of Lactation

Milk	G_{M3} (μg/ml)	G_{D3} (μg/ml)
Human		
Colostrum (day 3–5)	2.0	8.1
Mature milk (day 31–60)	8.2	3.7
Terminal milk (day 241–482)	13.9	2.1
Bovine		
Colostrum (day 3)	0.8	19.9
Mature milk	0.7	8.8
Terminal milk (day 423)	ND	10.4

ND means not detected.

Table III. The inhibitory Effects of Gangliosides and Their Derivatives on the Adhesion of *E. coli* Pb-176 to Caco-2 Cells

Inhibitor	Adhesion rate (%)	Level (Student's *t*-test)
G_{M1}	17.6±4.6	<0.01
G_{M3}	31.4±7.5	<0.01
G_{D3}	83.9±17.2	NS
G_{D3} lactone	108.5±11.6	NS
CDH	96.8±9.8	NS
NeuAc	110.4±12.1	NS
PBS	100.0±14.1	

Values represent the means±SD of four determinations, each of which was assayed in duplicate.

p values were calculated as compared to the adhesion rate of the control (PBS) group. NS means not significant.

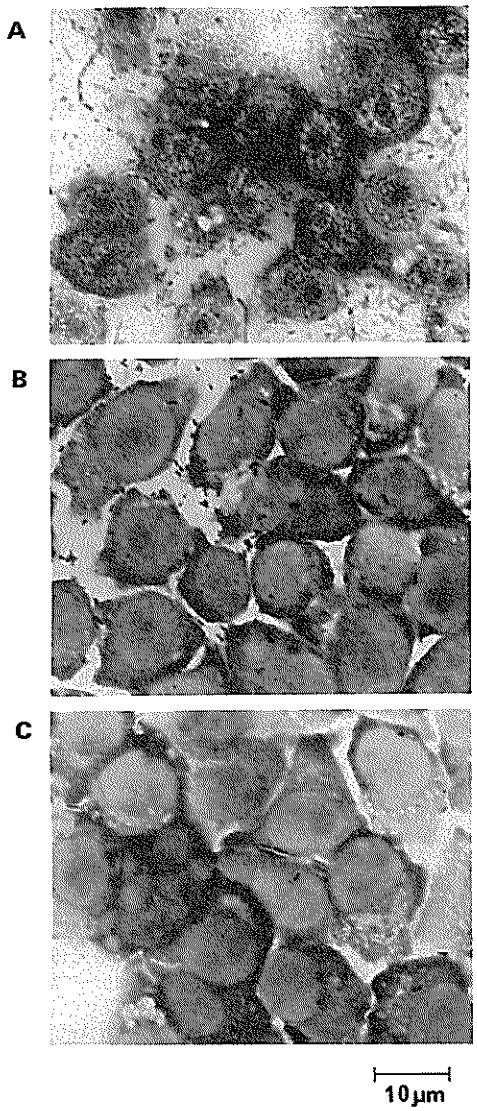

Fig. 1. Micrographs of the Adhesion of *E. coli* Pb-176 to Human Intestinal Carcinoma Cell Line Caco-2.

E. coli was added to Caco-2 cell cultures, after being incubated at 37°C for 30 min in the absence of inhibitors (A), and in the presence of G_{M3} (B) and G_{M1} (C). Other assay conditions are described in Materials and Methods.

gangliosides in human and bovine milk collected at different stages of lactation. The predominant ganglioside in human colostrum was G_{D3}, but in mature and terminal milk it was G_{M3}. During lactation, the content of G_{M3} increased from 2.0 μg/ml to 13.9 μg/ml and that of G_{D3} decreased from 3.1 μg/ml to 2.1 μg/ml. On the other hand, bovine milk contained only a slight amount of G_{M3}, and its content of G_{D3} was more than that in human milk. These data suggest that the content of G_{M3} correlates with the extent of inhibition by human and bovine milk of ETEC Pb-187 adhesion to Caco-2 cells. Table III summarizes the adhesion rates of ETEC Pb-176 to Caco-2 cells in the presence of gangliosides and their derivatives (1 mg/ml). The most effective inhibitor was G_{M1}; the adhesion rate of ETEC Pb-176 was less than 20% of the positive control in the presence of G_{M1}. *E. coli* adhesion was also depressed to 31.4% by G_{M3}. The inhibitory effects of G_{D3} were less than that of G_{M3}, indicating that G_{M3} performs more effectively as an inhibitor of *E. coli* adhesion than G_{D3}. On the other hand, ganglioside derivatives such as G_{D3} lactone, CDH,

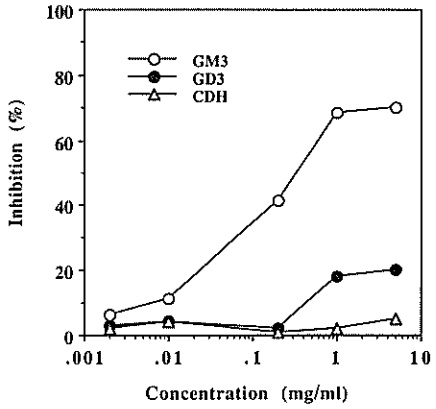

Fig. 2. Effects of G_{M3}, G_{D3}, and Ceramide Lactoside on the Adhesion of *E. coli* Pb-176 to Caco-2 Cells.

E. coli was incubated with inhibitors at concentrations between 0.002 and 5 mg/ml. The adhesion assay was done as described in Materials and Methods.

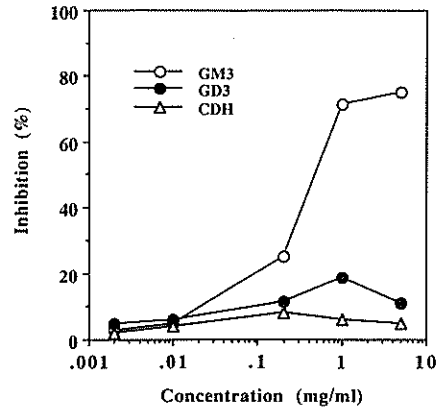

Fig. 3. Effects of G_{M3}, G_{D3}, and Ceramide Lactoside on the Adhesion of *E. coli* ATCC29552 to Caco-2 Cells.

E. coli was incubated with inhibitors at concentrations between 0.002 and 5 mg/ml. The adhesion assay was done as described in Materials and Methods.

and NeuAc did not affect *E. coli* adhesion to human intestinal carcinoma cells. As shown in Fig. 1, ETEC Pb-176 adhesion was inhibited when the bacteria were incubated with G_{M1} and G_{M3}, while a number of *E. coli* bound to the surface of Caco-2 cells after incubation in the absence of inhibitors. These microscopic observations support the data obtained in the adhesion assay with FITC-labeled *E. coli*. The inhibitory effects of G_{M3} and G_{D3} on *E. coli* adhesion to Caco-2 cells were investigated at concentrations between 0.002 and 5 mg/ml using different serotype strains of ETEC Pb-176 (Fig. 2) and EPEC ATCC29552 (Fig. 3) to see whether it was dose-dependent. The inhibition by G_{M3} of ETEC Pb-176 adhesion was similar to that of EPEC ATCC29552. G_{M3} inhibited over 65% of the adhesion of both strains at concentrations of one mg/ml and above, but G_{D3} inhibited less than 20% of their adhesion at the same concentrations.

Discussion

The results obtained here demonstrated that G_{M1} and G_{M3} significantly inhibited the adhesion of *E. coli* to human intestinal carcinoma cells. Ashkenazi and Mirelman[13] reported that the non-immunoglobulin fraction, which they prepared by passing human defatted milk through an immunosorbent column immobilized with antibodies

against human IgG and IgA, inhibited *E. coli* adhesion to the intestinal mucosa of guinea pigs. Because the inhibitory activity was resistant to trypsin digestion but completely abolished by periodate treatment, they suggested that carbohydrate residues were probably involved in the inhibition process. Milk gangliosides are considered to be present particularly in the membrane fraction of the fat globule derived from the apical plasma membrane of the secretory cells in the lactating mammary gland, but they are distributed in various milk fractions, *i.e.*, fats, defatted milk, and whey.[14,15] This suggests that gangliosides in defatted milk may be responsible for the inhibitory activity detected by Ashkenazi and Mirelman.[13]

Of the gangliosides tested in this study, G_{M1} was the most effective inhibitor. Traces of G_{M1} were detected in human milk by a sensitive immunoassay using an aluminium sheet TLC plate,[16] while we could not confirm the presence of G_{M1} in the lipid extract from human milk by thin layer chromatography. Consequently, G_{M1} and/or G_{M3} seemed to be responsible for the inhibitory activity of human milk, and to be more effective than G_{D3}. The lack of inhibitory activity of bovine milk may be due to the reduced content of G_{M1} and/or G_{M3} in bovine milk. Although the content of *N*-acetylneuraminic acid in human milk decreased during lactation,[17] the total content of gangliosides gradually increased (Table II). It is well known that immunological components such as secretory IgA and lactoferrin are plentiful in colostrum and their contents decrease according to the progress of lactation.[18] Gangliosides may be a component for host defense in milk over all the stages of lactation.

Willemsen and De Graaf[19] demonstrated that *N*-glycolyl-G_{M3} derived from pig intestinal mucosa binds to the K99 fimbriae of enterotoxigenic *E. coli* isolated from diarrheic calves. K99 fimbriae do not recognized *N*-acetyl-G_{M3}, which is the predominant structure of human milk G_{M3}. However, the origin of K99$^+$ *E. coli* is a calf and that of *E. coli* used in this study is human. *E. coli* infection appears to be host-specific as described by Deneke *et al.*[20] We assessed the adhesion of *E. coli* strains pathogenic for human in a homologous system using intestinal carcinoma cells of human origin. Because *N*-glycolyl neuraminic acid is absent in humans,[21] the colonization factor antigens of ETEC Pb-176 and EPEC ATCC29552 may recognize *N*-acetyl-G_{M3}. So far, several NeuAc-specific fimbriae of *E. coli* have been discovered.[22-25] Korhonen *et al.*[23] detected fimbriae that recognize sialyl-galactosides in *E. coli* isolated from neonatal patients with meningitis or sepsis. Hemagglutination by the fimbriae was inhibited by *N*-acetylneuraminyl-lactose (NeuAc(α2→3)Gal(β1→4)Glc), which corresponds to the carbohydrate moiety of G_{M3}. Rolsma *et al.*[26] reported that G_{M3} inhibited the binding of group A rotavirus binding to porcine enterocytes and the African green monkey kidney cell line MA-104. We demonstrated that G_{M3} inhibits the adhesion of different serotypes of *E. coli* to human intestinal carcinoma cells. Further studies are required to fully clarify the physiological role of milk gangliosides in infants and to reveal the relationship between the structure of ganglioside and its activity, but on the basis of this study we can say that G_{M3} possibly performs as a physiological component in the intestinal tract of infants to protect them against enteric infections.

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