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A mouse B16 melanoma mutant deficient in glycolipids

(glucosyltransferase/glucosylceramide/glucocerebroside)

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ABSTRACT **Mouse B16 melanoma cell line, GM-95 (formerly designated as MEC-4), deficient in sialyllactosylceramide was examined for its primary defect. Glycolipids from the mutant cells were analyzed by high-performance TLC. No glycolipid was detected in GM-95 cells, even when total lipid from 10^7 cells was analyzed. In contrast, the content of ceramide, a precursor lipid molecule of glycolipids, was normal. Thus, the deficiency of glycolipids was attributed to the first glucosylation step of ceramide. The ceramide glucosyltransferase (EC 2.4.1.80) activity was not detected in GM-95 cells. There was no significant difference of sialyllactosylceramide synthase activity, however, between GM-95 and the parental cells. The deficiency of glycolipids in GM-95 cells was associated with changes of the cellular morphology and growth rate. The parental cells showed irregular shapes and tended to overlap each other. On the other hand, GM-95 cells exhibited an elongated fibroblastic morphology and parallel arrangement. The population-doubling times of GM-95 and the parental cells in serum-free medium were 28 hr and 19 hr, respectively.**

Glycolipids are a group of membrane components that embed the lipid portion in the outer leaflet of plasma membrane and extend the sugar chain to the outer environment. In animal cells, most glycolipids exist as glycosphingolipids. Most glycosphingolipids are derived from the simplest glycolipid, glucosylceramide (GlcCer), which serves as the base for >300 glycosphingolipids. GlcCer and its derived glycosphingolipids are present essentially in all animal cells. Another group of glycolipids is the galactosphingolipids, characterized by the attachment of galactose to ceramide; they are mainly expressed in brain and kidney.

It is postulated that glycolipids serve as signal molecules because of the localization on the cell surface and structural diversity of the carbohydrate moieties attached to them. Several lines of evidence suggest the importance of glycolipids in various cellular processes. For example, neurite extention of neuroblastoma cells by gangliosides, such as G_{M1} and G_{Q1b}, differentiation of HL-60 leukemia cells to macrophage by sialyllactosylceramide (G_{M3}), growth regulation of cells by G_{M3} and GlcCer, and implications of various glycolipids to cell–cell recognition have been reported (for review, see refs. 1 and 2). However, most of these results were obtained under artificial conditions—i.e., by an addition of glycolipids in culture medium, an inhibition of transferases by specific inhibitors, a removal of sugar chains from glycolipids by enzymatic digestion, or a blockade of glycolipids with antibodies or toxins. Although these approaches are very useful to study the functions of glycolipids, one cannot exclude the intrinsic side effect.

To study the biological functions of glycolipids under physiological conditions, mutant cells lacking a specific glycosyltransferase would provide an ideal tool. Although several glycosylation mutant cells have been isolated by treatment with a mutagen followed by selection with lectins, defects in these mutants were involved in either glycoprotein syntheses (for review, see ref. 3), nucleotide sugar syntheses, or nucleotide sugar transporters (4). Mutants defective in glycolipid-specific transferases have rarely been found. Recently, Tsuruoka *et al.* (5) isolated a mouse mammary carcinoma mutant that gained G_{M3} expression by selection with antilactosylceramide (anti-LacCer) monoclonal antibody (mAb) in combination with complement (5).

In this report, we demonstrate that a mutant B16 melanoma cell line, GM-95 [formerly designated as MEC-4; ref 6] lacking in G_{M3} has a primary defect in ceramide glucosyltransferase (UDP-glucose: *N*-acylsphingosine D-glucosyltransferase, EC 2.4.1.80; GlcT-1). GM-95 cells contained virtually no glycolipid and exhibited altered morphology and growth properties.

MATERIALS AND METHODS

Materials. mAb GMR-6 (anti-G_{M3}; IgM) was provided by T. Tai of the Tokyo Metropolitan Institute of Science (7). Fluorescein isothiocyanate labeled anti-mouse goat IgM(μ) was purchased from Kirkegaard & Perry Laboratories. Tetramethylrhodamine-conjugated myosin subfragment was obtained from Wako Pure Chemical, Osaka. CMP-sialic acid [cytidine 5′-monophosphate, sialic acid (*sialic*-4,5,6,7,8,9,-^{14}C monophosphate), specific activity 5.55 GBq/mmol] and UDP-Glc {uridine diphosphate glucose [*glucose*-^{14}C (U)], specific activity 11 GBq/mmol} were purchased from NEN. Ceramide prepared from bovine brain sphingomyelin, unlabeled nucleotide sugars, and NBD-GlcCer (12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1, 3-diazoyl-4-yl)]-aminodecanoylsphingosyl β-D-glucoside) were obtained from Sigma. Lactosylceramide was prepared from buttermilk. The Hoechst stain kit for mycoplasma test was obtained from Flow Laboratories. Horseradish peroxidase-conjugated lectins were from Seikagaku Kogyo, Tokyo. All other reagents used were of analytical grade. Mouse B16 melanoma cells were from M. Taniguchi of Chiba University.

Cell Lines and Culture Conditions. G_{M3}-deficient GM-95 cells were isolated from MEB-4 cells. MEB-4 cells do not express a melanoma-specific antigen recognized by mAbs M562 and M622 but express another melanoma antigen recognized by M2590 mAb (anti-G_{M3}, IgM) (8). To obtain clones lacking G_{M3}, MEB-4 cells were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (1.5 μg/ml). G_{M3}-negative cells were selected by killing with M2590 mAb and rabbit complement. After 10 times selection of the mutagenized cells with M2590 mAb, 100 clones were isolated by limiting

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Abbreviations: GlcCer, glucosylceramide or glucocerebroside; LacCer, lactosylceramide; G_{M3}, sialyllactosylceramide; GlcT-1, ceramide glucosyltransferase; mAb, monoclonal antibody; HPTLC, high-performance TLC.

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dilution. The clones were then tested for reactivity toward M2590 mAb by indirect immunostaining, and four clones with low or nonreactivity with the antibody were isolated. These clone were designated as MEC-1, MEC-2, MEC-3, and MEC-4 (6); they were renamed as GM-20, GM-70, GM-89, and GM-95, respectively, because they are G_{M3} mutants. These cell lines were tumorigenic, and their metastatic properties have been characterized (6). In the present study, GM-95 cells, which express no G_{M3}, were examined to determine the deficient point in G_{M3} synthesis.

The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO)/10% fetal calf serum under 5% CO_2/95% air. When serum-free conditions were required, E-RDF medium supplemented with RD-I (Kyokuto Pharmaceutical, Tokyo) was used (9). All cell lines were negative in mycoplasma contamination when tested with the Hoechst 33258 staining kit (10).

Indirect Immunofluorescent Staining. Cells were allowed to attach for 24 hr to glass coverslips. The cells were washed with phosphate-buffered saline (PBS), fixed with 10% (vol/vol) formaldehyde, blocked for 1 hr with 10% fetal calf serum, and treated with GMR-6 mAb (1:1, culture supernatant). After being washed with PBS, the cells were poststained with fluorescein isothiocyanate-labeled anti-mouse IgM.

Lipid Analyses. Cells grown in E-RDF medium were collected by scraping at subconfluency, washed twice with PBS, and lyophilized. Total lipids were extracted with 20 vol of chloroform/methanol, 2:1 (vol/vol), filtrated, and evaporated to dryness. The total lipids were then redissolved into a small volume of chloroform/methanol, 2:1 (vol/vol) and chromatographed on a precoated silica-gel high-performance TLC (HPTLC) plate (E. Merck) in chloroform/methanol/water, 65:25:4 (vol/vol). Glycolipids were visualized with orcinol-H_2SO_4 reagent.

TLC immunostaining was done according to Higashi *et al.* (11). Total lipids were applied onto Polygram SIL G plate (Macherey & Nagel), developed in chloroform/methanol/water, 5:4:1 (vol/vol), and stained as described (11).

For ceramide analysis, total lipids were spotted on a silica-gel HPTLC plate and were developed 2 cm from the spotted origin with the solvent [chloroform/methanol/water, 60:35:8, (vol/vol)]. After drying the plate, the lipids were chromatographed in chloroform/methanol/acetic acid, 90:2:8 (vol/vol). The plate was dried completely and sprayed with cupric acetate/phosphoric acid reagent to visualize lipids (12, 13).

Lectin Blot Analyses. Lectin blot analyses were done according to the method of Kijimoto-Ochiai *et al.* (14) Cell lysates (40 μg of protein) were subjected to SDS/PAGE (10% gel) (15), transblotted onto nitrocellulose membrane (16), and treated with horseradish peroxidase-conjugated Con A or wheat germ lectin. Glycoproteins bound to lectins were detected by using the Konica immunostaining kit (Konica, Tokyo).

Enzyme Assay. GlcT-1 activity was measured according to Basu *et al.* (17) with slight modifications. Standard incubation mixture (50 μl) was 0.5% Triton X-100/20 mM Tris·HCl buffer, pH 7.5/500 μM [^{14}C]UDP-Glc (3×10^5 dpm per reaction)/0.3 mM ceramide/600 μg of cell protein as an enzyme source. The mixture was incubated for 2 hr at 30°C. The reaction was stopped with 500 μl of chloroform/methanol, 2:1 (vol/vol). After mixing, the lower phase was washed with chloroform/methanol/0.1 M KCl, 3:48:47, (vol/vol), dried, and subjected to scintillation counting or analyzed by TLC followed by autoradiography.

G_{M3} synthase (CMP-sialic acid: LacCer sialytransferase) activity was measured as follows. The reaction mixture (25 μl) was 150 mM cacodylate·HCl buffer, pH 6.5/10 mM $MgCl_2$/10 mM $MnCl_2$/1 mM 2-mercaptoethanol/0.3% Triton CF-54/0.3 mM LacCer/1 mM [^{14}C]CMP-sialic acid (2.5×10^5 dpm per reaction)/80 μg of cell protein. The reaction mixture was incubated for 2 hr at 37°C. After incubation, the reaction was stopped with 500 μl of water, and the sample was applied to Bond Elute RP 18 column (Analytichem International, Harbor City, CA) equilibrated with water. The column was washed with water, and the glycolipids were eluted with chloroform/methanol, 2:1. The eluent was dried and subjected to TLC followed by autoradiography.

Glucocerebrosidase activity was measured according to the method of Dinur *et al.* (18).

Cell Growth Assay. Cells were maintained in E-RDF medium supplemented with RD-I 2 weeks before the growth assay. Cells (1×10^4) were plated in 96-well plates (Primaria; Beckton Dickinson Labware). The cell number was determined after staining with crystal violet by a colorimetric assay described by Kung *et al.* (19).

Protein Assay. Proteins were quantitated by using Micro BCA protein assay reagent kit (Pierce) (20).

RESULTS

Morphological Characterization of G_{M3}-Deficient GM-95 Cells. B16 melanoma cells and its mutagenized subclone MEB-4 cells have been shown to express G_{M3} on the cell surface (6). We have isolated a G_{M3}-deficient mutant, GM-95, from MEB-4 cells. Indirect immunofluorescent staining of MEB-4 and GM-95 cells are shown in Fig. 1. MEB-4 cells expressed G_{M3} strongly (Fig. 1*A*), whereas GM-95 cells did not express G_{M3} (Fig. 1*B*). Actin filament was stained with tetramethylrhodamine-conjugated myosin subfragment to visualize all cells in the same field (Fig. 1 *C* and *D*). MEB-4 cells show irregular shapes and tended to overlap each other (Fig. 2*A*), whereas G_{M3}-deficient GM-95 cells exhibited an elongated fibroblastic morphology and parallel arrangement (Fig. 2*B*). Both mutants produced melanin pigment, indicating they kept the melanoma phenotype.

Identification of a Primary Defect in G_{M3} Biosynthesis in GM-95 Cells. To identify a primary defect of GM-95 cells in the G_{M3} synthetic pathway, glycolipids and ceramide from the mutant cells were analyzed by HPTLC. Total lipids extracted by chloroform/methanol, 2:1 (vol/vol) were chromatographed on a silica-gel plate, and glycolipids were visualized with orcinol/H_2SO_4 reagent (Fig. 3*A*). As reported (21), the main glycolipids isolated from B16 cells were GlcCer and G_{M3} (lane 3). A small amount of LacCer was also detected when 10^7 of B16 cells were analyzed (data not shown). A broad band migrating slightly slower than LacCer was identified as phosphatidylethanolamine, which was brown in color, differing from the red glycolipid band. As shown in Fig. 3, the glycolipid pattern of MEB-4 cells was basically similar to that of B16 cells (lane 4). The contents of GlcCer and G_{M3} were 15 nmol and 10 nmol per 10^7 cells, respectively. In contrast, all three species of glycolipids were absent in GM-95 cells. No glycolipid was detected in GM-95 cells, even when total lipids from 10^7 cells were analyzed (lane 5). Considering the detection limit of the orcinol/H_2SO_4 method ($\approx$100 pmol per lane), the amount of GlcCer in GM-95 cells was, if any, <100 pmol per 10^7 cells. The G_{M3} quantity was also measured by the TLC immunostaining method with which $\approx$5 pmol of G_{M3} was detectable (Fig. 3*B*). No G_{M3} was detected, however, when total lipid from 10^7 of GM-95 cells was analyzed (lane 5). Therefore, the amount of G_{M3} in GM-95 cells was <5 pmol per 10^7 cells. Fig. 4 shows TLC analysis of ceramide in B16, MEB-4, and GM-95 cells (lanes 2–4, respectively). All three cell lines contained comparable amounts of ceramide, indicating that the deficiency of glycolipids in GM-95 cells was not caused by the aberration of ceramide synthesis. The synthetic pathway of G_{M3} is shown in Fig. 5 (22, 23). To find out why GM-95 cells were deficient in glycolipids, we examined activity of GlcT-1 using GM-95

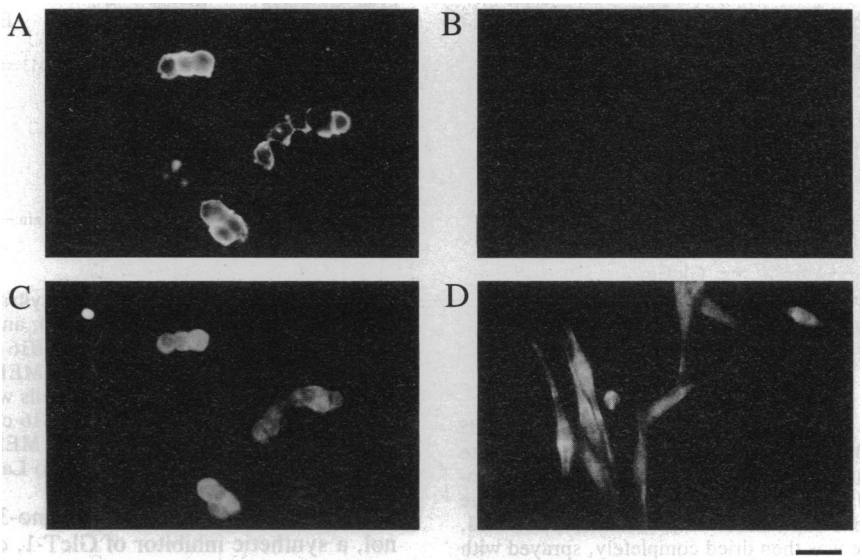

FIG. 1. Immunofluorescent staining of mouse melanoma cell lines with anti-G_{M3} mAb. Melanoma cells were incubated with anti-G_{M3} mAb (GMR-6) and poststained with fluorescein-conjugated goat anti-mouse IgM. MEB-4 cells (*A*) were labeled with the antibody strongly, whereas GM-95 cells (*B*) had no fluorescence. Actin filament was stained with tetramethylrhodamine-conjugated myosin subfragment to visualize all cells in the same field as *A* and *B* (*C* and *D*, respectively). MEB-4 cells do not stick onto glass surface and have a circular shape. (×280; bar = 30 μm.)

cell lysate. If the glycolipid synthesis is blocked at the stage of GlcCer synthesis, no complex glycolipids derived from GlcCer would accumulate. The enzyme activity of GlcT-1 was measured by incorporation of [^{14}C]glucose into ceramide. Likewise, G_{M3} activity was measured as a control. Fig. 6*A* shows an autoradiogram of the reaction products catalyzed by crude cell lysates. GlcCer was synthesized in B16 (lane 1) and MEB-4 (lane 3) cell lysates but not in GM-95 (lane 5) cell lysates. Even were ceramide not added exogenously, B16 (lane 2) and MEB-4 (lane 4) cells also synthesized GlcCer due to a relatively high content of endogenous ceramide. The specific activities of GlcT-1 in B16 and MEB-4 total cell lysates were 28 and 18 pmol/mg of protein per hr, respectively. There was no significant difference of G_{M3} synthase activity, however, between these three cell lines (Fig. 6*B*, lanes 1, 3, and 5). No G_{M3} was detected in GM-95 cell lysate when LacCer was not added to the incubation mixture, indicating a lack of endogenous LacCer (lane 6). Because we used crude cell lysate for the enzyme assay, the apparent loss of GlcT-1 activity could be caused by the glucocerebrosidase. The activity of this enzyme was determined by using a fluorescent substrate, C_{12} NBD-GlcCer (19). Activities in all three cell lines were at the same level (data not shown). It is also possible that the loss of GlcT-1 activity in GM-95 lysate could be from the presence of an inhibitor or the absence of an activator. GlcT-1 activity in MEB-4 cell lysate was inhibited by GM-95 cell lysate weakly when equal amounts of the cell lysates were mixed (data not shown). Because the inhibitory activity was very weak, we believe that this was due to the nonspecific effect of cell lysate.

Glycoprotein synthesis was not affected by the mutation because the lectin blot analyses using Con A and wheat germ lectin as probes showed no significant difference between B16 and GM-95 cells (data not shown). This result suggests

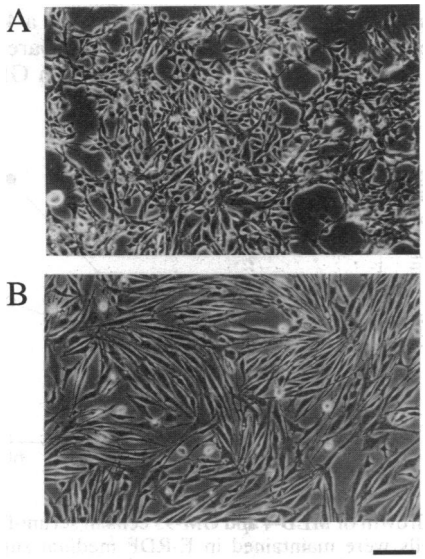

FIG. 2. Phase-contrast micrographs of MEB-4 and GM-95 cells cultured in serum-free-defined medium. (*A*) MEB-4 cells show an irregularly oriented and densely packed pattern. (*B*) GM-95 cells are elongated and exhibit a parallel fibroblastic orientation. Falcon 3003 culture dishes were used. (×55; bar = 100 μm.)

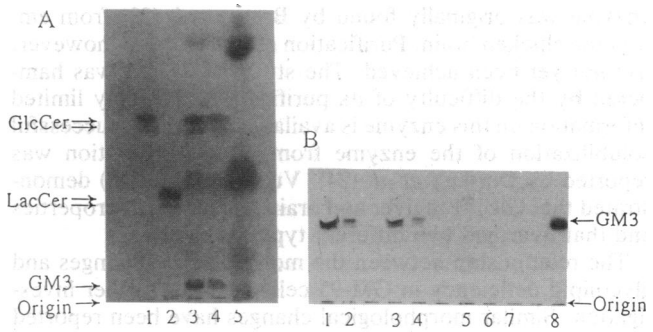

FIG. 3. Glycolipid pattern of B16, MEB-4, and GM-95 cells determined by TLC. Total lipids from the cells were prepared and analyzed as described. (*A*) Visualization of glycolipids with orcinol/H_2SO_4 reagent. Lanes: 1, GlcCer (1 μg); 2, LacCer (5 μg); 3 and 4, total lipids equivalent to 10^6 of B16 and MEB-4 cells, respectively; 5, total lipid equivalent to 10^7 of GM-95 cells. (*B*) TLC immunostaining of G_{M3} with GMR-6 anti-GM3 mAb. Lanes: 1 and 2, total lipids equivalent to 10^6 and 10^5 of B16 cells; 3 and 4, total lipids equivalent to 10^6 and 10^5 of MEB-4 cells, respectively; 5–7, total lipids equivalent to 10^7, 10^6, and 10^5 of GM-95 cells, respectively; 8, G_{M3} standard (1 nmol).

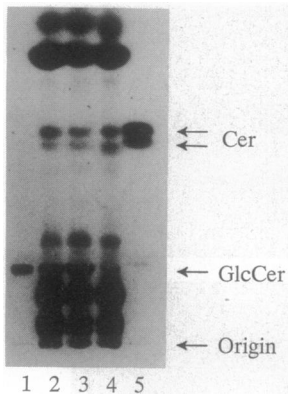

FIG. 4. TLC analysis of ceramide in B16, MEB-4, and GM-95 cells. Total lipids from 10^6 cells were spotted on the silica-gel plate and developed 2 cm from the spotted origin with a solvent [chloroform/methanol/water, 60:35:8 (vol/vol)]. After drying the plate, the lipids were chromatographed in chloroform/methanol/acetic acid, 90:2:8 (vol/vol). The plate was then dried completely, sprayed with cupric acetate/phosphoric acid reagent, and heated to visualize lipids. Lanes: 1, GlcCer standard (1 μg); 2–4, total lipids from B16, MEB-4, and GM-95 cells, respectively; 5, ceramide standard (1 μg).

that the synthetic pathway for UDP-Glc and its transporting system is not affected by the mutation.

Growth of MEB-4 and GM-95 Cells in Serum-Free Medium. It is possible that small amounts of glycolipids necessary for the survival and growth of GM-95 cells were supplied from serum in the culture medium. To assess glycolipid requirements in cell growth, we used serum-free-defined medium, E-RDF. Both MEB-4 and GM-95 cells could be maintained in E-RDF medium >2 months. The population-doubling times of MEB-4 and GM-95 cells were 19 and 27 hr, respectively (Fig. 7). The growth rate of each cells in RPMI medium/10% serum has been reported (6). The doubling times of MEB-4 and GM-95 cells in the medium were 18 and 30 hr, respectively. The data indicate that exogenously supplied glycolipids from serum have little effect on cell growth.

DISCUSSION

Our data demonstrate that the mutation in GM-95 cells disrupts the first glycosylation step of glycolipid biosyntheses. The mutation in GM-95 cells had a primary enzymatic basis because the cells exhibit no GlcT-1 activity. The enzyme was originally found by Basu *et al.* (23) from embryonic chicken brain. Purification of this enzyme, however, has not yet been achieved. The study of GlcT-1 was hampered by the difficulty of its purification, and only limited information on this enzyme is available. Recently, successful solubilization of the enzyme from rat Golgi fraction was reported by Durieux *et al.* (24). Vunnam *et al.* (25) demonstrated that GlcT-1 in liver and brain had different properties and that liver had two different types of enzymes.

The relationship between the morphological changes and glycolipid deficiency in GM-95 cells requires further investigation. Similar morphological changes have been reported on glycolipid-depleted cells (26–29) by the treatment with

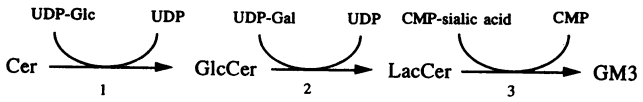

FIG. 5. The synthetic pathway of G_{M3} (22, 23). Reactions: 1, GlcT-1, UDP-glucose: ceramide glucosyltransferase; 2, LacCer synthase, UDP-galactose: glucosylceramide galactosyltransferase; 3, G_{M3} synthase, CMP-sialic acid: LacCer sialyltransferase. Cer, ceramide.

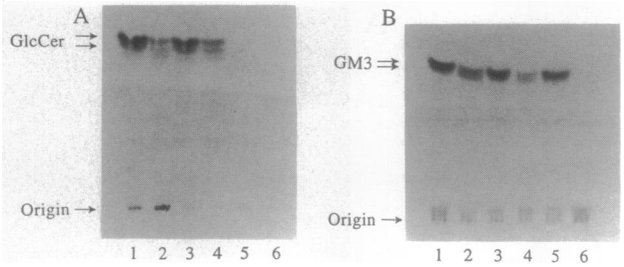

FIG. 6. Autoradiograms of glycosyltransferase products as catalyzed by cell lysates of B16, MEB-4, and GM-95 cells as enzyme sources. (*A*) GlcT-1 assay. Lanes: 1, B16 cells; 2, B16 cells with no ceramide addition; 3, MEB-4 cells; 4, MEB-4 cells with no ceramide addition; 5, GM-95 cells; 6, GM-95 cells with no ceramide addition. (*B*) G_{M3} synthase assay. Lanes: 1, B16 cells; 2, B16 cells with no LacCer addition; 3, MEB-4 cells; 4, MEB-4 cells with no LacCer addition; 5, GM-95; 6, GM-95 with no LacCer addition.

D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, a synthetic inhibitor of GlcT-1, or with endoglycoceramidase (29), which cleaves the linkage between ceramide and oligosaccharide. The morphological changes of GM-95 cells were not caused by the accumulation of ceramide because its content in the cells was normal. The morphology was not reverted by exogenously added GlcCer in the culture medium. It is known that the effect of an exogenously supplied glycolipid is not always the same as when endogenously generated, but the explanation for this is unknown (29).

Many studies have shown the importance of glycolipids on growth regulation. For instance, modulation of cell growth by GlcCer (30) or gangliosides (31) has been reported. Furthermore, Hanada *et al.* (32) demonstrated that sphingolipids were essential for the growth of Chinese hamster ovary cells. Dickson *et al.* (33) isolated a mutant strain of yeast (*Saccharomyces cerevisiae*) which contained little or no sphingolipids and did not require sphingolipids for growth. This deficient strain, however, had a slower growth rate than that of the parental strain and could use exogenous phytosphingosine to make sphingolipids. It is likely that glycolipids are also absolutely necessary for the survival and growth of animal cells. However, this hypothesis was not substantiated by our experiment. GM-95 cells, which contained virtually no glycolipid, could be maintained in serum-free-defined medium >2 months. The only difference observed was a 40% reduction of the growth rate compared with the parent MEB-4 cells. The difference of growth rates between GM-95 cells

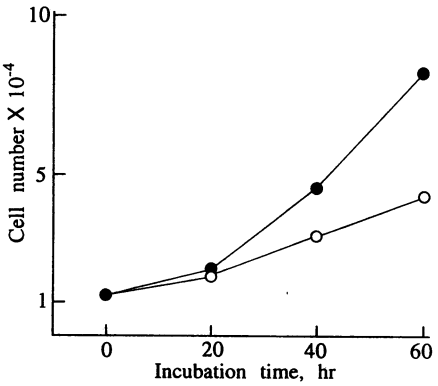

FIG. 7. Growth of MEB-4 and GM-95 cells in serum-free-defined medium. Cells were maintained in E-RDF medium supplemented with RD-I 2 weeks before the growth assay. Cells (1×10^4) were plated in 96-well plates. After 20-, 40-, or 60-hr incubation, cell number was estimated after staining with crystal violet by a colorimetric assay described by Kung *et al.* (19). Values are the means of three determinations; SDs were within 15% of mean values. ●, MEB-4 cells; ○, GM-95 cells.

and MEB-4 cells was at the same level as reported (6) for these mutants cultured under serum-supplemented conditions. One might expect that lipids other than glycolipids were newly expressed in GM-95 cells to compensate for the deficiency of glycolipids. However, lipid patterns of MEB-4 and GM-95 cells were similar except for glycolipids (Figs. 3*A* and 4 and data not shown). These results suggest that glycolipids are not an essential factor for the growth of B16 cells.

Fenderson *et al.* (34) cultured freshwater killi fish (medaka) embryos in the presence of D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol. Although glycolipids were reduced dramatically in their experiment, no evidence of developmental abnormality was observed up to the final differentiation stage. A question arising from their experiment is whether or not glycolipids are essential components for animal survival. This question was discussed by Radin (35). One of the best ways to examine the physiological functions of glycolipids in animals is to introduce a null mutation in a specific glycosyltransferase by using recently developed gene-targeting technology (36). However, isolation of the gene for a glycosyltransferase is necessary for this strategy. Recently, a cloning method based on the expression of the target gene in animal cells has been developed (37, 38); the method allows isolation of the gene without purifying a protein. GM-95 cells are ideal host cells for the expression cloning of GlcT-1 because these cells do not express the enzyme. Because GlcT-1 catalyzes the first step of glycolipid synthesis, it might be possible to produce mice without glycolipids except for GalCer and derived analogs by targeting GlcT-1, which would reveal the physiological significance of glycolipids in intact animals.

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