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Inhibition of Cholera Toxin by Human Milk Fractions and Sialyllactose

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The effects of human milk fractions on clolera toxin B subunit binding to monosialoganglioside 1 (G_{M1}) were investigated. Human milk, human defatted milk, whey, and a low-molecular-weight fraction of human milk inhibited the binding, but casein did not inhibit it. The inhibitory activity of whey from bovine-milk-based infant formula was less than that of whey from human milk. Differences in composition between human and bovine whey seemed to influence the extent of the inhibitory activity. Sialylated oligosaccharides were considered to be the possible components that inhibited cholera toxin. The effects of sialyllactose, a predominant sialylated component of human milk, on cholera toxin-induced diarrhea were investigated by the rabbit intestinal loop method. Sialyllactose inhibited the cholera toxin inducing fluid accumulation, although neither sialic acid nor lactose had an effect on it. The results suggest that sialyllactose is responsible for the inhibitory activity of milk on cholera toxin.

Mother's milk is the best food for infants. It contains antimicrobial factors such as immunoglobulins, as well as all the nutrients which support the growth of infants.

Infection starts with the adhesion of a pathogen to the target cell. Many pathogens recognize the carbohydrate structure on the surface of cells as a receptor.[1,2] Some human milk oligosaccharides have the same structures as those of receptors found on the surface of cells, and are regarded as potential inhibitors of infection, because they are the soluble receptor analogues for the pathogens.[1,2]

Several bacterial and viral pathogens such as *Escherichia coli*, *Campylobacter pylori*, and influenza virus A and B recognize a sialic-acid-containing carbohydrate structure on the target cells as a receptor.[1–6] Sialylated components in human milk may help to prevent infection of these pathogens as soluble receptor analogues.[1,2]

Enterotoxins from *Vibrio cholerae* and enterotoxigenic *Escherichia coli* recognize monosialoganglioside 1 (G_{M1}) on the cell surface as a receptor, adhere to the intestinal mucosa, and cause diarrhea.[7,8] G_{M1} is a glycolipid containing the sialylated carbohydrate structure shown in Fig. 1. The ganglioside fractions from human milk and lactoferrin have been demonstrated to inhibit the action of these enterotoxins.[8–11]

Human milk contains a large amount of sialic acid compared with bovine milk.[12,13] Sialic acid bound to oligosaccharides accounts for 72 to 77% of total sialic acid in human milk.[14] These sialylated oligosaccharides seems to behave as potential inhibitors against the enterotoxins produced by these bacteria. Sialyllactose is a predominant sialylated substance in human milk,[14] but the role of this trisaccharide has not been defined well. 3'-Sialyllactose and G_{M1} have partially the same sequence of carbohydrates, namely the NeuAcα2-3Galβ1-4Glc (Fig. 1). If cholera toxin recognizes this sequence, 3'-sialyllactose may behave as a receptor analogue for cholera toxin. To examine this possibility, we investigated the inhibitory effect of sialyllactose on cholera toxin by the rabbit intestinal loop method.

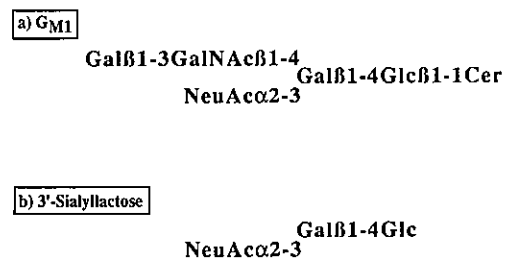

Fig. 1. The Carbohydrate Structure of G_{M1} and 3'-Sialyllactose.

Materials and Methods

Chemicals. Cholera toxin, G_{M1}, and neuramin-lactose (sialyllactose) from bovine milk were purchased from Sigma Chemicals (St. Louis, MO). Cholera toxin B subunit conjugated to horse radish peroxidase (HRP-CTB) and sialic acid were purchased from Biological Labolatories and Seikagaku-Kogyo (Tokyo, Japan), respectively. All other reagents were analytical grade.

Fractionation of human milk. Human milk samples were obtained at about 50 days postpartum from healthy volunteer mothers living in Japan. Human defatted milk was prepared by centrifugation of human milk at 30,000 × *g* for 15 min at 4°C. Casein and whey were separated by adjusting the pH of milk to 4.6 with 1 N HCl and allowing it to stand at room temperature for 30 min, after which it was centrifuged at 30,000 × *g* for 15 min at 4°C. The casein precipitate was lyophilized, and the pH of whey was adjusted to 7.4 with 1 N NaOH. The low-molecular-weight fraction was prepared by ultrafiltration using a Mol-cut II module (fractionating molecular weight: 10,000; Nippon Millipore Ltd., Tokyo, Japan).

Bovine-milk-based infant formula powder was dissolved in warm water at a concentration of 13% (w/v). The whey fraction of infant formula was obtained by the same method used for human milk. All milk fractions were stored below −20°C until use.

Competitive inhibition assay for cholera toxin binding to G_{M1}. HRP-CTB was diluted 1,000-fold with one percent bovine serum albumin (BSA) in potassium phosphate buffered saline (PBS, pH 7.4). Casein was dissolved in PBS at a concentration of one percent (w/v). Human milk, dissolved infant formula, and milk fractions except for casein were thawed at room temperature. All the samples were mixed with an equal volume of HRP-CTB solution and incubated for 30 min at room temperature. Competitive inhibition assays using G_{M1} coated microtitration plates were done with these sample solutions, and the extent of inhibition was

Abbreviations: monosialoganglioside 1 (G_{M1}), *N*-acetyl-neuraminic acid (NeuAc), galactose (Gal), glucose (Glc).

calculated.[11]

Rabbit intestinal loop method. Sialyllactose was dissolved in PBS at concentrations of 50, 100, 150, and 200 μg/ml. Sialic acid and lactose were dissolved in PBS at concentrations of 100 and 200 μg/ml. Cholera toxin was dissolved in PBS at concentrations of 0.5, 1.0, and 2.0 μg/ml. Sialyllactose, sialic acid and lactose solutions were mixed with the same volume of cholera toxin solution. As a positive control, cholera toxin diluted with PBS at the same concentrations of sample solutions was used. These samples were placed on ice to prevent activity loss.

KBL-Jw male rabbits, aged 8 to 10 weeks, were used for the test. After 24 h without food, the abdomen was opened under anesthesia with pentobarbital, and 8 to 10 loops were made per rabbit. One ml of sample solutions were injected in each intestinal loop.[15] To avoid regional differences in response, the order of the injections was randomized.

About 20 h later, the rabbits were killed by pentobarbital injection, and the volume of fluid accumulated and the length of each loop was measured. The extent of inhibition was calculated as

$$\text{Inhibition (\%)} = (1 - A/B) \times 100$$

A: Volume of fluid in the presence of test substances
B: Volume of fluid in the positive control

Results

Inhibitory effects of milk and milk fractions on cholera toxin binding to G_{M1}

As shown in Fig. 2, human milk and human defatted milk inhibited approximately 75% of the binding of cholera toxin B subunit to G_{M1}. The inhibitory activity of whey and the low-molecular-weight fraction from human milk was 80 and 25%, respectively, but casein did not inhibit the binding.

Boving-milk-based infant formula inhibited 70% of the binding of cholera toxin B subunit to G_{M1}. Whey from infant formula also inhibited this binding, but the extent of inhibition was about 40%, which is much lower than the inhibition caused by whey from human milk.

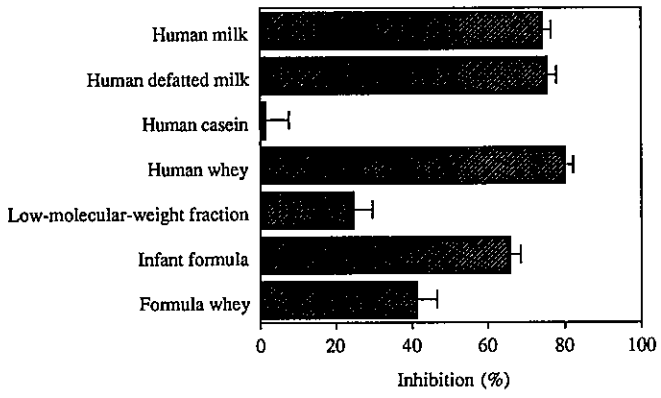

Fig. 2. Inhibition of Cholera Toxin B Subunit Binding to G_{M1} by Human milk, Infant Formula, and Milk Fractions.
Data are presented as mean ± SD ($n=5$).

Table Fluid Accumulation Induced by Cholera Toxin in the Rabbit Intestinal Loops

Cholera toxin (μg/ml)	Fluid accumulation[a] (ml/cm)
0.025	n.d.[b]
0.10	n.d.
0.25	1.385 ± 0.082
0.5	1.088 ± 0.158
1.0	1.188 ± 0.066

[a] Data are presented as mean ± SD ($n=8$).
[b] Not detected.

Effects of sialyllactose on fluid accumulation induced by cholera toxin in rabbit intestinal loops

As shown in Table I, cholera toxin induced fluid accumulation in the intestinal loops of all rabbits at concentrations equal to or higher than 0.25 μg/ml. Fluid accumulation in the loops was not affected by the amount of cholera toxin injected at concentrations between 0.25 and 1.0 μg/ml. Fluid accumulation induced by 0.25 μg/ml of cholera toxin was clearly reduced by more than 50 μg/ml of sialyllactose, as shown in Fig. 3. Fluid accumulation induced by 0.5 and 1.0 μg/ml of cholera toxin were reduced by more than 75 and 100 μg/ml of sialyllactose, respectively. However, sialic acid and lactose had no effect on fluid accumulation induced by cholera toxin as shown in Fig. 4.

Sialyllactose did not cause fluid accumulation in the rabbit intestine in the absence of cholera toxin (data not shown).

Discussion

Enterotoxins produced by *Vibrio cholerae* and enterotoxi-

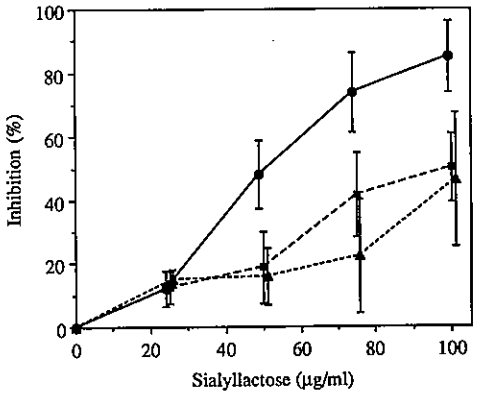

Fig. 3. Inhibitory Activity of Sialyllactose on Fluid Accumulation Induced by Cholera Toxin in the Rabbit Intestine.
Data are presented as mean ± SD ($n=8$).
—●— Cholera toxin 0.25 μg/ml; ---■--- Cholera toxin 0.5 μg/ml; ---▲--- Cholera toxin 1.0 μg/ml.

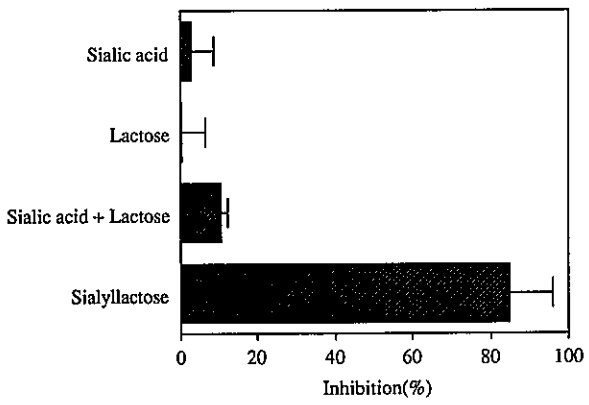

Fig. 4. Inhibitory Activity of Sialic Acid, Lactose and Sialyllactose on Fluid Accumulation Induced by Cholera Toxin in the Rabbit Intestine.
Data are presented as mean ± SD ($n=5$). The concentrations of sialic acid, lactose, and sialyllactose were 100 μg/ml, and the concentration of cholera toxin was 0.25 μg/ml.

genic *Escherichia coli* bind to ganglioside G_{M1} on the target cell surface. It has already been demonstrated that the genglioside fraction from human milk inhibits cholera toxin.[7,8] Lægreid *et al.* suggested that G_{M1} in human milk might contribute to this inhibitory activity.[10]

From the results shown in Fig. 2, it was suggested that the inhibitory activity of human milk on cholera toxin might exist in fractions other than the lipid fraction. Some components of whey were responsible for the inhibitory activity of defatted milk on the binding of cholera toxin to G_{M1}, because casein did not inhibit the cholera toxin B subunit binding to G_{M1}, while whey of human milk inhibited the binding. It also seemed that the low-molecular-weight components contribute at least in part to the inhibitory activity of whey. Sialylated oligosaccharides seemed to correlate with the inhibitory activity, because some of them have partially the same sequence of the carbohydrate moiety of G_{M1} such as 3'-sialyllactose (Fig. 1), and might be soluble receptor analogues for cholera toxin. In addition to this, most of sialylated oligosaccharides are found in the low-molecular-weight fraction.

The amount of sialic acid in the oligosaccharide fraction of human milk is more than twice as that in bovine-milk-based infant formula.[12] This suggests that differences in sialylated oligosaccharides might contribute to the difference in inhibitory activity between human and infant formula whey. The whey of infant formula showed much less inhibitory activity than that of human milk. This finding supports the idea that sialylated oligosaccharides might contribute to the inhibitory activity.

It has already been reported that the ganglioside fraction from bovine milk contains G_{M1} in trace amounts, and affects the cholera toxin binding to G_{M1}.[10] This sialylated glycolipid in bovine milk apparently contributed to the inhibitory activity of infant formula.

Cholera toxin induced fluid accumulation in the intestine at concentrations more than 0.25 μg/ml. The extent of fluid accumulation did not depend on the concentration, indicating that there is an optimum concentration for the response to the toxin in rabbits.

The rabbit intestinal loop method is considered suitable for the test of cholera toxin, because it is possible to investigate this phenomenon as it occurs in the human intestine.[15] The results shown in Fig. 3 suggest the potential role of sialyllactose in human milk as an inhibitor of cholera toxin. The content of 3'-sialyllactose in human milk is 100 to 170 μg/ml,[14] which is enough to inhibit the fluid accumulation induced by cholera toxin. Neither sialic acid nor lactose affected fluid accumulation induced by cholera toxin (Fig. 3), suggesting that the structure of sialyllactose may be important for the inhibitory activity, especially the carbohydrate sequence of NeuAcα2-3Gal seems to be essential.

It has reported that the amount of free sialic acid in feces of breast-fed infants increases, while the amount of glycosidically bound sialic acid decreases according to the progress of maturation.[16] This means that the activity of intestinal sialidase is low in newborn infant, and suggests that sialylated components in human milk may exist in a native form, and may play a role in the physiological function in the intestine of newborn infants.

Thus, this study suggests that sialyllactose possibly performs as a physiological component in the intestinal tract of infants to protect them against enteric infection.

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