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Establishment of cells exhibiting mutated glycolipid synthesis from mouse thymus by immortalization with SV-40 virus

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Immortalization with simian virus-40 and cloning of immortalized cells from mouse thymus were performed to establish cell lines for characterization of the mode of glycolipid expression in the thymic cells. Among the 25 cell lines obtained, three lines with different morphologies were established, that is, epithelial (IMTH-E), fibroblastic (IMTH-F), and asterisk-like (IMTH-I) cells, and their glycolipids, together with those in the thymus, were determined systematically. The major glycolipids in mouse thymus were the globo- and ganglio-series, both of which, were co-expressed in the three cell lines established. However, the mode of modification of the globo- and ganglio-series was distinct for each cell line. As to the globo-series, the structures with the longest carbohydrate chain for IMTH-E, -F, and -I cells were Gb_3Cer, Gb_4Cer, and Forssman antigen, respectively, having stepwise shorter carbohydrates at the nonreducing termini. Although the acidic glycolipids in IMTH-E cells comprised GM3 and GM2, and their sulfated isomers, IMTH-F and -I cells expressed GMIb and GDIc for the α-pathway, and up to GDI a for the a-pathway of ganglio-series glycolipids. GMIb-GalNAc present in the thymus was not detected in IMTH-F and -I cells, probably due to the lower synthetic activity for the metabolic intermediate Gg_4Cer. The results indicate that the immortalization technique is useful for obtaining individual cells having unique glycolipid profiles for analysis of the functional significance and metabolism of glycolipids in the thymus.
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Abbreviations: **Glycolipids are abbreviated according to the recommendations of the IUPAC-IUBMB Commission on Biochemical Nomenclature. *Eur J Biochem* 79, 11-21 (1977). The ganglioside nomenclature of Svennerholm [1] is employed throughout, except that GMIb, GMIb-GalNAc, GMIb-GalNAc-Gal, GDIc, and GDI α denote IV^3 NeuAcα-Gg_4Cer, IV^4GalNAcβ, IV^3NeuAcα-Gg_4Cer, IV^4(Galβ-3GalNAcβ),IV^3NeuAcα-Gg_4Cer, IV^3NeuAcα_2-Gg_4Cer, and III^6 NeuAcα, IV^3NeuAcα-Gg_4Cer, respectively. BSA, bovine serum albumin; PBS, phosphate-buffered saline; FCS, fetal calf serum; SV, simian virus; FABMS, fast atom bombardment mass spectrometry; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Hex, hexose: HexNAc, *N*-acetylhexosamine.**

Introduction

Glycosphingolipids are ubiquitous in various types of mammalian tissues and cells, and their carbohydrate chains are to be expressed in species- and organ-specific manners. On comparison of the ganglioside compositions of various organs of rabbit by the ganglioside-mapping procedure [2], the thymus exhibited unique features regarding the carbohydrate and ceramide structures of gangliosides [3–5]. The major carbohydrates of rabbit thymus gangliosides were found to be lacto-*N-neo*tetraose and lacto-*N-nor*hexaose, which are neolactoseries gangliosides, and their ceramide and sialic acid were preferentially composed of *N*-palmitoyl sphingosine and *N*-glycolylneuraminic acid, respectively [5]. These thymus-specific glycolipids were localized in the cortisone-sensitive immature thymocytes in the cortex, but were not observed in the thymuses of man, cow, rat and mouse [6], whose thymuses contained distinctly different glycolipids, and did not exhibit the tissue-characteristic profile like that in rabbit thymus [6]. In the case of mouse thymus, the major glycolipids with more than three carbohydrates were asialo GM1 (Gg_4Cer)

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and Forssman glycolipids belonging to the ganglio- and globo-series, respectively, which were widely distributed in various mouse tissues [7,8], and minor gangliosides, such as GDlc, GMlb-GalNAc and GMlb-GalNAc-Gal, were thought to be markers of thymocytes as alternative tissue-characteristic glycolipids for rabbit thymus [9–17], although their detailed distributions in various tissues of mouse have not been clarified yet. Since both GM1 and asialo GM1, but not Forssman glycolipids were dramatically reduced in the thymuses of mice 48 h after the administration of dexamethasone, they were preferentially distributed in the dexamethasone-sensitive thymocytes in the cortex [7], but their reactivities toward antibodies greatly changed during development, being higher in immature than mature thymocytes [17–20]. In a similar way, the cytolytic T lymphocyte precursors in mouse were serologically characterized as to the reactivity of globoside toward the antibody without analysis of its concentration in the cells [21]. Accordingly, although the functional significance of glycolipids in the thymus and thymocytes was evident on immunochemical analysis, the relevance of the metabolism and the antigenic properties of glycolipids in individual cells is obscure, because isolation of individual cell populations in a homogeneously enough state for chemical analysis of glycolipids is quite difficult, particularly from thymic tissues constructed of heterogeneous cells. To elucidate the synthetic potential of glycolipids in individual cells of the thymus, we established cell lines from mouse thymus by means of immortalization with simian virus, and determined their glycolipid compositions.

Materials and methods

Materials

Glycolipids were purified from various sources in our laboratory: GM2 and GM1 from bovine brain, GlcCer, LacCer, Gb_3Cer, Gb_4Cer and GM3 from human erythrocytes, Forssman glycolipid from equine kidney, Gg_3Cer from guinea pig erythrocytes, and GMlb from rat ascites hepatoma cells. Gg_4Cer was prepared by treatment with *Arthrobactor ureafaciens* sialidase [22], and *N*-stearoyl derivatives of GlcCer, LacCer, Gb_3Cer and Gg_3Cer, as standards for quantitation of the respective glycolipids on thin layer chromatography (TLC), were synthesized by deacylation of the purified glycolipids with sphingolipid ceramide *N*-deacylase (*Pseudomonas sp*. TK4), followed by reacylation with stearoyl chloride. GMlb-GalNAc and GMlb-GalNAc-Gal were kindly donated by Dr. K. Horikawa, The University of Kumamoto, Kumamoto, and Dr. A. Suzuki, Riken, Wako, respectively.

Carbohydrate-specific antibodies

Rabbit polyclonal antibodies toward GM1, Gg_4Cer and Gg_3Cer were generated by immunizing rabbits intradermally with an emulsion prepared with 1 mg of each purified glycolipid and 0.5 mg of methylated bovine serum albumin (BSA) in 1 ml of phosphate-buffered saline (PBS), and 1 ml of Freund's complete adjuvant (Sigma, St. Louis, MO, USA), with subsequent monitoring of the antibody titer by means of an enzyme-linked immunosorbent assay, the titers being 1:100,000 for anti-GMl, 1:600,000 for anti-Gg_4Cer, and 1:300,000 for anti-Gg_3Cer antibodies, respectively. No cross reaction with structurally related glycolipid derivatives of individual antigens was observed [23]. Anti-cholera toxin antibody was also generated by immunization of a rabbit with cholera toxin (Sigma) as described above, the titer being 1:5,000. Monoclonal anti-l^3SO_3-GalCer antibody (TCS-1) was generated in our laboratory [7]. Monoclonal antibodies against GM2 (NeuGc) (Pyk-2), and GM2 plus GMlb-GalNAc (YHD-06) were provided by Dr. M. Yamasaki, Konica Co., Tokyo [9]. Monoclonal anti-GM3 (M2590) antibody was obtained from Seikagaku Co., Tokyo.

Immortalization of cells from mouse thymus

Mice (HR-1, female, 5 weeks old) were bred in our animal laboratory, and housed at $25 \pm 1^{\circ}C$ and 65–75% humidity. The animal care and experimental protocols were in accord with the guidelines of Kinki University. The thymus was cut into small pieces (2 mm cube), which were washed once with FCS-free α-MEM medium, followed by infection with SV-40 tsA255 (7×10^7 PFU/ml) at $37^{\circ}C$ for 2 h. After washing of the pieces with FCS-free α-MEM, they were put on dishes (35 mm) in α-MEM, supplemented with 10% FCS, 10 μg/ml insulin, 30 ng/ml epidermal growth factor, 100 U/ml penicillin and 0.1 mg/ml streptomycin, in a humidified incubator at $37^{\circ}C$ under a 5% CO_2 atmosphere. The colonies produced were isolated with cloning chambers (5 mm, Iwaki Glass, Tokyo), and the isolated cells were cultured at least up to the 70th generation.

Separation and quantitation of glycolipids

After homogenization and lyophilization of thymus or cells, total lipids were extracted from the lyophilized powders with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1, by vol.), and then the concentrations of cholesterol and lipid-bound phosphorus in the total lipid extracts were determined by gas liquid chromatography with 5α-cholestane as an internal standard and Bartlett's method, respectively [25]. Then, the lipid extracts were fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; Pharmacia, Uppsala, Sweden). The neutral glycolipids were separated from unabsorbed neutral lipid fraction by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the gangliosides were prepared from the absorbed acidic lipid fraction by cleavage of the ester-containing lipids, followed by dialysis [5,23]. The gangliosides and neutral glycolipids thus obtained were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) and chloroform/methanol/ water (65:35:8, by vol.), and then visualized with resorcinol-HCl and orcinol-H_2SO_4 reagents, respectively. The density of spots was determined at analytical wavelengths of 580 nm for resorcinol-HCl-positive spots and 420 nm for orcinol-H_2SO_4-positive spots, respectively, using a

dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto). Standard curves were essentially obtained by using the same glycolipids as those detected in the thymuses: 0.1 to 1.5 μg of GalCer (18:0), LacCer (18:0), Gb_3Cer (18:0), Gg_3Cer, Gb_4Cer, Gg_4Cer, GM3 and GM1, on the same plate.

TLC-immunostaining

Lipids were applied on plastic-coated TLC plates, which were then developed successively with n-hexane/diethyl ether (4:1, by vol.) and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.). Each plate was incubated with a blocking buffer (1% polyvinylpyrrolidone (PVP) and 1% ovalbumin in PBS) at 4°C overnight and then with anti-carbohydrate antibodies in 3% PVP in PBS at 37°C for 2 h. Mouse monoclonal antibodies were used at the concentration of approximately 0.2 μg/ml, and rabbit antisera were usually diluted to 1:500 (by vol.). Afterward, the plates were washed 5 times with 0.1% Tween 20 in PBS, and the antibodies bound to the TLC plates were detected using peroxidase-conjugated anti-rabbit IgG + M or anti-mouse IgG + M antibodies (Cappel Laboratories, Cochranville, PA), diluted 1:1000 (by vol.) with 3% PVP in PBS, and with enzyme substrates H_2O_2 and 4-chloro-l-naphthol, as described previously [7, 8]. The density of spots was also determined using 10 ng to 100 ng of the respective glycolipids as standards for quantitation with a TLC-densitometer as described above, the limit of detection being 5 ng of glycolipids.

Structural analysis of glycolipids

The individual glycolipids were purified using a silica gel (latrobeads 6RS8060; latron Lab., Tokyo) column, with gradient elution with chloroform/isopropyl alcohol/water (85:15:0.2 and 40:60:2, by vol.) for mono- to trihexaosyl ceramides in the neutral GSLs, and for sulfatides and GM3 in the acidic glycolipids, followed by a gradient of chloroform/methanol/water (70:30:4 and 10:90:4, by vol.) for tetra- to hepta-hexaosyl ceramides in the neutral and acidic glycolipids. The purified glycolipids were characterized by negative ion FABMS (JMS-700TKM; JEOL Ltd, Tokyo) with triethanolamine as a matrix solvent, and by TLC-immunostaining with anti-Gg_3Cer, anti-Gg_4Cer, anti-Forssman, anti-I^3SO_3GalCer (TCS-1), anti-GM3 (M2590), anti-GM2 (YHD-06), and anti-GMl antibodies. GlcCer and GalCer in the ceramide monohexosides were separated on a borate-impregnated TLC plate with chloroform/methanol/water/15M ammonia (280:70:6:1, by vol.), and characterization of sulfoglycolipids was performed by TLC and TLC-immunostaining of the products after solvolysis with dimethylsulfoxide-methanol (9:1, by vol.) containing 9 mM sulfuric acid at 80°C for 1 h. Also, linkage analysis of carbohydrate residues was performed by permethylation of glycolipids, acetolysis, reduction and acetylation to generate partially methylated aldohexitol acetates and *N*-methyl acetamido aldohexitol acetates [5,23], which were analyzed by GLC-mass spectrometry on a ECNSS-M column (QP-5050A; Shimadzu, Kyoto).

Results

Glycolipids in mouse thymus

Glycolipids in the thymus of a mouse (HR-1 strain, 5 weeks old), which was used for preparation of immortalized cells in this experiment, were firstly characterized by isolation of individual glycolipids, comparison of their mobilities on a TLC plate with those of standard glycolipids and their reactivities with the respective antibodies, and analysis by negative ion FABMS with triethanolamine as the matrix. As shown in Figure 1A–D, the neutral glycolipids in the thymus were found to be Gb_3Cer, Gb_4Cer and Forssman glycolipid of the

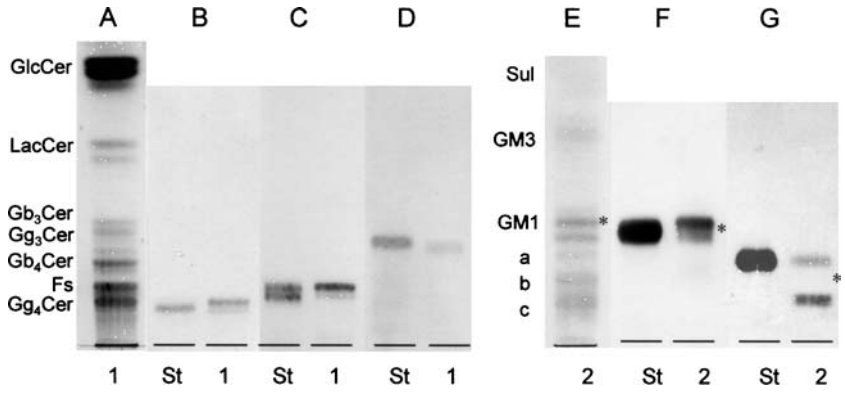

Figure 1. TLC of neutral (1) and acidic (2) glycolipids from mouse thymus. The glycolipids, corresponding to 1 mg of dry weight, were developed on TLC plates with chloroform/methanol/ water (65:35:8, by vol.) for A-D, and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) for E-G, and were detected with orcinol-H_2SO_4 (A and E), anti-Gg_4Cer (B), anti-Forssman (C), anti-Gg_3Cer (D), anti-GMl (F), and anti-GM2 (G) antibodies. Sts were the standard glycolipids for the respective antibodies, i.e., Gg_4Cer for B, Forssman (Fs) glycolipids for C, Gg_3Cer for D, GM1 for F, and GM2 for G, and the differences in mobility between standards and glycolipids from mouse thymus were due to their ceramide structures. Asterisks show the position of GM1.

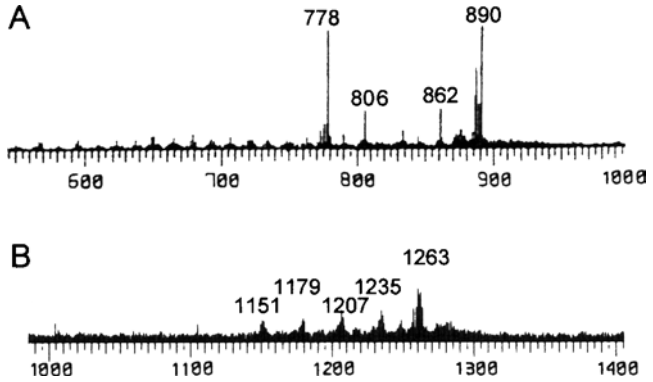

Figure 2. Negative ion FABMS spectra of acidic glycolipids in mouse thymus. Acidic glycolipids with the same mobilities as those of I^3SO_3-GalCer (A) and GM3 (B), designated as Sul and GM3 in Figure 1E, were analyzed by negative ion FABMS with triethanolamine as the matrix, and their molecular ion regions are presented.

globo-series, and Gg_3Cer and Gg_4Cer of the ganglio-series. Ceramide monohexoside, which was the major one, comprised GalCer (3%) and GlcCer (97%). While the amounts of acidic glycolipids were lower than those of neutral glycolipids (Figure 1). The major acidic glycolipid GM1 was confirmed from its reactivity toward anti-GMl antibody, and the upper and lower bands of GM1 on TLC-immunostaining in Figure 1F were *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid-containing ones, respectively. Rabbit polyclonal anti-GMl antibody did not react with GMlb-GalNAc-Gal, which was reactive toward cholera toxin. Acidic glycolipid a in Figure 1E, whose mobility on the TLC plate was lower than that of GM1, was determined to be $IV^3NeuAc\text{-}Gg_4Cer$ (GMlb) from its susceptibility to *V. cholerae* neuraminidase and characterization of the product as Gg_4Cer on TLC-immunostaining with anti-Gg_4Cer antibody. Also, glycolipid *b* was eluted in the monosialyl fraction from the DEAE-Sephadex column with 0.05 M ammonium acetate in methanol, and exhibited a positive reaction on TLC-immunostaining with anti-GM2 antibody YHD-06 (Figure 2G), indicating that its structure is GMlb-GalNAc [9]. Glycolipid c was separated into monosialo- and disialo-gangliosides, the former being thought to be GMlb-GalNAc-Gal from its reactivity toward cholera toxin as reported [17], and the latter to be GDlc from the following evidence. Its negative ion FABMS spectrum exhibited a molecular ion, (M-H), at *m/z* 1951 and fragment ions cleaved sequentially at the glycosidic linkages, $(M\text{-}NeuGc)^-$ at *m/z* 1644, $(Hex\text{-}HexNAc\text{-}Hex\text{-}Hex\text{-}Cer\text{-}H)^-$ at *m/z* 1337, $(HexNAc\text{-}Hex\text{-}Hex\text{-}Cer\text{-}H)^-$ at *m/z* 1175, $(Hex\text{-}Hex\text{-}Cer\text{-}H)^-$ at *m/z* 972, $(Hex\text{-}Cer\text{-}H)^-$ at *m/z* 810, and $(Cer\text{-}H)^-$ at *m/z* 648, and permethylation analysis yielded 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol, 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol, 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol, and 4,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-1,3,5-tri-*O*-acetylgalactitol in a ratio of 1:1:1:1, showing that the structure is NeuGcα2-8NeuGcα2-3Galβl-3GalNAcβ1-4Galβl-4Glcβl-l'ceramide, CDlc. In mouse thymocytes, GMlb, GMlb-GalNAc, GMlb-GalNAc-Gal and GDlc have been previously characterized well [9–16]. In addition, the bands of Sul and GM3 in Figure 1E were identified as I^3SO_3-GalCer and $II^3NeuAc\text{-}LacCer$ on TLC-immunostaining with anti-I^3SO_3-GalCer and anti-GM3 antibodies, and on negative ion FABMS (Figure 2). The deprotonated molecular ions in Figure 2 were as follows, *m/z* 890, 862, 834, 806 and 778, for Figure 2A, and *m/z* 1263, 1235, 1207, 1179 and 1151 for Figure 2B, corresponding to I^3SO_3-GalCer and $II^3NeuAc\text{-}LacCer$ with 24:0, 22:0, 20:0, 18:0 and 16:0 as fatty acids, and with sphingosine (18d:l), respectively. The concentrations of glycolipids in the thymus determined on TLC-densitometry and TLC-immunostaining are compiled in Table 1. The glycolipid present in the highest concentration was GlcCer, and the relative concentrations of the ganglio- and globo-series carbohydrates in the neutral glycolipids were similar, being 0.76 and 0.61 μg/mg of dry weight, respectively. While the basic carbohydrate of gangliosides belonged to the ganglio-series, among which the structures modified on Gg_4Cer (α-pathway) were present at higher concentrations than those on GM1 (a-pathway), reflecting the molecular heterogeneity of carbohydrates in the thymus.

Table 1. Concentrations of glycosphingolipids in the thymuses of mice

Glycolipid	*Concentration (μg/mg of dry weight)*
GalCer	0.05 ± 0.01
GlcCer	1.40 ± 0.15
LacCer	0.09 ± 0.03
Gb_3Cer	0.11 ± 0.02
Gg_3Cer	0.03 ± 0.02
Gb_4Cer	0.25 ± 0.01
Gg_4Cer	0.50 ± 0.03
Forssman	0.40 ± 0.03
GM3	0.04 ± 0.01
GM1	0.09 ± 0.03
GM1b	0.06 ± 0.02
GM1b-Ga1NAc	0.04 ± 0.01
GM1b-Ga1NAc-Gal	0.02 ± 0.01
GD1c	0.06 ± 0.01
I^3SO_3-Ga1Cer	0.03 ± 0.01

Mean values for five tissue specimens are presented.

Immortalization of thymic cells

Infection of mouse thymus with SV-40 virus yielded 25 cell lines, which were classified into three types of cells on the basis of their morphological features. Typical ones, having epithelial-like, fibroblast-like, and asterisk-like morphologies, were established as cell lines, IMTH-E (Figure 3A), IMTH-F (Figure 3B), and IMTH-I (Figure 3C), respectively.

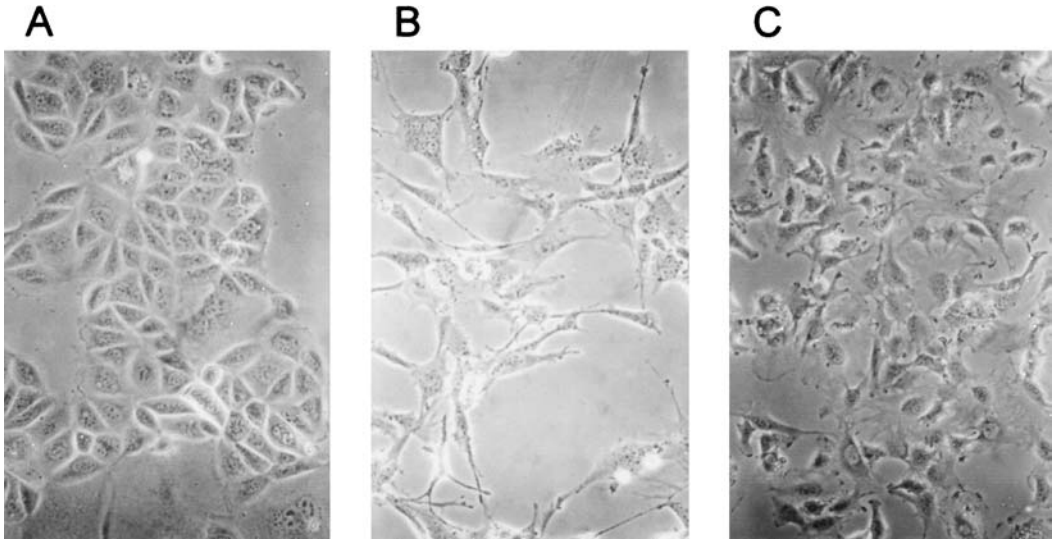

Figure 3. Cells immortalized from mouse thymus. A, IMTH-E; B, IMTH-F; C, IMTH-I.

Glycolipids in cells immortalized from mouse thymus

Figure 4 shows the TLC and TLC-immunostaining of neutral glycolipids in IMTH-E, -F and -I cells. On comparison of their mobilities with those of standard glycolipids, GlcCer, LacCer, Gb_3Cer, Gb_4Cer and Forssman glycolipid, on a TLC plate, IMTH-E, -F and -I cells were found to contain 2, 4 and 5 neutral glycolipids, respectively (Figure 4). The negative ion FABMS spectra of tetra- and pentahexaosyl ceramides in IMTH-F and -I cells exhibited ions for HexNAc-Hex-Hex-

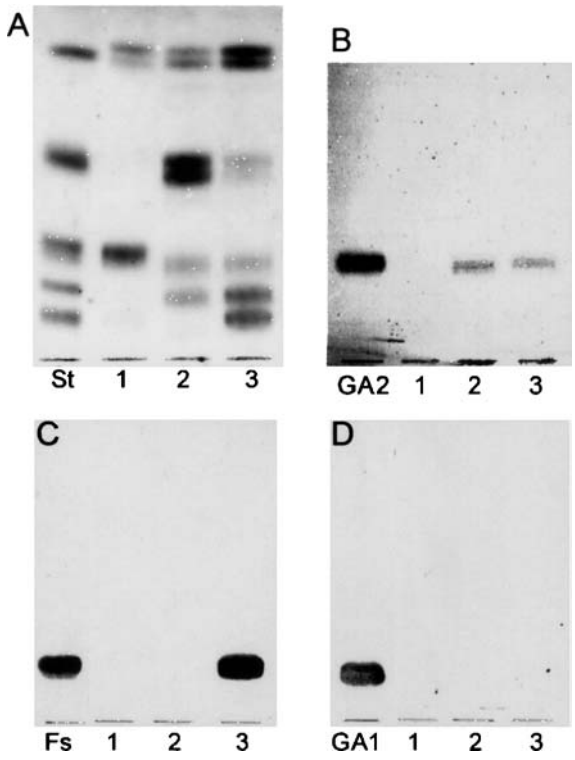

Figure 4. TLC of neutral glycolipids in IMTH-E (1), IMTH-F (2), and IMTH-I (3) cells. The glycolipids, corresponding to 0.5 mg of dry weight, were developed on TLC with chloroform/methanol/water (65:35:8, by vol.), and were visualized with orcinol-H_2SO_4 (A), anti-Gg_3Cer (B), anti-Forssman (C), and anti-Gg_4Cer (D) antibodies. St, standard glycolipids. For A, glycolipid mixture, GlcCer, LacCer, Gb_3Cer, Gb_4Cer and Forssman glycolipids from the top. The standard glycolipids for B, C and D were GA2 (Gg_3Cer), Fs (Forssman), and GA1 (Gg_4Cer), respectively.

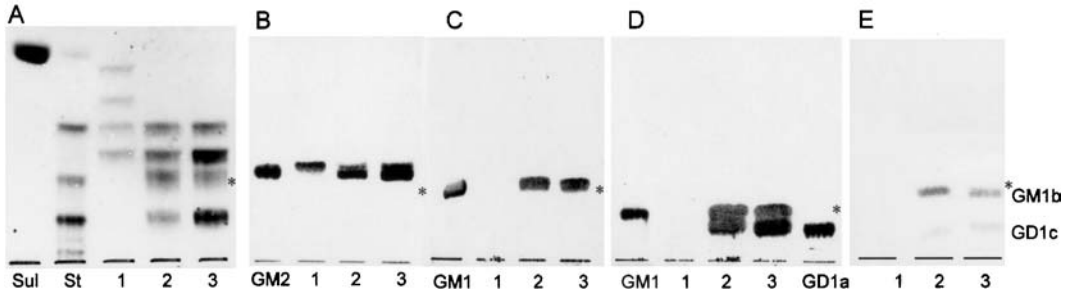

Figure 5. TLC of acidic glycolipid in IMTH-E (1), IMTH-F (2), and IMTH-I (3) cells. The glycolipids, corresponding to 0.5 mg of dry weight, were developed as described in the legend to Figure 1, and were visualized with orcinol-H_2SO_4 (A), anti-GM2 (B), anti-GM1 (C, D), and anti-Gg_4Cer (E) antibodies. For D and E, the plates, after development and blocking, were incubated with *V. cholerae* sialidase at 37°C for 2 hrs, and then immunostained with anti-GM1 and anti-Gg_4Cer antibodies, respectively. St, standard glycolipid mixture, I^3SO_3-GalCer, GM3, GM1 and GD1a from the top. Sul, I^3SO_3-GalCer. Asterisks show the position of GM1.

Hex-Cer and HexNAc-HexNAc-Hex-Hex-Hex-Cer, showing that they are globoside and Forssman glycolipid, respectively, and the latter was positive with anti-Forssman antibody (Figure 4C). But the Hex-HexNAc-Hex-Hex-Cer structure was not found in the tetrahexaosyl ceramide fractions for Lc_4Cer and Gg_4Cer in all cells on negative ion FABMS, and the cells were negative with anti-Gg_4Cer antibodies on TLC-immunostaining (Figure 4D). The trihexaosyl ceramide in IMTH-E cells was proven to be Gb_3Cer by FABMS (Figure 6A), showing molecular ions in the range between *m/z* 1022 and *m/z* 1134 for palmitic (16:0) and lignoceric (24:0) acid-containing molecules, and fragment ions cleaved sequentially at the glycosidic bonds, $(Hex\text{-}Hex\text{-}Cer)^-$, $(Hex\text{-}Cer)^-$ and Cer^-. But, the trihexaosyl ceramides in IMTH-F and IMTH-I cells carried the HexNAc-Hex-Hex-Cer structure, which was proven to be Gg_3Cer by TLC-immunostaining (Figure 4B), showing the occurrence of the initial step of biosynthesis of ganglio-series glycolipids along with the major globo-series glycolipids in both types of cells.

On the other hand, acidic glycolipids in all cells gave four bands (Figure 5A), two of which obtained for all cells as common ones, and were characterized as GM3 and GM2 on comparison of their mobilities and reactivities toward their antibodies on TLC with those of standards (Figure 5B), and on negative ion FABMS. GMlb-GalNAc, which was positive with anti-GM2 antibody and was contained in mouse thymus (Figure 1G), was not detected in the cells even in a trace amount (Figure 5B). The other two bands for IMTH-E cells were of sulfoglycolipids, whose structures were shown to be II^3SO_3-LacCer (Figure 6B) and II^3SO_3-Gg_3Cer (Figure 6C) on negative ion FABMS, respectively, exhibiting ions at *m/z* 1255 for $(II^3SO_3Cer\text{-}l)^-$, *m/z* 1052 for $(II^3SO_3\text{-}LacCer\text{-}1)^-$, *m/z* 810 for $(LacCer\text{-}1)^-$, and *m/z* 648 for $(GlcCer\text{-}1)^-$. While the two bands that migrated to the same positions as those of GM1 and GD1 a in IMTH-F and -I cells were proven to be GM1 and GDla from their positive reactions toward anti-GMl antibody after treatment of the plate with *V. cholerae* sialidase, which was able to cleave the terminal sialic acid, but not the internal sialic acid of GDla to give GM1 (Figure 5D). In addition, IMTH-F and -I cells were found to contain GMlb and GDlc, whose sialic acids were completely removed by *V. cholerae* sialidase to yield Gg_4Cer (Figure 5E). The glycolipid compositions of the cells immortalized from mouse thymus are summarized in Table 2. The amounts of glycolipids with more than four carbohydrates in the thymus-derived cells were higher than those in the tissue, but Gg_4Cer was not present in the cells, although GMlb and GDlc modified to Gg_4Cer with sialic acid were present in trace amounts, suggesting that the α-pathway for ganglio-series in IMTH-F and -I cells was active at Gg_3Cer. In contrast, the a-pathway for ganglio-series in IMTH-F and -I cells was active up to GDla. Also, instead of I^3SO_3-GalCer in the tissue, II^3SO_3-LacCer and II^3SO_3-Gg_3Cer were present in IMTH-E cells as the major acidic glycolipids.

Table 2. Concentrations of glycosphingolipids in cells immortalized from mouse thymus

	Concentrations (μg/mg of dry weight)		
Glycolipid	*IMTH-E*	*IMTH-F*	*IMTH-I*
G1cCer	0.65	0.87	1.49
LacCer	0.06	2.48	0.46
Gb_3Cer	1.03	–	–
Gg_3Cer	–	0.39	0.37
Gb_4Cer	–	0.42	0.84
Forssman	–	–	0.86
GM3	0.20	0.73	0.80
GM2	0.35	0.75	1.21
GM1		0.63	0.61
GM1b		0.07	0.07
GD1a		0.32	1.08
GD1c		0.03	0.12
II^3SO_3-LacCer	0.31	–	–
II^3SO_3-Gg_3Cer	0.20	–	–

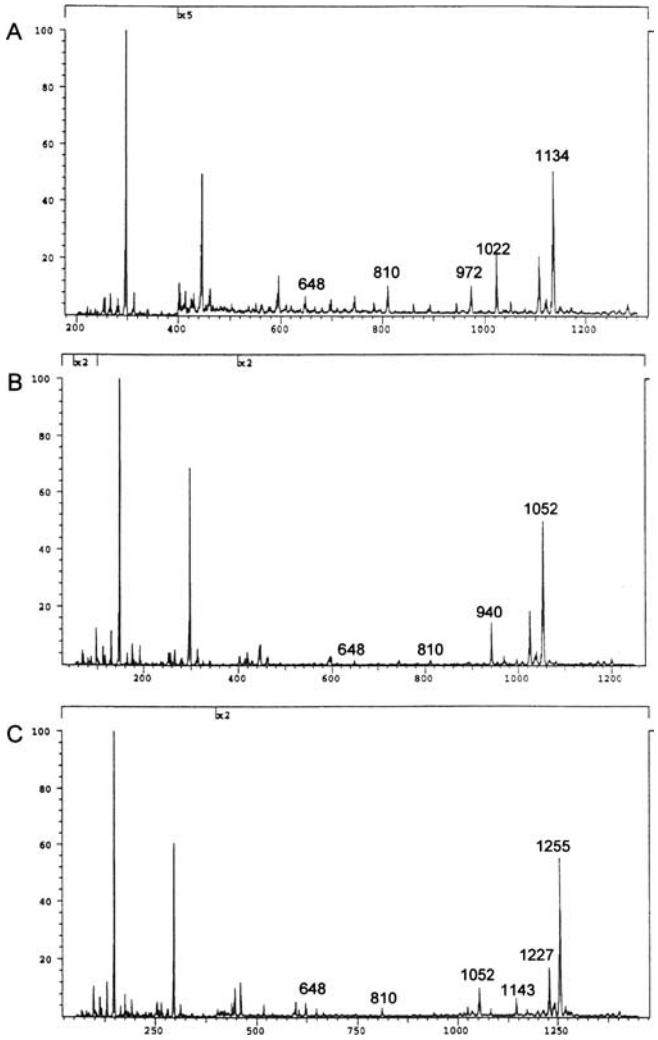

Figure 6. Negative ion FABMS spectra of glycolipids from IMTH-E cells. Neutral trihexaosyl ceramide (lane 1 in Figure 4A) (A), and acidic glycolipids that migrated to the first (B) and second (C) positions from the top (lane 1 in Figure 5A) were analyzed by negative ion FABMS with trimethylamine as the matrix.

Discussion

As shown in this paper, cells established from mouse thymus by immortalization with SV-40 virus maintained the major metabolic pathways, globo- and ganglio-series, in the original tissues, but their glycolipid compositions were found to differ among the cells established, probably due to the selection and proliferation of a single cell population from the tissue (Figure 7). Interestingly, three cell lines established contained the different structures for globo-series glycolipids, that is, Gb_3Cer in IMTH-E, Gb_4Cer in IMTH-F and Forssman glycolipid in IMTH-I cells. As to the a-pathway for ganglio-series glycolipids, IMTH-F and -I cells expressed gangliosides up to GD1 a, similar to in the thymus, but GM2 was the terminal product of the a-pathway in IMTH-E cells. Instead of sialylated glycolipids, IMTH-E cells contained sulfated glycolipids, II^3SO_3-LacCer and II^3SO_3-Gg_3Cer, which were structural isomers of GM3 and GM2, respectively, and sulfotransferase to LacCer, and GalNAc transferase to II^3SO_3-LacCer were thought to compete with the syntheses of GM3 and GM2 to give similar amounts of sialylated and sulfated glycolipids in the cells. Similar metabolic competition for the syntheses of GM3 and II^3SO_3LacCer was observed in human uterine endometrial carcinoma cells [20], whose morphologies, particularly those of the well-differentiated type of carcinoma cells, were very similar with that of IMTH-E cells [21–23], showing that IMTH-E cells with sulfoglycolipids have the nature of epithelial cells. In addition, a change in the sulfoglycolipid structure, from I^3SO_3-GalCer in mouse thymus to II^3SO_3-LacCer in IMTH-E cells, was similarly observed in uterine endometrial adenocarcinoma cells, when cancerous cells were transferred into the culture, due to the diminished synthesis of GalCer in the cultured cells [20]. The new expression of II^3SO_3-LacCer in both human uterine endometrial carcinomal cells and mouse IMTH-E cells led to the synthesis of II^3SO_3-Gg_3Cer by GalNAc transferase from II^3SO_3-LacCer.

As to the α-pathway for ganglio-series glycolipids, Gg_3Cer, as an initial molecule of the step, was present in IMTH-F and -I cells, but not in IMTH-E cells, in relatively high concentrations. On the other hand, although Gg_4Cer was contained in mouse thymus at a significantly high concentration, it was not detected in either IMTH-F or -I cells, even in a trace amount. Since GMlb and GDlc synthesized from Gg_4Cer by sialyltransferase were present in both IMTH-F and -I cells, Gg_4Cer in both types of cells seemed to be consumed for the syntheses of GMlb and GDlc. One can suggest that the relatively higher concentration of Gg_3Cer and lower concentration of Gg_4Cer in both types of cells than in the tissues are due to the lower synthetic activity for Gg_4Cer in comparison with that in the thymus, resulting in the accumulation of Gg_3Cer in IMTH-F and -I cells. The low amounts of Gg_4Cer in IMTH-F and -I cells might lead to a lower supply of GMlb for the synthesis of GMlb-GalNAc, which was detected in the thymus and thymocytes [9], because GM3 and GMlb were found to be the substrates for GalNAc transferase for the syntheses of GM2 and GMlb-Ga1NAc, respectively [27].

Thus, the glycolipid profiles in the tissue and cells should be determined as to the metabolic flow through the whole synthetic pathway. In fact, transfection and targeted deletion of fucosyltransferase genes caused characteristic changes of glycolipids, not only at the step of fucose-transfer, but also at the branching pathways sharing the same precursors [24, 25]. In this respect, IMTH-I cells with synthetic activity for Forssman glycolipids were thought to be originated from the dexamethasone-resistant cells in the thymus, because GM1 and Gg_4Cer, but not Forssman glycolipids have been selectively removed from the thymus of mice 48 h after administration of dexamethasone [7]. To clarify the cellular properties of cells immortalized from the thymus in comparison with those of thymic cell populations, biological

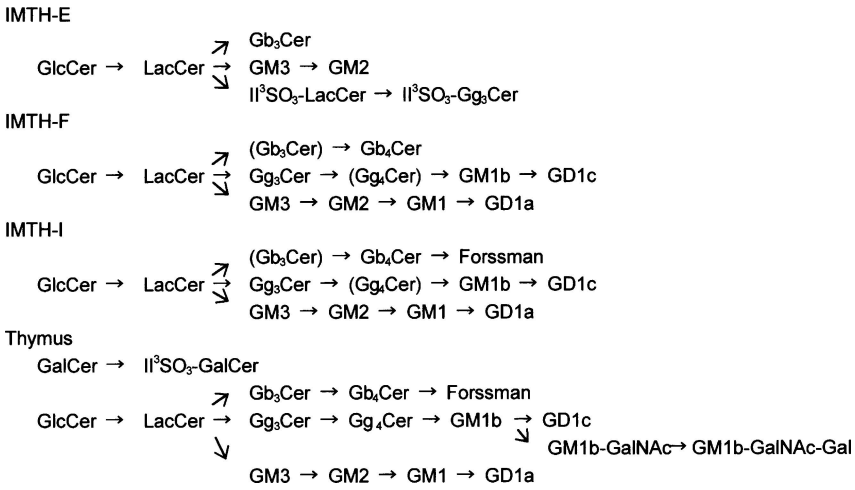

Figure 7. Metabolic pathways for glycolipids in cells immortalized from mouse thymus in comparison with those in mouse thymus. The glycolipids in parenthesis were not detected on TLC with chemical and immunochemical procedures, or on negative ion FABMS.

and immunological analyses with several markers of glycolipids and CD antigens are now in progress in our laboratory.

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