A Novel Method for the Production of Glycosphingolipids

by **Yumiko Shimura**[a]), **Junya Suzuki**[a]), **Maria Carmelita Z. Kasuya**[b]), **Koji Matsuoka**[c]), and **Kenichi Hatanaka***[b])

[a]) Japan Chemical Innovation Institute, 2-11-9 Iwamotocho, Chiyoda-ku, Tokyo 101-0032, Japan
[b]) Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan (phone: +81-3-5452-6562; fax: +81-3-5452-6806; e-mail: hatanaka@iis.u-tokyo.ac.jp)
[c]) Division of Material Science, Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan

Neolacto-series ganglioside sialylparagloboside (SPG) is a ganglioside species present in various human tissues, and used in many important studies. In this study, four ganglioside analogs, GM3, GD3, SPG, and NeuAc-Gal-GlcNAc-Gal-GlcNAc-Gal-Glc-Cer, were synthesized by the saccharide-primer method using MDCK cells and β-lactoside primer with different aglycons. As compared to former methods for producing SPG, the primer method was rapid and convenient. Moreover, the yield of SPG was much higher than that obtained by former methods. The production of gangliosides with an azido group in the aglycon moiety was also achieved by using MDCK cells.

Introduction. – Gangliosides, glycosphingolipids containing sialic acid, are present on cell surface membranes and are involved in cell-to-cell interactions, antigenic attachment sites, and receptors [1][2]. Neolacto-series ganglioside sialylparagloboside (SPG; NeuAc(α2–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc-Cer) has been recognized in various human organs, such as spleen [3], erythrocytes [4], liposarcoma [5], and castration-resistant prostate cancer cells [6]. SPG is a receptor of influenza A virus [7]. The influenza A virus binds most effectively to SPG followed by GD1a, GM3, and GM4 as assessed by inhibition of the cytopathy of *Madine–Darby* canine kidney (MDCK) cells due to infection with the influenza A virus [8]. SPG increases during granulocytic differentiation of human myelogenous leukemia cell line HL-60 induced by retinoic acid, and HL-60 cells are differentiated into mature granulocytes when the cells are cultivated with SPG [9]. *Helicobacter pylori* binds to SPG, whereas lacto- and ganglio-series gangliosides do not [10]. In *Tay–Sachs* disease, SPG in cerebrum cells is synthesized instead of a specific blockage of GM1a synthesis from GM2 [11].

SPG is an extremely important oligosaccharide. However, production of SPG is very time-consuming. Chemical synthesis of SPG is difficult and requires several reaction steps [12]. SPG can be isolated from human plasma [13], brain microvascular endothelial cells [14], and colon adenocarcinoma [15]. But, it is very difficult to get human tissues. Extraction from cultured HL-60 cells is also possible, although the amount of SPG that can be obtained is low [9][16]. Enzymatic production of SPG using sialyltansferases requires rare enzymes. Isolation of paragloboside substrate from bovine erythrocytes is necessary [17][18].

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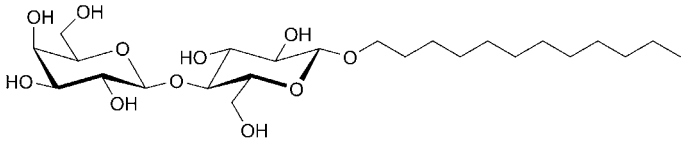
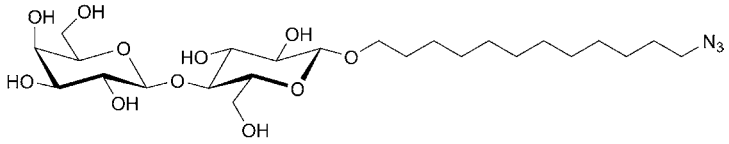
We employed biocombinatorial synthesis of sialylated oligosaccharides by using saccharide primers and cells [19]. Saccharide primers are synthetically accessible amphiphilic glycoside derivatives [20][21]. They resemble intermediates in the biosynthetic pathway of glycolipids and act as substrates for cellular enzyme-catalyzed glycosylation. By using a variety of cells, various kinds of oligosaccharides can be constructed from a single saccharide primer. For example, incorporation of β-lactoside primer into B16 melanoma cells afforded α2-3 sialyl lactoside, which has the same oligosaccharide structure as GM3, the main ganglioside synthesized by B16 cells [19][22]. On the other hand, incorporation of β-lactoside primers into Vero cells afforded Gb3-, Gb4-, and GM3-type oligosaccharides [23].

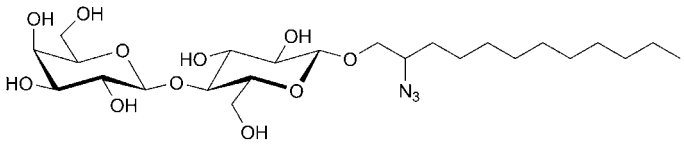
In this study, we synthesized SPG and other gangliosides by the saccharide primer method. Three kinds of pimers, namely dodecyl β-lactoside (Lac-C12 primer), 12-azidododecyl β-lactoside (Lac-12N_3 primer), and 2-azidododecyl β-lactoside (Lac-2N_3 primer) were administered into renal epithelial *Madin–Darby* canine kidney (MDCK) cell line to act as substrate for the synthesis of various oligosaccharides.



Lac-C12: 12-Dodecyl β-D-lactoside



Lac-12N_3: 12-Azidododecyl β-D-lactoside



Lac-2N_3: 2-Azidododecyl β-D-lactoside

Results and Discussion. – *Separation of the Glycosylated Primer.* The HPTLC results (*Fig. 1*) of the lipid extract from the cell homogenates and the culture media, obtained after incubation of cells with 50 μM Lac-C12, Lac-12N_3, and Lac-2N_3 primers and purified using *Sep-Pak C18* column, showed three kinds of new bands corresponding to putative glycosylated products. Further purification of the culture medium fraction using InertSep SAX, followed by stepwise elution with MeOH and H_2O using *Sep-Pak C18* column (*Fig. 2,a*) led to the separation of overlapping bands X3 and X4 (30 and 40% MeOH/H_2O), increasing the number of glycosylated products to four.

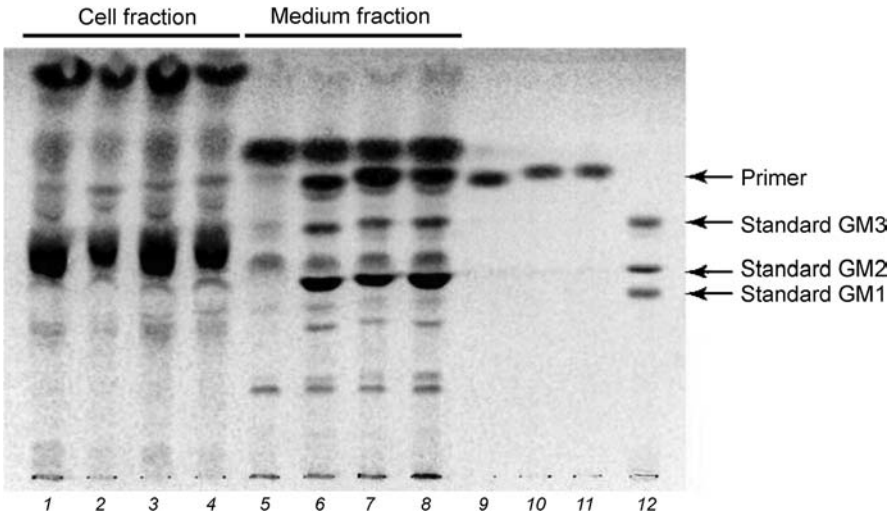


Fig. 1. *Profile of lipids obtained from incubation of MDCK cells with β-lactoside primers.* MDCK Cells (2.0×10^6, 100-mm dish) were incubated for 48 h in serum free TI-DF in the presence of 50 μM each primer. Lipids from the culture media were purified with *Sep-Pak C18* column and separated by HPTLC. Bands were visualized by spraying with resorcinol. Lipid included in each lane is equivalent to 1.0 ml of medium. The experiment was repeated three times. *Lanes 1* and *5:* control (DMSO in TI-DF only) for the cell and culture medium fractions; *Lanes 2* and *6:* treated (50 μM Lac-C12 primer) for the cell and culture medium fractions; *Lanes 3* and *7:* treated (50 μM Lac-$12N_3$ primer) for the cell and culture medium fractions; *Lanes 4* and *8:* treated (50 μM Lac-$2N_3$ primer) for the cell and culture medium fractions; *Lane 9:* Lac-C12 primer (5 nM); *Lane 10:* Lac-$12N_3$ primer (5 nM); *Lane 11:* Lac-$2N_3$ primer (5 nM); and *Lane 12:* GM3, GM2, and GM1a standard (0.5 μg each).

Structure of the Glycosylated Primer. Mass spectrum of X1 obtained from Lac-C12 primer showed a peak at *m*/*z* 800.3 (*Fig. 2,b*). Fragmentation of X1 showed fragment-ion peaks at *m*/*z* 509.1 and 289.9, corresponding to Lac-C12 primer and *N*-acetylneuraminic acid (NeuAc), respectively. So, X1 was assumed to be NeuAc-Lac-C12. By monosaccharide analysis, X1 was found to contain Glc, Gal, and NeuAc in a molar ratio of 1.0 : 1.0 : 1.1. Other monosaccharides were not detected. By enzyme assay, X1 was hydrolyzed by α-2,3-neuraminidase, α2-3,6-neuraminidase, and α2-3,6,8-neuraminidase. Accordingly, X1 has a terminal α2-3 sialic acid moiety. From these results, X1 was identified as a GM3 analog (NeuAc(α2-3)Lac-C12).

Mass spectrum of X2 obtained from Lac-C12 primer showed a peak at *m*/*z* 1165.4 (*Fig. 2,b*). Fragmentation of X2 led to peaks at *m*/*z* 874.4, 712.3, and 509.2, that were identified to correspond to hexose-hexNAc-Lac-C12, hexNAc-Lac-C12, and Lac-C12, respectively. So, X2 was assumed to be a hexose-hexNAc-Lac-C12 linked to a NeuAc somewhere. By monosaccharide analysis, X2 was found to contain Glc, Gal, GlcNAc, and NeuAc in a molar ratio of 1.0 : 2.0 : 1.0 : 1.1. Other monosaccharides were not detected. By enzyme assay, X2 was hydrolyzed by α-2,3-neuraminidase, α2-3,6-neuraminidase, and α2-3,6,8-neuraminidase, indicating that X2 has a terminal α2-3 sialic acid. MS Fragmentation of X2 hydrolyzed by α2-3,6,8-neuraminidase led to a fragment-ion a peak at *m*/*z* 874.5 (data not shown), identified to correspond to hexose-

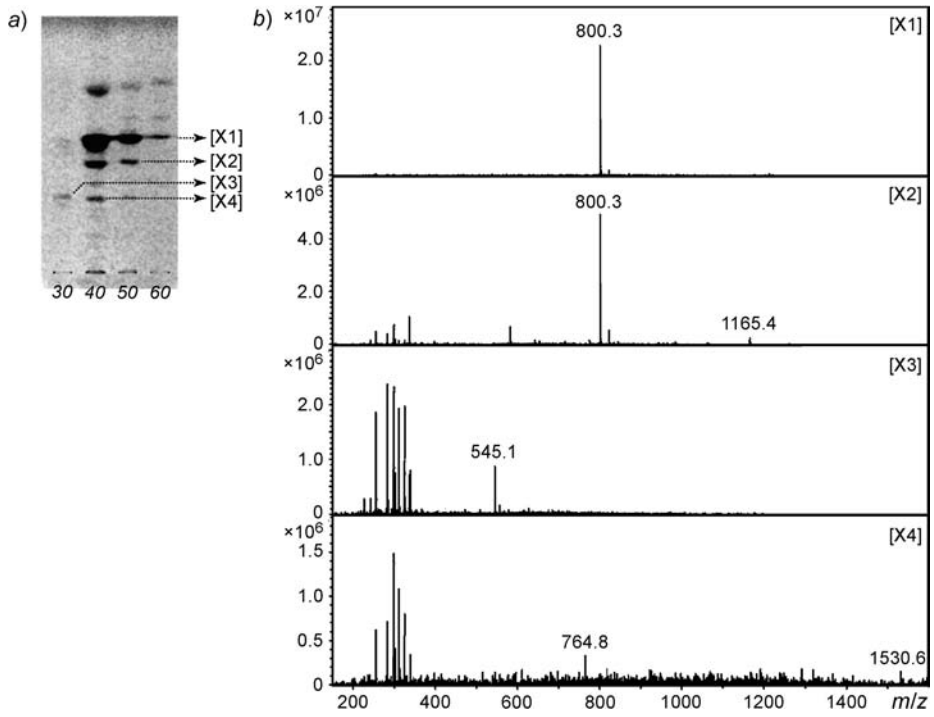


Fig. 2. *Mass spectra of lipids obtained from incubation of MDCK cells with β-lactoside primers. a*) MDCK Cells (5.0×10^6, 150 mm dish) were incubated for 48 h in serum-free TI-DF in the presence of 50 μM each primer. Lipid fractions from MDCK cell medium were purified with *Sep-Pak C18* column and InertSep SAX. These fractions were purified by stepwise elution with MeOH and H_2O using *Sep-Pak C18* column. Lipids were separated by HPTLC. Bands were visualized by spraying with resorcinol. Lipid included in each lane is equivalent to 5.0 ml of medium. The experiment was repeated three times. *Lane number:* concentration of eluted buffer (percent MeOH). *b*) MALDI-TOF mass spectra of the glycosylated products obtained from Lac-C12 primer.

hexNAc-Lac-C12. X2 hydrolyzed by α2-3,6-neuraminidase was further hydrolyzed by β-1,4-galactosidase but not by β1-3,6-galactosidase, indicating that X2 has a terminal Sia(α2-3)Gal(β1-4). From these results, X2 was identified as a SPG analog (NeuAc(α2-3)Gal(β1-4)GlcNAc-Lac-C12).

Mass spectrum of X3 obtained from Lac-C12 primer exhibited a peak at *m*/*z* 545.1 (*Fig. 2*,*b*). However, fragmentation of X3 gave rise to peaks at *m*/*z* 800.3, 581.0, 509.2, and 290.0. Empirically, the mass spectra of gangliosides with two sialic acid residues exhibit a peak at a *m*/*z* value equivalent to half of the primary *m*/*z*. The primary *m*/*z* value of X3 is 1090.2. Fragmentation of X3 led to peaks at *m*/*z* 800.3, 581.0, 509.2, and 290.0, which were identified to correspond to NeuAc-Lac-C12, NeuAc-NeuAc, Lac-C12 primer, and NeuAc, respectively. So, X3 was considered to be NeuAc-NeuAc-Lac-C12. By monosaccharide analysis, X3 was found to contain Glc, Gal, and NeuAc in a molar ratio of 1.0 : 1.0 : 2.1. Other monosaccharides were not detected. By enzyme assay, X3 was hydrolyzed by α2-3,6,8-neuraminidase but not by α2-3,6-neuraminidase,

indicating that X3 has an α2-8 or a branched sialic acid. From these results, X3 was identified as a GD3 analog (NeuAc-NeuAc-Lac-C12).

Mass spectrum of X4 obtained from Lac-C12 primer showed a peak at *m*/*z* 1530.6 (*Fig. 2*,*b*). Fragmentation of X4 gave rise to peaks at *m*/*z* 1239.6, 1077.5, 874.4, and 509.2, which were attributed to hexose-hexNAc-hexose-hexNAc-Lac-C12, hexNAc-hexose-hexNAc-Lac-C12, hexose-hexNAc-Lac-C12, and Lac-C12 primer, respectively. So, X4 was assumed to be a hexose-hexNAc-hexose-hexNAc-Lac-C12 linked to a NeuAc somewhere. By monosaccharide analysis, X4 was found to contain Glc, Gal, GlcNAc, and NeuAc in a molar ratio of 1.0 : 3.0 : 1.9 : 1.2. Other monosaccharides were not detected. By enzyme assay, X4 was hydrolyzed by α-2,3-neuraminidase, α2-3,6-neuraminidase, and α2-3,6,8-neuraminidase, evidencing that, X4 has a terminal α2-3 sialic acid. MS Fragmentation of X4 hydrolyzed by α2-3,6,8-neuraminidase led to a peak at *m*/*z* 1239.5 (data not shown), identified to correspond to hexose-hexNAc-hexose-hexNAc-Lac-C12. X4 hydrolyzed by α2-3,6-neuraminidase was further hydrolyzed by β-1,4-galactosidase but not by β1-3,6-galactosidase, indicating that X4 has a terminal Sia(α2-3)Gal(β1-4). From these results, X4 was determined as a NeuAc(α2-3)Gal(β1-4)GlcNAc-Gal-GlcNAc-Lac-C12 analog.

MDCK Cells express ganglioside GM3. On the other hand, the expression of GD3 was out of the limit of detection in MDCK cells [24]. The expression of neolacto-series gangliosides was not observed in MDCK cells. In this study, we detected four kinds of ganglioside analogs by using β-lactoside primers and MDCK cells. It is noteworthy that neolacto-series gangliosides were found to be highly expressed by MDCK cells administered with the saccharide primer.

Comparison of the Amount of SPG Produced by the Saccharide Primer Method with That Obtained by Different Methods. SPG was first identified from bovine and human spleen [3]. Conventionally, the methods of preparing SPG include chemical synthesis [12], extraction from human tissues [13–15] or cultured cells [9][16], and enzyme synthesis [17][18]. However, these methods are complicated, and they require technical expertise, and the use of rare enzymes and glycosyl donors. In addition, the yield of SPG is low. For example, the amount obtained from human myelogenous leukemia cell line HL-60 (1.0×10^8 cells) was only *ca*. 1.22 μg [9].

In contrast, the amount obtained by using Lac-C12 primer and MDCK cells was 33 μg (from 250 ml of medium; it is equivalent to 1.0×10^8 cells for our method; *Fig. 3*). By this method, the yield of SPG was *ca*. 27 times higher than that achieved by former methods. By administering the saccharide primer with dodecyl aglycon into the cell culture medium, glycosylated products were released from the cell to the culture medium, such that the cell synthesized the product in more than the usual quantity [19–21]. The saccharide primer could be repeatedly administered and harvested every 48 h, continuously for *ca*. 5–6 times [23][25]. We were able to produce SPG (22 μg), GM3 (940 μg), GD3 (12 μg), and NeuAc-Gal-GlcNAc-Gal-GlcNAc-Lac (10 μg) analogs from 1.0 l of MDCK cell culture medium by continuous administering Lac-12N_3 primer.

Effect of the Position of the N_3 Group on the Amount of Cellular Glycosylation. The advantage of the saccharide primer method is that a functional group can be introduced to the primer. To establish the effect of the position of N_3 group on cellular glycosylation, MDCK cells were incubated with N_3-containing primers for 48 h. The

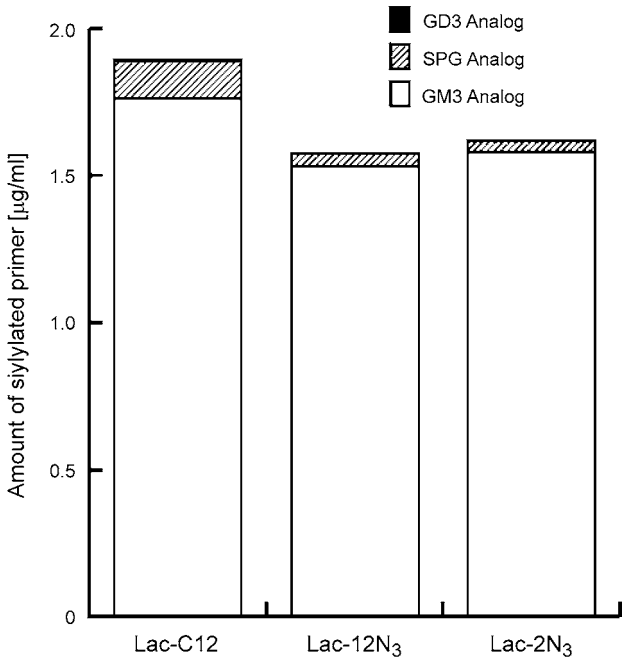


Fig. 3. *Amount of sialylated primer obtained after incubation of MDCK cells.* MDCK Cells (5.0×10^6, 150-mm dish) were incubated for 48 h in serum-free TI-DF in the presence of 50 μM Lac-C12 primer. Lipids from the culture media were purified using *Sep-Pak C18* column with MeOH, followed by *Inertsep SAX* and finally *Sep-Pak C18* column by stepwise elution with MeOH and H_2O. The culture medium fractions were analyzed by HPTLC. HPTLC Plate was sprayed with resorcinol, and heated to detect the separated glycolipids that were later quantified using a densitometer.

lipid fractions extracted from the respective cell and culture medium were analyzed by HPTLC. As shown in *Fig. 1*, most of the sialylated products were secreted to the culture medium. Quantification of the sialylated primers with a densitometer showed that the relative amount of the sialylated products were in order of Lac-C12 > Lac-$2N_3$ > Lac-$12N_3$ primer. As shown in *Fig. 3*, the amount of GM3 analog from Lac-C12, Lac-$12N_3$, or Lac-$2N_3$ primer was found to be 1.8, 1.5, and 1.6 μg/ml, respectively. SPG Analog was found to be 130, 44, and 36 ng/ml, respectively, and GD3 analog was found to be 2.5, 0.67, and 2.1 ng/ml, respectively. On the other hand, the amount of NeuAc(α2-3)Gal(β1-4)GlcNAc-Gal-GlcNAc-Lac-C12 analog was below the limit of detection.

These results indicate that the Lac-$12N_3$ and Lac-$2N_3$ primers were taken up by MDCK cells and transported to the *Golgi* apparatus to serve as acceptors for glycosylation. However, the results suggested that the oligosaccharide production from MDCK cells with Lac-$12N_3$ and Lac-$2N_3$ primer were lower than with Lac-C12 primer.

Conclusions. – In this study, we detected four kinds of ganglioside analogs by the saccharide primer method. By using β-lactoside primers and MDCK cells, the production of GM3, GD3, SPG, and NeuAc-Gal-GlcNAc-Gal-GlcNAc-Lac analogs

were accomplished. For producing SPG, it is noteworthy that the primer method gave higher yields as compared to former methods. Moreover, it was indicated that the production of gangliosides by introduction of an N_3 group in the aglycon moiety can be achieved by using MDCK cells.

This work was supported by *NEDO* (*New Energy and Industrial Technology Development Organization)* of Japan.

Experimental Part

General. The products obtained from the culture medium and cell fractions were separated by HPTLC (silica gel *60*; *E. Merck*) with $CHCl_3$/MeOH/0.25% KCl (aq.) 5 : 4 : 1 (*v*/*v*/*v*). HPTLC Plates were sprayed with resorcinol and heated (120°) to detect the separated glycolipids which were later quantified using *CS-9300PC* dual-wavelength flying spot scanning densitometer (*SHIMADZU*) [26].

Materials. Madin–Darby canine kidney (MDCK) cells were purchased from *ATCC* (Manassas, USA). Three kinds of primers (*Fig. 1*) were synthesized according to literature [20][26]. *Eagle*'s Minimal Essential Medium (EMEM), *Dulbecco*'s Modified Eagle Medium:Nutrient Mixture F-12 (D-MEM/F-12), sodium pyruvate and antibiotic-antimycotic were from *Wako* (Tokyo, Japan). Insulin-transferrin-sodium selenite-X (ITS-X) was from *GIBCO Invitrogen* (California, USA). Fetal bovine serum (FBS) was from *JRH biosciences* (Lenexa, USA). *Sep-Pak C18* (reversed-phase column) was from *Waters* (Massachusetts, USA). *InertSep SAX* (anion exchange column) was from *GL Science* (Tokyo, Japan). α-2,3-Neuraminidase (from *Macrobdella decora*), α2-3,6-neuraminidase (from *Clostridium perfringens*), β1-3,6-galactosidase (from *Escherichia coli*), and β-1,4-galactosidase (from *Streptococcus pneumoniae*) were purchased from *E. Merck* (D-Darmstadt). α2-3,6,8-Neuraminidase (from *Arthrobacter ureafaciens*) was from *Nacalai Tesque* (Kyoto, Japan). Gangliosides GM3, GM2, and GM1a were purchased from *Matreya LLC* (Pennsylvania, USA). *p*-Aminobenzoic acid ethyl ester (ABEE) labeling kit, *Honenpak C18* column, and monosaccharide standard mixture (including D-galactose (Gal), D-mannose (Man), D-glucose (Glc), L-arabinose (Ara), D-ribose (Rib), D-xylose (Xyl), *N*-acetyl-D-mannosamine (ManNAc), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), L-fucose (Fuc), and L-rhamnose (Rha)) were from *J-Oil Mills Inc*. (Tokyo, Japan).

Cell Culture. MDCK Cells were cultured in EMEM supplemented with 1.0 mM sodium pyruvate, 1% antibiotic–antimycotic and 10% FBS at 37° in a humidified atmosphere of 5% CO_2. The culture medium was changed every 3–4 d. When the cells were 80–90% confluent, they were passaged using trypsin/EDTA.

Incubation of Cells with Lactoside Primer Cells (2×10^6) were seeded into 100-mm culture dishes containing 10 ml of medium and incubated for 48 h. Then, the cells were washed with TI-DF (D-MEM/F12 containing 1% ITS-X without phenol red) and were incubated with 5.0 ml of primer dissolved in DMSO at a concentration of 50 μM at 37° for 48 h. After incubation, culture media were collected, and cells were washed with PBS (–), harvested using a spatula, and the suspension centrifuged at 1000 rpm for 5 min [26]. The lipids were extracted from the cell pellet with $CHCl_3$/MeOH 2 : 1 (*v*/*v*), then with $CHCl_3$/i-PrOH/H_2O 7 : 11 : 2 (*v*/*v*/*v*), in a sonicated bath. Lipids from the culture media were purified using *Sep-Pak C18* column [20] and *InertSep SAX*.

Separation of Gangliosides by InertSep SAX. Gangliosides could be extracted and purified from the collected culture media by *InertSep SAX*, silica-based solid phase. *InertSep SAX* was activated by MeOH, followed by H_2O, 0.8M AcONa (aq.), and H_2O, and then equilibrated with $CHCl_3$/MeOH/H_2O 5 : 10 : 1 (*v*/*v*/*v*). The gangliosides were eluted using $CHCl_3$/MeOH/4.0M $AcONH_4$ (aq.) 5 : 10 : 1 (*v*/*v*/*v*).

Mass Spectra. Mass spectra (negative-ion mode) were recorded on *HCTultra* (high-capacity ion trap mass spectrometer; *Bruker Daltonics*, D-Bremen). The lipid extract from the culture medium fraction was separated by HPTLC. Individual spots were scraped off the plates, extracted by sonication for 30 min using 1.0 ml MeOH, dried, and the mass spectrum was recorded.

Monosaccharide Analysis. The lipid extract from the culture medium fraction that was separated by HPTLC was hydrolyzed with 4M TFA at 100° for 3 h, and the monosaccharides obtained were *N*-

acetylated, labeled with ABEE, and analyzed by HPLC using *Honenpak C18* column as described in [27][28]. By this method, NeuAc and *N*-glycoylneuraminic acid were detected as ManNAc and *N*-glycoylmannosamine, resp. [28].

Treatment with Enzymes. All enzyme digestions were carried out on the lipid extract from the culture medium fraction separated by HPTLC. For α-2,3-neuraminidase from *M. decora*, lipids were dissolved in 50 µl of neuraminidase soln. (10 mU/ml of α-2,3-neuraminidase in 50 mM sodium phosphate, pH 6.0) and incubated at 37° for 4 h. α2-3,6-Neuraminidase from *C. perfringens* hydrolyzes only terminal α2-3 and α2-6 sialic acid [29][30]. For α2-3,6-neuraminidase, lipids were dissolved in 50 µl of neuraminidase soln. (50 mU/ml of α2-3,6-neuraminidase in 50 mM sodium phosphate, pH 6.0) and incubated at 37° for 4 h. α2-3,6,8-neuraminidase from *A. ureafaciens* hydrolyzes α2-3, α2-6 and α2-8 linked sialic acids regardless of their binding positions [31]. For α2-3,6,8-neuraminidase, lipids were dissolved in 100 µl of neuraminidase soln. (10 mU/ml of α2-3,6,8-neuraminidase in 50 mM AcONa, pH 5.0) and incubated at 37° for 3 h [32][33]. For β1-3,6-galactosidase from *E. coli*, lipids were dissolved in 50 µl of galactosidase soln. (2.0 U/ml of β1-3,6-galactosidase in 50 mM sodium phosphate, pH 6.0) and incubated at 37° for 4 h. For β-1,4-galactosidase from *S. pneumoniae*, lipids were dissolved in 50 µl of galactoidase soln. (100 mU/ml of β-1,4-galactosidase in 50 mM sodium phosphate, pH 6.0) and incubated at 37° for 4 h.

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Received April 18, 2011