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Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin

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Abstract To elucidate the potential of mammalian milk as to protection of infants from infections, we determined the ganglioside compositions of human, cow and goat milk in relation with cholera toxin and botulinum type A neurotoxin-receptors. Gangliosides accounted for 1 to 2 μmol of lipid-bound sialic acid (LSA) in 100 ml of milk, and GD3 comprised about 69% of LSA in all milk samples. Among the milk samples examined, goat milk was found to contain an amount of gangliosides belonging to the b-pathway representing 15.8% of the total LSA. Accordingly, botulinum neurotoxin bound to GT1b and GQ1b in goat milk, but not to any gangliosides in human or cow milk. On the other hand, GM1, the cholera toxin receptor, was found to be present in all milk samples at concentrations of 0.02% to 0.77% of the total LSA and to be maintained at a relatively constant level in human milk during the postpartum period. Gangliosides from 1 ml of pooled human milk exhibited the ability to attenuate the binding of cholera toxin (30 ng) to GM1 by 93%, and those from 500 μl of goat milk completely inhibited the binding of botulinum type A neurotoxin 1.5 μg to GT1b.

Keywords GM1 ganglioside · GQ1b ganglioside · b-pathway · TLC-immunostaining · Ganglioside mapping

The glycolipid nomenclature is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [1]. The ganglioside nomenclature of Svennerholm is employed throughout [2]. PVP, polyvinylpyrrolidone; LSA, lipid-bound sialic acid.

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Introduction

Glycosphingolipids are ubiquitous in mammalian tissues and cells, but their carbohydrate structures change in association with cellular proliferation and differentiation. A typical example is the characteristic change of glycosphingolipids in the mammary gland, in which epithelial cells differentiate to produce milk under regulation by several stimulators including prolactin, epidermal growth factor, steroids and insulin [3, 4]. When the glycolipids in murine mammary glands were examined, gangliosides were found to change during the pregnancy and lactation periods [5]. That is, although GM3, GM1 and GD1a are the predominant components in the glands of nonpregnant mice, the synthesis of GD1α abruptly starts during the mid-trimester of gestation and GD1α becomes the predominant ganglioside as a component of the milk fat globule membrane in the lactating glands after delivery [5], but the reason why GD1α needs to be supplied to neonates is unknown. One can suggest that GD1α plays a role by facilitating neural development in neonates, because its

distribution in murine tissues has been demonstrated to be restricted to cerebellar Purkinje cells [6].

Also, as reported previously [7], human colostrum and cow milk exhibit a unique ganglioside composition with GD3 as the major constituent. Since GD3 is expressed in glial cells [8–11], its maternal supply may be involved in neuronal development and immunological education in neonates, but the bioactivity of gangliosides remains uncertain, although there have been several reports on the activity of exogeneous gangliosides [12].

Among several features of gangliosides reported in the literature, the receptor activities on their carbohydrate structures are well established, *i.e.*, GM1 for cholera toxin and GT1b for botulinum neurotoxin [12], and the presence of gangliosides with cholera toxin-receptors has been revealed in human and cow milk by an inhibition experiment on their biological activity toward rabbit intestine, demonstrating the functional significance of the gangliosides in mammalian milk for protection of neonates from cholera toxin-induced diarrhea [13, 14]. GM1 is a well characterized receptor for cholera toxin [15, 16], but its metabolic pathway is distinct from that of GD3 in human and cow milk. In general, gangliosides with gangliotetraose are mainly distributed in the neural tissues of man and cow, and their synthesis in non-neural tissues is low, raising the possibility that the neutralizing activity against cholera toxin in human and cow milk really depends on GM1. Accordingly, to evaluate the real ability of gangliosides in mammalian milk to neutralize bacterial toxins, we determined the detailed ganglioside compositions of human, cow and goat milk by means of ganglioside-mapping analysis on the basis of our previous reports [7, 17], and their inhibitory activities toward the cholera toxin and botulinum neurotoxin on their binding to receptors. In this experiment, the uniqueness of the gangliosides in mammalian milk was again shown through analysis of gangliosides in goat milk, in which there were ones in the b-pathway for neural tissues.

Materials and methods

Materials

Standard gangliosides from various sources were purified in our laboratory: GM3 from human erythrocytes, GD3 from chicken brain, and GM1, GD1a, GD1b, GT1b and GQ1b from bovine brain. Cholera toxin B subunit was purchased from Sigma (St. Louis, Mo, USA) and used to immunize rabbits (New Zealand White; Japanese Biological Materials, Tokyo) together with Freund's complete adjuvant (Sigma) to prepare anti-cholera toxin B subunit antibodies. Botulinum type A neurotoxin and rabbit anti-botulinum type A neurotoxin antibodies were kindly donated by Prof. S. Kozaki, Osaka Prefectural University, Osaka. Anti-Gg_3Cer, anti-Gg_4Cer and anti-GM1 antibodies were also prepared by immunizing rabbits as described above, and monoclonal antibodies, Y916, which cross-reacted with GD3, GT3 and $IV^6NeuAc\alpha$-nLc_4Cer were prepared by immunization with the ganglioside mixture prepared from cow milk in our laboratory. Monoclonal antibodies against GM2 (YHD06) and GD3 (R24), and GT1b (GMR5) and GQ1b (GMR13) were kindly donated by Dr. M. Yamazaki, Konica Co., Tokyo, and Dr. T. Tai, Tokyo Metropolitan Institute of Medical Science, Tokyo, respectively. Human milk samples at 2 to 390 d postpartum were kindly provided by the maternity ward, University Hospital, the University of Tokyo, and pooled milk samples from cows (Holstein) at 10 to 30 days postpartum and goats (Japanese Saanen) at 5 to 25 days postpartum were obtained from Yakult Co., Tokyo. All the milk samples were dialyzed against distilled water and lyophilized.

Analysis of gangliosides

The extraction of lipids from the freeze-dried milk was performed by incubation with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1:0, *v*/*v*/*v*), the volume of the combined extracts being adjusted with chloroform/methanol (1:1, *v*/*v*/*v*). After determination of GM3 and GD3 in the extracts by TLC with resorcinol–HCl reagent, and TLC-immunostaining with anti-GM3 and anti-GD3 antibodies, the extracts were fractionated into neutral and acidic lipids on a DEAE–Sephadex column (A-25, acetate form; GE Healthcare Bioscience, Piscatway, NJ, USA), and the gangliosides were prepared from the absorbed acidic lipid fraction by cleavage of the ester-containing lipids, followed by dialysis [5, 18]. The amount of lipid-bound sialic acid (LSA) in each fraction thus obtained was determined by the thiobarbituric acid procedure after treatment of the gangliosides with *Arthrobacter ureafaciens* neuraminidase as described previously [19], and by the resorcinol–HCl procedure [20]. The partially purified gangliosides were developed on TLC plates (Merck, Darmstadt, Germany) with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, *v*/*v*/*v*) and chloroform/methanol/2.5 N ammonia (60:40:9, *v*/*v*/*v*), and the spots were visualized with resorcinol–HCl or orcinol–H_2SO_4 reagent. Standard gangliosides, GM3, GD3, GM1 and GT1b (0.1 to 1.5 μg), were developed on the same plate, and the density of spots visualized with resorcinol–HCl was determined at the analytical wavelength of 580 nm and the control wavelength of 700 nm using a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto) for the preparation of standard curves for quantitation. Then, after analysis by the ganglioside-mapping procedure [21], individual gangliosides were

purified by silica gel column chromatography (Iatrobeads, 6RS8060; Iatron, Tokyo) with gradient elution from chloroform/methanol/water (55:45:2, *v*/*v*/*v*) to (20:80:2, *v*/*v*/*v*) for monosialogangliosides, and from chloroform/methanol/water (55:45:4, *v*/*v*/*v*) to (10:90:4, *v*/*v*/*v*) for di-, tri- and tetrasialogangliosides, respectively [22]. The structures of the purified gangliosides were elucidated by comparison of their positions with those of standards on a ganglioside-map, linkage analysis of carbohydrate chains by GLC-mass spectrometry on a ECNSS-M column (QP-5050A; Shimadzu, Kyoto) using partially methylated aldohexitol acetates and *N*-methyl acetamido aldohexitol acetates, hydrolysis with sialidase (*V. cholerae*, Sigma, or *A. ureafaciens*, Markin Bio, Kyoto)[22], and TLC-immunostaining with anti-GM3 (M2590), anti-GM2 (YHD-06), anti-GD3 (R24), anti-GT1b (GMR5), anti-GQ1b (GMR13), anti-GM1 (rabbit polyclonal), and anti-Gg_4Cer (rabbit polyclonal) antibodies.

TLC-immunostaining

Gangliosides were developed on a plastic-coated TLC plate (Marchery-Nagel, Düren, Germany), and then the plate was incubated in a blocking buffer [1% polyvinylpyrrolidone (PVP) and 1% ovalbumin in PBS] at 4°C overnight. Then, the plate was incubated with 1.5 μg of botulinum type A neurotoxin or 30 ng of cholera toxin in PBS, the toxin bound on the plate being detected with rabbit anti-botulinum type A neurotoxin (1:500) or anti-cholera toxin (1:500) antibodies and peroxidase-conjugated goat anti-rabbit IgG antiserum (1:1,000) in 3% PVP in PBS [23, 24]. Peroxidase on the plate was visualized with H_2O_2 and 4-chloro-1-naphthol as substrates, and the density of spots was determined with a TLC densitometer as described above. Gangliosides GM1 (0.05–5 ng) and GT1b (10–100 ng) as the receptors of the cholera toxin and botulinum neurotoxin, respectively, were developed on the same TLC plate for the preparation of standard curves for quantitation. For inhibition of toxin-binding to gangliosides on a TLC plate, the total gangliosides, corresponding to 1 ml of milk, were mixed with 100 μg of cholesterol (Sigma) and 100 μg of egg phosphatidylcholine (Sigma), and after evaporation, the residue was suspended in 1 ml of PBS by sonication to prepare liposomes. Then, cholera toxin (30 ng) and botulinum neurotoxin (1.5 μg) were mixed with 10 μl, 100 μl, 500 μl or 1,000 μl of liposomes, and the volume of the solution was adjusted to 2 ml with PBS. After standing on ice for 10 min, the solution was centrifuged at 2000×*g* for 30 min, and the resultant supernatant was diluted to 3 ml with PBS, and then reacted with 200 pg of GM1 for cholera toxin or 20 ng of GT1b for botulinum neurotoxin on a TLC plate as described above.

Table 1 Concentrations of gangliosides in pooled human, cow and goat milk

Days after delivery	Human		Cow		Goat	
	3–4		10–30		5–25	
LSA	1.79±0.28 (μmol/100 ml of milk)		1.02±0.05 (μmol/100 ml of milk)		1.05±0.05 (μmol/100 ml of milk)	
	Percent of the total LSA	nmol/100 ml of milk	Percent of the total LSA	nmol/100 ml of milk	Percent of the total LSA	nmol/100 ml of milk
GM3	29.2	522.7	12.3	125.5	4.1	43.1
GM2	–	–	3.2	32.6	–	–
IV^3NeuAcα, II^6(Galβ1-4GlcNAcβ)-nLc_4Cer	–	–	2.1	21.4	–	–
IV^6NeuAcα-nLc_4Cer	1.6	28.6	–	–	–	
GD3	68.7	614.9	69.8	356.0	68.6	360.2
GD2	–	–	–	–	8.8	46.2
GD1b	–	–	–	–	3.9	20.5
IV^3NeuAcα, II^6(NeuAcα2-3Galβ1-4GlcNAcβ)-nLc_4Cer	–	–	0.9	4.6	–	–
GT3	0.5	3.0	5.2	17.7	10.9	38.2
GT1b	–	–	–	–	2.0	7.0
GQ1b	–	–	–	–	1.1	2.9
Others	–	–	6.5	–	0.6	–
GM1	0.02	0.40	0.07	0.72	0.77	8.12

Concentrations of GM1 were measured by TLC-immunostaining with cholera toxin

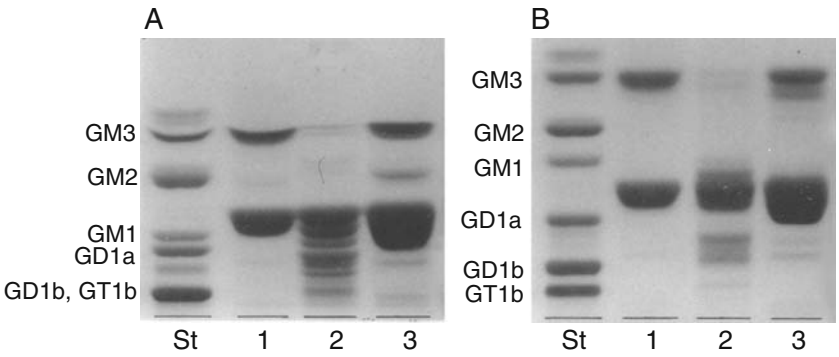

Fig. 1 TLC of gangliosides from pooled human (*1*), goat (*2*) and cow (*3*) milk. Ganglioside, (10–15 μg of LSA) were developed with chloroform/methanol/2.5 N ammonia (60:40:9, *v*/*v*/*v*) (**A**) and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, *v*/*v*/*v*) (**B**), and detected with resorcinol–HCl reagent. *St* standard ganglioside mixture, *i.e.*, GM3, GM2, GM1, GD1a, GD1b, and GT1b

Results

Concentrations of gangliosides in human, cow and goat milk

Pooled (three samples each) human milk at 3 to 4 days postpartum, cow milk at 10 to 30 days postpartum and goat milk at 5 to 25 days postpartum were used for the preparation of gangliosides, whose concentrations expressed as LSA were in the range of 0.97 to 2.07 μmol in 100 ml of milk (Table 1). Also, the molar ratios of LSA to lipid-bound phosphorus ranged from one-tenth to one-fifteenth, and were found to be identical in the cream and skim milk fractions in the separate experiment. As shown in Fig. 1, the major gangliosides in human milk at 3 to 4 days postpartum were GM3 and GD3, which together comprised about 98% of LSA, as reported previously [7]. Similarly, GD3 was also the major ganglioside in cow and goat milk, though minor components differed between them. Of the gangliosides in human, cow and goat milk, those in goat milk exhibited the most complex composition.

Gangliosides in goat milk

On ganglioside-mapping, at least eight gangliosides were found to be present in goat milk (Fig. 2). Judging from their positions on the map, G1, G2 to G5, G6 and G7, and G8 were located at the positions of mono-, di-, tri- and tetrasialogangliosides, respectively, and G1, and G2 and G3 were concluded to be GM3 and GD3 from their positions on the map, and their reactivities with anti-GM3 and anti-GD3 antibodies, respectively. GM1, as a cholera toxin receptor, was not clearly detected in the monosialoganglioside region on the map with resorcinol–HCl reagent. To determine the structures of gangliosides G1 to G8, they were purified by silica gel column chromatography as described under Experimental Procedures and the mobilities of the purified gangliosides on a TLC plate were compared with those of standard ones (Fig. 3). Also, permethylation analysis of gangliosides revealed the following core structures: G1, G2, G3 and G6, NeuAcα_n2–3Galβ1–4Glcβ1–1′Cer; G4, GalNAcβ1–4(NeuAc$\alpha_2$2–3)Galβ1–4Glcβ1–1′Cer; G5, Galβ1–3GalNAcβ1–4(NeuAc$\alpha_2$2–3)Galβ1–4Glcβ1–1′Cer; and G7 and G8, NeuAcα_n2–3Galβ1–3GalNAcβ1–4(NeuAcα_m2–3)Galβ1–4Glcβ1–1′Cer (Table 2). In addition, *V. cholerae* neuraminidase converted them into the following products; LacCer from G1, G2, G3 and G6, GM2 from G4, and GM1 from G5, G7 and G8 (Fig. 4), and *A. ureafaciens* neuraminidase yielded the following products; Gg_3Cer from G4, and Gg_4Cer from G5, G7 and G8, respectively, of which GM2, GM1, Gg_3Cer and Gg_4Cer were confirmed by TLC-immunostaining with the respective antibodies. Also, TLC-immunostaining with monoclonal anti-GT1b and anti-GQ1b antibodies revealed that G7 and G8 were GT1b and GQ1b, respectively (Fig. 5A and B). Thus, the structures of gangliosides in goat milk were concluded to be GM3 for G1, GD3 for G2 and G3, GD2 for G4, GD1b for G5, GT3 for G6, GT1b for G7, and GQ1b for G8, indicating that the gangliosides in goat milk are composed

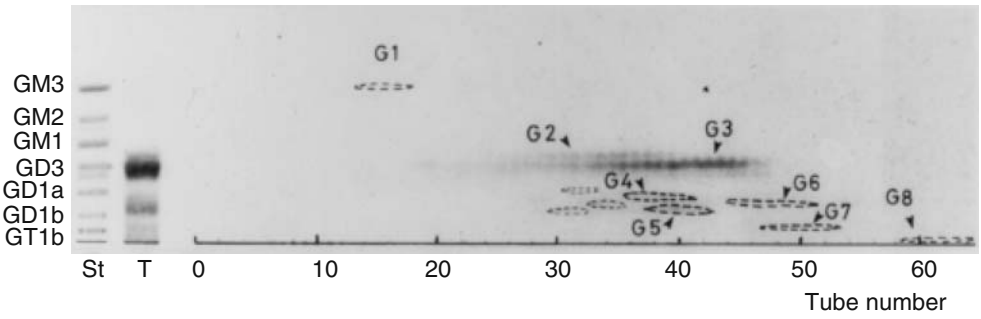

Fig. 2 Ganglioside-map of the gangliosides from goat milk. The developing solvent for TLC was chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, *v*/*v*/*v*), and the spots were visualized with resorcinol–HCl reagent. *St* standard ganglioside mixture, *T* total gangliosides from goat milk

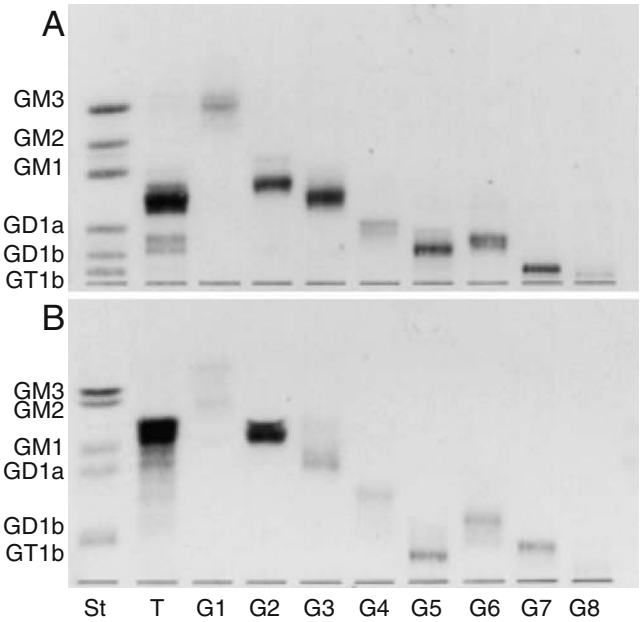

Fig. 3 TLC of gangliosides purified from goat milk. Gangliosides were developed with chloroform/methanol/2.5 N ammonia (60:40:9, *v*/*v*/*v*) (**A**) and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, *v*/*v*/*v*) (**B**), and detected with resorcinol–HCl reagent. G1 to G8 correspond to those on the ganglioside-map (Fig. 2). *St* standard ganglioside mixture, *T* total gangliosides from goat milk

of ones with LacCer, *i.e.*, GM3, GD3 and GT3, and ones in the b-pathway for ganglio-series gangliosides, *i.e.*, GD2, GD1b, GT1b and GQ1b. *N*-Glycolylneuraminic acid in gangliosides amounted to less than 10%, and difference in the mobilities of G2 and G3 was found to be due to their fatty acid compositions, lignoceric and palmitic acids being the predominant fatty acids in G2 and G3, respectively.

Ganglioside compositions of human, cow and goat milk

Ganglioside-mapping analyses and TLC-immunostaining with monoclonal antibody Y916 of gangliosides in human and cow milk revealed the presence of GT3 in addition to the components reported previously [7, 17]. The concentrations of gangliosides in human, cow and goat milk are compiled in Table 1. The common components in human, cow and goat milk were GM3, GD3 and GT3, all of which carried LacCer as a core structure, and they comprised 98.4, 87.3 and 83.6% of the total LSA, respectively. Among them, GD3 was the major one, comprising 69% of the total LSA in all milk samples. On TLC with resorcinol–HCl or orcinol–H_2SO_4 reagent, ganglio-series gangliosides were detected only in goat milk, comprising 15.8% of the total LSA, and the minor components other than LacCer-containing ones in human and cow milk were of the lacto-series without the receptor activities toward the cholera toxin and botulinum neurotoxin.

Receptors for cholera toxin and botulinum neurotoxin in human, cow and goat milk

On sensitive TLC-immunostaining with cholera toxin, GM1 was detected in all milk samples (Fig. 5C), the highest concentration being observed in goat milk, amounting to 8.12 nmol in 100 ml of milk, which was 11-fold and 20-fold those in cow and human milk, respectively (Table 1). Also, binding of cholera toxin to GD1b in goat milk was detected, but the relative receptor activity was less than one-hundredth that of GM1. The structure of GM1 was elucidated on the basis of its mobility on a TLC plate, and the change in reactivity after treatment of total gangliosides with *V. cholerae* and *A. ureafaciens* neuraminidases, which resulted in increased reactivity of gangliosides from goat milk with *V. cholerae* neuraminidase and in disappearance of the reactivity of all gangliosides with *A. ureafaciens* neuraminidase. Thus, the concentrations of GM1 in human, cow and goat milk were less than 1% of the total LSA, this being outside the limit of detection on TLC with chemical reagents.

Then, the concentrations of GM1 in human milk during various lactation periods were measured by TLC-immunostaining. As shown in Fig. 6, although the concentrations of GD3, IV^{6}NeuAcα-nLc$_4$Cer and GT3 in human milk at 2 to 4 days postpartum were significantly higher than those at 14 to 390 days postpartum, GM1 was detected in relatively constant concentrations during the periods examined, amounting to 0.14 to 0.90 nmol in 100 ml of milk, corresponding to 0.01 to 0.08% of the total LSA (Table 3). The band of GM1 migrating to a slightly lower position

Table 2 Partially methylated aldohexitol acetates and *N*-methyl acetamino aldohexitol acetates derived from gangliosides of goat milk

	G1	G2	G3	G4	G5	G6	G7	G8
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol	1	0.9	0.9	–	–	0.8	0.8	0.9
1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl galactitol	–	–	–	1.1	1.0	–	0.9	0.8
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl galactitol	–	–	–	–	0.9	–	–	–
1,3,5-tri-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)-4,6-di-*O*-methyl galactitol	–	–	–	–	0.8	–	0.7	0.8
1,5-di-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)-3,4,6-tri-*O*-methyl galactitol	–	–	–	0.7	–	–	–	–

The ratios of partially methylated aldohexitol acetates to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol (=1.00) are presented

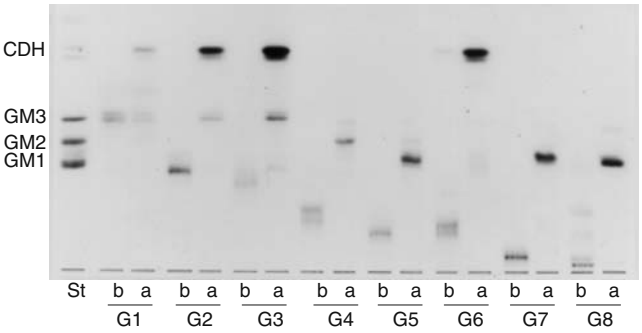

Fig. 4 TLC of gangliosides before (*b*) and after (*a*) treatment with *V. cholerae* neuraminidase. Gangliosides purified from goat milk (1–2 μg) were suspended in water, hydrolyzed with 0.01 units of *V. cholerae* neuraminidase at 37°C for 30 min, evaporated to dryness and then developed on a TLC plate with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, *v*/*v*/*v*). The spots were visualized with orcinol-H_2SO_4 reagent. *St* standard glycolipid mixture, *i.e.*, LacCer (*CDH*), GM3, GM2 and GM1

observed from 40 days postpartum was judged to represent palmitic acid-rich molecules on comparison with the positions of standards, indicating changes in the molecular species of GM1 during the lactation periods.

On pretreatment of cholera toxin (30 ng) with liposomes containing gangliosides corresponding to 10 to 1,000 μl of human milk, followed by TLC-immunostaining, the toxin that bound to GM1 on a TLC plate decreased in a dose-dependent manner, as shown in Fig. 7, and 1 ml of pooled human milk containing about 4 pmol of GM1 was found to exhibit the ability to attenuate the binding of cholera toxin by 93%. Similar inhibitory activity to that of 1 ml of human milk was obtained with gangliosides from 500 μl of cow milk and 40 μl of goat milk, this being nearly parallel with the concentrations of GM1. Thus, human, cow and goat milk were shown to have the ability to neutralize cholera toxin.

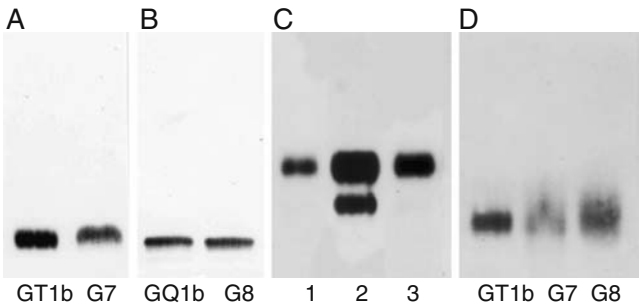

Fig. 5 TLC-immunostaining with anti-GT1b and anti-GQ1b antibodies, cholera toxin and botulinum neurotoxin. Gangliosides were developed with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, *v*/*v*/*v*), and the spots were visualized by TLC-immunostaining with anti-GT1b (**A**) and anti-GQ1b (**B**) antibodies, cholera toxin (**C**) and botulinum neurotoxin (**D**). GT1b and GQ1b, standard gangliosides from bovine brain; *G7* and *G8* gangliosides purified from goat milk; *1*, *2* and *3* total gangliosides from 1 ml of pooled milk samples of human, goat and cow, respectively

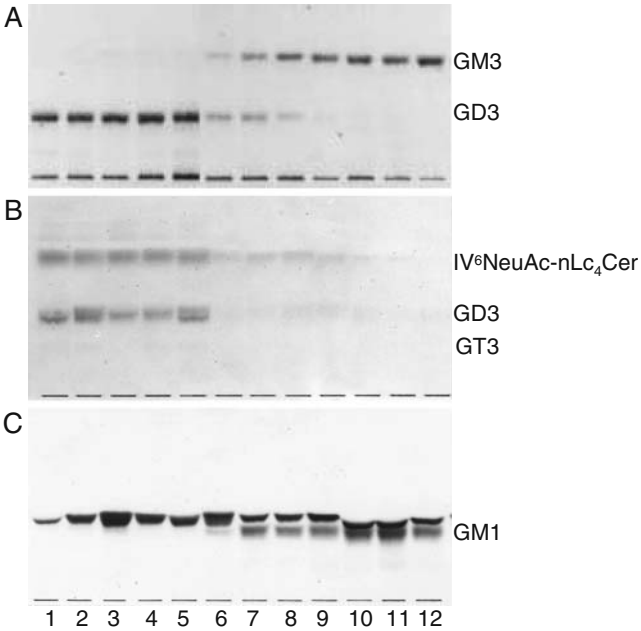

Fig. 6 TLC of gangliosides from human milk in various lactation periods. Gangliosides (10 μg of LSA) were developed with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, *v*/*v*/*v*), and detected with resorcinol-HCl reagent (**A**), monoclonal antibodies, Y916 (**B**), and cholera toxin (**C**). Lactation periods: *1* 2 days, *2* and *3* 3 days, *4* and *5* 4 days, *6* 14 days, *7* 40 days, *8* 60 days, *9* 120 days, *10* 195 days, *11* 270 days, and *12* 390 days

On the other hand, botulinum type A neurotoxin reacted with gangliosides G7 (GT1b) and G8 (GQ1b) in goat milk, but not with those in human and cow milk (Fig. 5D). On TLC-immunostaining, 1.5 μg of botulinum neurotoxin was found to be completely removed by pre-treatment with liposomes containing gangliosides from 500 μl of goat milk, which contained 35 pmol of GT1b and 16 pmol of GQ1b as the receptors. However, since free fatty acids have been reported to exhibit receptor activity in the presence of NaCl [23] and were present in all milk samples in significantly high concentrations, they might contribute to the neutralization of botulinum neurotoxin as well.

Discussion

The ganglioside composition of the milk fat globule membrane has been determined in association with the change in the metabolism in epithelial cells of the mammary gland during the pregnancy and lactation periods [25, 26]. In the case of murine mammary glands, GD1α, which is not present in the glands of nonpregnant mice, characteristically increases during the mid-trimester of gestation and becomes the predominant ganglioside as a component of the milk fat globule membrane in the lactating gland [5], indicating that a new metabolic pathway, which does not operate in the resting glands, is

Table 3 Concentrations of LSA and GM1 in human milk during different lactation periods

Specimen	Lactation period (days)	LSA (μmol/100 ml of milk)	GM1 (nmol/100 ml of milk)	GM1 in the total LSA (%)
1	2	2.34	0.14	0.01
2	3	1.77	0.40	0.02
3	3	1.44	0.74	0.05
4	4	1.62	0.46	0.03
5	4	1.80	0.42	0.02
6	14	1.09	0.52	0.05
7	40	1.49	0.62	0.04
8	60	1.56	0.60	0.04
9	120	1.41	0.76	0.05
10	195	1.01	0.78	0.08
11	270	1.07	0.90	0.08
12	390	1.10	0.68	0.06

The specimen numbers correspond to those in Fig. 6

activated in the lactating gland for the production of milk. Consequently, the a-pathway for ganglio-series gangliosides, which is the major route for ganglioside synthesis in the nonpregnant gland, is greatly suppressed, but operates at a trace level in the lactating gland [5].

Similarly, GD3, as the major ganglioside in human, cow and goat milk, was thought to be synthesized through a metabolic shift in the lactating gland, in which sialyltransferase to GM3 seems to be activated. As a result, GM3, GD3 and GT3, all of which contain LacCer as the core structure, are exclusively present in human, cow and goat milk, as shown in Fig. 1 and Table 1, and as reported by others [13, 27]. However, it is obscure which metabolic route is maintained with the drastic metabolic shift in human, cow and goat lactating glands. Biologically important glycolipids, such as those with receptor activity toward bacterial toxins were thought to be probably synthesized even that their metabolic route competed with the major pathway.

In human and cow milk, the occurrence of cholera toxin receptor GM1 has been well recognized on enzyme-linked immunosorbent assaying and with the *in vivo* intestinal loop method [13, 14]. However, since the synthesis of GM1 competes with that of GD3, active synthesis of GD3 in human, cow and goat milk results in suppression of the metabolic route to GM1. In addition, the metabolism of ganglio-series gangliosides in human and bovine tissues is known to be restricted to neural tissues, reflecting the extremely low concentrations of GM1 in human and cow milk. In fact, the concentrations of it in human, cow and goat milk amounted to less than 1% of the total LSA, but the relatively low concentration is constantly maintained in human milk during different lactation periods, although metabolism of the major glycolipids greatly changes from 14 days of lactation. On TLC-immunostaining, the binding of 30 ng of cholera toxin B-subunit (MW 58 kDa, 0.5 pmol) to GM1 was shown to be inhibited by 93% with the total gangliosides from 1 ml of human milk containing 4 pmol of GM1. In the literature, 1 ml of human milk has been reported to inhibit 100 ng of cholera toxin *in vivo* and *in vitro*, and its inhibitory activity is five to ten times higher than that of cow milk [13, 28]. Our results revealed that inhibition of the cholera toxin-binding to GM1 was essentially dependent on the amount of GM1 in the ganglioside preparation from mammalian milk, and was in the order of goat, cow and human milk. The difference in the results seemed to be probably due to the procedure involving liposomes.

On the other hand, gangliosides exhibiting receptor activity toward botulinum neurotoxin were only detected

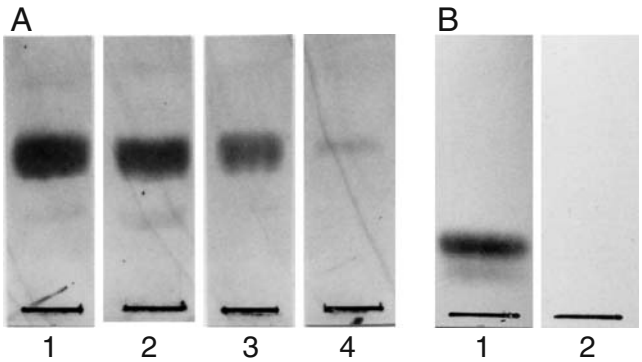

Fig. 7 TLC-immunostaining with cholera (**A**) and botuliunum (**B**) toxins of GM1 (**A**) and GT1b (**B**) gangliosides. **A** Cholera toxin (30 ng) was mixed with liposomes containing gangliosides from 10 μl (*2*), 100 μl (*3*), and 1,000 μl (*4*) of human milk in 2 ml of PBS, and then left to stand on ice for 10 min. After centrifugation at 2,000×*g* for 30 min, each supernatant was reacted with 200 pg of GM1 on a TLC plate. *1* the toxin without any treatment was subjected to TLC-immunostaining. **B** Botulinum toxin (1.5 μg) was mixed with liposomes containing gangliosides from 500 μl of goat milk (*2*) in 2 ml of PBS, and then left to stand on ice for 10 min. After centrifugation at 2,000×*g* for 30 min, the supernatant was reacted with 20 ng of GT1b on a TLC plate. *1* the toxin without any treatment was subjected to TLC-immunostaining

in goat milk. As reported previously [23], botulinum neurotoxin bound to GQ1b, GT1b and GD1a, but not to GT1a, GD1b, GD3, GM1, GM2 or GM3, among which GQ1b was the most potent receptor. Accordingly, preincubation of botulinum neurotoxin (1.5 μg, 10 pmol) with gangliosides from 500 μl of goat milk containing 35 pmol of GT1b and 16 pmol of GQ1b abolished the binding of toxin to GT1b on a TLC plate. But the inhibitory activity of gangliosides toward botulinum neurotoxin was significantly lower than that toward cholera toxin, due to the higher affinity of cholera toxin to GM1 than that of botulinum neurotoxin to GT1b. Thus, mammalian milk was proven to exhibit the ability to protect infants from bacterial infections, as demonstrated also by epidemiologic studies [29, 30]. Although gangliosides from human and cow did not exhibit inhibitory activity toward botulinum neurotoxin, free fatty acids in both milk samples might be responsible for the inhibitory activity, as reported previously [23].

In addition, detailed analysis of the ganglioside composition of mammalian milk again disclosed the similarity with that in neural tissues. In particular, GD1α, as the major ganglioside in murine milk, was selectively distributed in cerebellar Purkinje cells, not in other tissues or cells [6]. Also, GD3, whose expression in mammalian tissues is not characteristic like that of GD1α in murine tissues, is known to be present in significantly high concentrations in the fetal and neonatal brains of vertebrates including man, and is abundant in a reactive microglial lineage [8–11]. Moreover, it was surprising that GQ1b, which is abundant in the occipital cortex of the human brain [31], and in the migrating neurons in mice [32], was present in goat milk. GQ1b is found predominantly during neural cell migration and initial fiber outgrowth, and is transported from the Golgi body to the growth cone of a neuron through cytoskeleton-directed axonal flow [33]. Furthermore, its bioactivity as to neurite outgrowth was shown on exogeneous addition of GQ1b to the culture medium of neuroblastoma cells [34]. These findings indicate that mammalian milk contains biologically active gangliosides for neuronal development, which are maternally supplied to neonates, though it is unclear whether gangliosides in neonates certainly exhibit bioactivity in neuronal tissues without degradation. The intake of gangliosides during the neonatal period is seemingly important for education of immunological systems for efficient establishment of a neuronal network.

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