Inhibition of Vero Cell Cytotoxic Activity in Escherichia coli O157:H7 Lysates by Globotriaosylceramide, Gb3, from Bovine Milk

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In order to clarify the presence and verotoxin (VT) inhibitory activity of globotriaosylceramide (Gb3) in bovine milk, we analyzed neutral glycosphingolipids (GSLs) from bovine milk and investigated the inhibitory effect of bovine milk Gb3 on the cytotoxicity of VT2. Five species of neutral GSLs, designated as N-1, N-2, N-3, N-4, and N-5, were separated on thin-layer chromatography (TLC). N-1, N-2, and N-3 showed the same mobility as glucosylceramide, lactosylceramide, and Gb3 on the TLC plate, respectively. N-4 and N-5 GSLs migrated below globoside on the TLC plate. N-3 GSL having the same TLC mobility as Gb3 from bovine milk was immunologically identified as Gb3 by monoclonal antibody against Gb3, anti-CD77 monoclonal antibody. Furthermore, the effect of bovine milk Gb3 on VT2-induced cytotoxicity was investigated. We found that treatment of VT2 with bovine milk Gb3 can reduce the cytotoxic effect of VT2.

Key words: bovine milk; Gb3; glycolipids; O-157:H7; verotoxin

A verotoxin (VT)-producing Escherichia coli (VTEC) strain, O157:H7, is now recognized as an important etiologic agent of hemorrhagic colitis (HC)1-4 and its role in the development of classical hemolytic uremic syndrome (HUS) is firmly confirmed.5-7 It has been well established that VTs, VT1 and VT2, play an important role in the progression of HC to serious systemic complications such as HUS and neurological manifestations leading to death.8-11

A cell membrane neutral glycosphingolipid (GSL), globotriaosylceramide (Gb3) is presently recognized as the only functional receptor for VTs, VT1 and VT2.12-15

Infection starts with the adhesion of a pathogen to the target cell. Many pathogens and bacterial toxins recognize the carbohydrate structure on the surface of cells as a receptor.16-18 Human milk is very rich in complex carbohydrates that are attached to protein or lipids backbones as glycoconjugates [glycoproteins (GP) and GSLs].19 Some human milk glycoconjugates have the same structures as those of receptors found on the surface of cells, and competitively inhibit microbial adhesion and enterotoxin binding, because they act as the soluble receptor analogues for the pathogens and bacterial toxins.16,17,20-24

In a previous study, it has been reported that human milk contains a VT receptor, GSL Gb3.25 As well as human milk, bovine milk also contains numerous glycoconjugates [glycoproteins (GP) and GSLs].19 Thus far, although analysis of bovine milk gangliosides, which are GSLs containing sialic acid, has been done,26-29 little is known about the composition of neutral GSLs in bovine milk.30,31

In this study, we investigated bovine milk factors (neutral GSLs) that might influence the pathogenesis of VTEC. The purposes of this study were to discover the presence, amount, and biologic activity of Gb3 in bovine milk.
Materials and Methods

Materials. Bovine milk specimen (Snow Brand Milk Products Co., Ltd., Tokyo, Japan) was commercially obtained. Dimyristoylphosphatidylethanolamine (DMPC), distearoylphosphatidylethanolamine (DSPC) (Nippon Oil & Fats, Tokyo, Japan), dipalmitoylphosphatidic acid (DPPA) (Nippon Fine Chemical, Osaka, Japan), and cholesterol (Chol) (Sigma Chemical Co., St. Louis, MO, USA) were commercial products. Glucosyleramide (GlcCer), lactosyleramide (LacCer), Gb3, and globotetraosyleramide (Gb4) were obtained from human erythrocytes. E. coli O157:H7 (J-2), which produces only VT2, was cultured in nutrient broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C overnight with shaking and used as the source of toxin. Anti-CD77 monoclonal antibody, which is specific for Gb3, was purchased from Cosmo Bio Co., Ltd., Tokyo, Japan.

Cell. Vero cells were used for the assay of Vero cell cytotoxicity. Cells were maintained in Eagle's minimal essential medium (MEM) (Nissui) supplemented with 10% fetal calf serum and antibiotics.

Extraction and purification of neutral GSLs. Bovine milk (2,000 ml) was placed in a flask and ethanol was added to a concentration of 70%. The mixture was stirred vigorously at room temperature for 1 hr and filtered through a filter paper. The insoluble portion was re-extracted sequentially with 10 volumes each of 70% ethanol, chloroform-methanol (2:1, v/v), (1:1, v/v), (1:2, v/v), and chloroform-methanol-water (30:60:8, v/v/v) at 50°C for 1 hr, and filtered. The filtrates (ethanol, chloroform-methanol, and chloroform-methanol-water extracts) were combined and dried by rotary evaporation. The dried lipid extracts were washed with acetone to remove neutral lipid. The residue was dissolved in a minimum amount of chloroform-methanol (1:1, v/v), and then incubated at room temperature for 3 hr to cleave the ester-containing lipids after adjusting the pH of the solution to 12 with 1 N sodium methylate (Wako Pure Chemical, Tokyo, Japan). The solution was neutralized with 1 N acetic acid in methanol and diazylized against distilled water. The dialysate was evaporated and the residue was dissolved in a minimum amount of mixture of chloroform-methanol-water (30:60:8, v/v/v/v) and put on a DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (acetate form) by the method of Ledeen et al. to separate neutral GSLs and gangliosides. The neutral GSLs were eluted successively with 10 column volumes of chloroform-methanol-water (30:60:8, v/v/v/v) and evaporated to dryness in vacuo. For purification of Gb3, the neutral GSL fraction obtained by the DEAE-Sephadex A-25 column chromatography was rechromatographed on an Iatrobeads (Iatrobeads 6RS-8060; Iatron Chemical Products, Tokyo, Japan) column (0.4 × 10 cm) with a linear gradient of chloroform-methanol (9:1 to 5:5, v/v). Fractions were collected and monitored by high-performance thin-layer chromatography (HPTLC).

Analytical procedure. The amount of lipid-bound hexose in the neutral GSL fraction was measured by the orcinol method. Neutral GSL solution containing 6 µg of lipid-bound hexose was put on an HPTLC plate (Merk, Darmstadt, Germany) and developed with chloroform-methanol-water (65:35:8, v/v/v). Neutral GSLs were colored by spraying the plate with 0.2% orcinol in 2 N H2SO4 followed by heating in an oven at 120°C for 5–10 min. The spots were scanned for measurement by a dual wavelength TLC densitometer (Shimadzu CS-930, Shimadzu, Kyoto, Japan) at a wavelength of 520 nm. The amounts of individual neutral GSLs were calculated from the amount of lipid-bound hexose in the neutral GSL fraction on the basis of the peak area ratios obtained from densitometric scanning of the TLC plate.

Preparation of crude verotoxin. Crude VT2 was prepared from E. coli O157:H7 (J-2) according to the method described previously. The resulting toxin was filter-sterilized through a 0.22-µm filter unit (Millex-GV, Millipore, Bedford, MA, USA) and stored at −20°C until use.

Preparation of liposomes. Liposomes for liposome immune lysis assay (LILA) were prepared from a lipid mixture solution containing DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol), and GSLs (0.05 µmol) as described previously. As the release marker, 0.05 m carboxyfluorescein (CF; Eastman Kodak, Rochester, NY, USA) was used. Unencapsulated CF was removed by repeated centrifugation at 20,000 × g for 20 min in gelatin veronal-buffered saline [0.1% gelatin, 10 mM veronal buffer (pH 7.4), and 145 mM NaCl]. The final pellet of liposomes was suspended in gelatin-veronal-buffered saline (1 ml) and stored at 4°C.

Liposomes for the adsorption test were prepared by the following method. DSPC (7 µmol), Chol (2 µmol), and bovine milk Gb3 (0.25, 0.5, or 1.0 mg), each dissolved in an organic solvent, were mixed in a conical flask. The lipids were dried on a rotary evaporator, followed by standing for 30 min under high vacuum in a desiccator. After addition of 500 µl of saline and incubation at an appropriate temperature for 3 min, the lipid film was dispersed by vigorous vortexing and the resulting liposome suspension was centrifuged at 16,000 × g for 20 min (4°C). After centrifugation, the final pellet of liposomes was used for the adsorption test.
LILA. LILA was done by the microplate method as described previously.\textsuperscript{36,37} Briefly, 25 µl of the antibody solution and 5 µl of the 50-fold-diluted suspension of stock liposomes were added to each well of a microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After the addition of 25 µl of 400-fold-diluted fresh guinea pig serum as the complement, the mixture was incubated at 37 for 1 hr in a moist chamber. The reaction was stopped by the addition of 100 µl of 10 mM sodium ethylenediaminetetraacetic acid-veronal-buffered saline. The fluorescence of each well was measured with a spectrophotometer (MTP-100F; Corona Electric, Hitachi-naka, Japan) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

Adsorption of VT by bovine milk Gb3. This was done to test the ability of bovine milk Gb3 to remove VT activity from \textit{E. coli} O157:H7 (J-2) lysates. Briefly, 400 µl of crude toxin from the \textit{E. coli} lysates (diluted 1:200 with cell culture medium) was incubated with bovine milk Gb3-containing liposomes or liposomes that were free from Gb3 at 37°C for 30 min. After that, the mixture was centrifuged at 16,000 × g for 20 min (4°C). After centrifugation, the supernatant (100 µl) was tested for cytotoxic activity on Vero cells.

The assay of Vero cell cytotoxicity. Vero cells (2 × 10^5) in a volume of 900 µl of culture medium per well were seeded into 24-well tissue culture plates (Sumitomo Bakelite) and cultured for 24 hr at 37°C. After cultivation, samples (100 µl) of each toxin adsorbed with Gb3-having liposomes or Gb3-free liposomes prepared as described above were added to the appropriate wells in 24-well tissue culture plates containing Vero cells and then cultured. After 2 days cultivation at 37°C, viable cells were counted. Vero cells non-treated with toxin served as controls. The results were expressed as percent Vero cell survival compared to survival of control cells treated with non-toxin. Calculation formula is as follows: % survival = (the number of cells treated with Gb3-containing liposome-adsorbed, Gb3-free liposome-adsorbed, or non-adsorbed toxin/the number of control cells) × 100.

Statistical analysis. Student's \textit{t} test was done for statistical evaluation of the results. Results are expressed as the arithmetic mean with the standard error of the mean (mean ± S.E.).

Results

Neutral GSLs profile of bovine milk

The neutral GSLs of bovine milk were analyzed by HPTLC. Figure 1 shows an HPTLC profile of the neutral GSLs obtained from bovine milk. Five kinds of neutral GSLs were identified. They are designated as N-1, N-2, N-3, N-4, and N-5 from top to bottom on TLC (Fig. 1A). N-1 GSL migrated with an \textit{Rf} similar to that of GlcCer and N-2 GSL, which was the predominant component in bovine milk, showed the same TLC mobility as the LacCer standard. On the other hand, the other GSL, N-3, was chromatographed with Gb3. Furthermore, more polar neutral GSLs, N-4, and N-5, migrated more slowly than Gb4 on TLC. N-1 and N-3 GSLs gave two spots on TLC. This is considered to be mainly due to a difference in their ceramide composition, especially fatty acid. The amounts of N-1, N-2, N-3, N-4, and N-5 GSL in bovine milk, measured by densitometric analysis, are shown in Table 1. To find whether N-3 GSL, with the same TLC mobility as Gb3, is Gb3, it was further purified by preparative
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TLC and the isolated N-3 (Fig. 1B) was tested immunologically.

**Immunological testing of N-3 GSL**

To identify the molecular species of N-3 from bovine milk, N-3 GSL was analyzed by LILIA. Results are shown in Fig. 2. Liposomes containing N-3 GSL corresponding to Gb3 standard in TLC mobility showed a reactivity with anti-CD77 monoclonal antibody which reacts specifically with Gb3. However, liposomes composed of DMPC, Chol, and DPPA had no reactivity to this antibody. This finding indicates that N-3 GSL is Gb3.

**Adsorption effect of bovine milk Gb3-containing liposomes on VT**

Gb3 is recognized as the only functional receptor for the VTs. Therefore, we investigated whether bovine milk Gb3 inhibits the cytotoxic effect of VT by adsorption. The results of this experiment are shown in Fig. 3.

As shown in Fig. 3, the adsorption effect of bovine milk Gb3 on VT was dose-dependent. On the other hand, when the crude toxin was incubated with Gb3-free liposomes, it showed 19.5±1.6% survival of Vero cells. Survival % of Vero cells treated with Gb3-free liposome-adsorbed toxin was similar to that of Vero cells treated with non-adsorbed toxin (16.5±0.6%). This suggests that bovine milk Gb3 would be beneficial to eliminate free VT.

**Discussion**

It has been shown that human milk contains GSL Gb3 which is the functional receptor for VTs, VT1 and VT2. Thus, it is also reasonable to postulate that GSLs of bovine milk could include a ligand to the VTs, such as Gb3, as a protective analogue of host receptor. So far, however, few reports on the neutral GSL composition of bovine milk have appeared.

In this study, 5 kinds of neutral GSLs, N-1, N-2, N-3, N-4, and N-5, were detected in bovine milk. N-1, N-2, and N-3 neutral GSLs had the same TLC migration rate as GcCer, LacCer, and Gb3, respectively. N-4 and N-5 (more polar neutral GSLs) migrated below Gb4 on TLC. In this result, the important point to note is the presence of N-3 GSL having the similar mobility as Gb3 on the TLC plate in bovine milk. N-3 represented 2.6 μg lipid-bound hexose per ml of bovine milk (Table 1). N-3 was recognized by an anti-CD77 monoclonal antibody which reacts specifically with Gb3 (Fig. 2), indicating that the N-3 GSL identified in bovine milk is Gb3 (Galα1-4Galβ1-4Glcβ1-1Cer).

Enterotoxins from *Vibrio cholerae* and enterotoxigenic *Escherichia coli* recognize GSL (ganglioside GM1) on the cell surface as a receptor, adhere to the
intestinal mucosa, and cause diarrhea. Before now, ganglioside GM1 from human milk has been demonstrated to inhibit the action of these enterotoxins. Globule with VTs, binding vine significance human milk could its vine whether VT1 and VT2. In milk, GSLs are found mainly in the fat globule membranes that are derived from mammary gland cell membranes. To examine whether theGb3 in bovine milk acts as a protective analogue of host receptors, therefore, Gb3 from bovine milk was inserted into liposomal membranes and its efficacy in removing VT activity from E. coli O157:H7 (J-2) lysates was assessed. In this study, we could confirm that liposomally inserted bovine milk Gb3 has a verotoxin inhibitory effect (Fig. 2). Thus, it is reasonable to postulate that the Gb3 of bovine milk may be a strong candidate for being a cell receptor analogue for the VTs.

With the exception of the description of GM1 in human milk, few data exist on the biological significance of glycolipids in other milk, such as bovine milk. This present study shows that the Gb3 in bovine milk is biologically active and capable of binding to the VTs. Thus, it could contribute to the protection of humans, especially infants, from the VTs, assuming that the Gb3-ligand complex is unsolubilized and rendered biologically inactive. The GSL, such as Gb3, found in milk is mainly associated with membranous structures, such as the milk fat globule membrane. We hypothesize that the VTs, when binding to the Gb3 of such structures, may become unavailable or less available for binding to the host receptors. Therefore, bovine milk Gb3 may provide protection from VTs, VT1 and VT2, released by VTEC. If so, this would protect the infants from VT-associated diarrhea and probably from the subsequent HUS that develops in some of these children. However, further studies are required to fully clarify the relationship between bovine milk Gb3 and the pathogenesis of VT-associated diseases.

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References

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