GM-95, a mutant cell line derived from mouse melanoma MEB-4 cells, is deficient in glycosphingolipids (GSLs) due to the lack of ceramide glucosyltransferase-1 activity (Ichikawa, S., Nakajo, N., Sakiyama, H., and Hirabayashi, Y. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2703–2707). In this study, we examined the involvement of the complex sphingolipids in cell to substratum adhesion. Immunofluorescent and chemical analyses revealed that the complex sphingolipids were significantly concentrated in the detergent-insoluble substrate attachment matrix of both GM-95 and MEB-4 cells. In spite of the absence of GSLs, GM-95 cells retained the ability to adhere to extracellular matrix (ECM) proteins such as fibronectin, collagen, and laminin. When both GM-95 and MEB-4 cells were treated with neutral sphingomyelinase, GM-95 cells were rounded up and detached from all ECM proteins examined. In contrast, neither the morphology nor the adherence of MEB-4 cells was altered. Under this treatment, sphingomyelin (SM) became undetectable in both cells. A similar inhibition was observed upon pretreatment of cells with fumonisin B1 or ISP-1, both of which block the synthesis of ceramide, a common precursor of both GSLs and SM. Stable transfectants expressing GSLs, which were established by transfection of glucosyltransferase-1 cDNA into GM-95 cells, became resistant to neutral sphingomyelinase-mediated rounding up and detachment from ECM proteins. In conclusion, the complex sphingolipids play critical roles in cell to substratum adhesion, and the presence of either GSLs or SM is sufficient for the adhesion.

Complex sphingolipids consist of glycosphingolipids (GSLs) and sphingomyelin (SM) that have different polar head groups, a sugar chain and phosphocholine, respectively. These lipids are ubiquitous components of the eukaryotic plasma membrane and comprise approximately 10% of the plasma membrane lipids. Complex sphingolipids have the hydrophobic residue, ceramide, which consists of sphingosine and fatty acids. Recently, ceramide and sphingosine, as the breakdown products from SM, are reported to be involved in intracellular signaling pathways for various cytokines. They have been recognized as bioactive compounds that modulate protein kinase or ion channel activities (1–3).

GSLs have been defined as tumor antigens and as regional and temporal markers in early embryogenesis (4, 5). Several lines of evidence have suggested that GSLs are involved in cell growth, differentiation, and adhesion (5, 6). However, in spite of a large number of studies done during the past few decades, the physiological functions of GSLs have still been elusive.

We have established a GSL-deficient mutant cell line, GM-95, from a B16 mouse melanoma cell line, MEB-4, and determined that the mutant is deficient in ceramide glucosyltransferase (UDP-glucose:N-acylsphingosine d-glucosyltransferase; GlcT-1) that catalyzes the first step of GSL synthesis (7). We have also found that GM-95 cells can proliferate in a GSL-free medium, clearly indicating that GSLs are not essential for cell growth (7). Recently, Hanada et al. (8) have established a temperature-sensitive mutant cell line (SPB-1 cells) that is deficient in serine palmitoyltransferase and is responsible for the initial step of ceramide synthesis. At a non-permissive temperature, SPB-1 ceased growing in a sphingolipid-free medium (8, 9). Although detailed mechanisms are not yet clear, their studies demonstrated that sphingolipids play critical roles in cell growth.

In spite of the physiological significance of sphingolipids, however, the biological function of membrane GSLs and SM is still unknown. GSLs have been implicated in cell to substratum adhesion, especially fibronectin (FN)-mediated adhesion (10–12). GM-95 cells are expected to be an ideal tool not only to investigate the physiological function of GSLs but also to assess the biological significance of SM. Very recently, we have cloned a cDNA encoding GlcT-1 by expression cloning and have established stable transfectants expressing GlcT-1 on the surface of GM-95 cells (13). In the current study, we examined the role of complex sphingolipids including GSLs and sphingomyelin for cell to substratum adhesion by using the mutant cells and the GlcT-1 cDNA transfectants.

EXPERIMENTAL PROCEDURES

Materials—E-RDF medium was purchased from Kyokuto Pharmaceutical Corp. (Tokyo). The medium is a complete serum-free medium that contains insulin and transferrin (7). FN (human plasma), type IV collagen (bovine placenta), and laminin (mouse Engelbreth-Holm-Swarm sarcoma) were purchased from Koch (Tokyo). Type I collagen (porcine skin) was obtained from Nitta Gelatin Co., Ltd. (Tokyo). Highly

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purified recombinant neutral sphingomyelinase (Bacidillus cereus) and phospholipase C (B. cereus) were purchased from Higeta Shouyu Co., Ltd. (Tokyo) and Boehringer Mannheim, respectively. Fumonisin B1 was purchased from Sigma. ISP-1 was generously donated by Dr. T. Kawasaki, Department of Biological Chemistry, Kyoto University, Japan. Mouse anti-GM3 ganglioside monoclonal antibody (mAb) M2590 was purchased from Meiji Seika Kaisha, Ltd. (Tokyo). Mouse anti-sphingomyelin mAb VJ41 was donated by Dr. K. Ono, Pharma Research Laboratory, Hoechst Japan Limited. All other chemicals were of the highest purity available.

Cells and Cell Culture—Mouse melanoma cell line MEB-4 and its GSL-deficient mutant GM-95 (7, 14) were cultured in minimum Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cell to Substratum Adhesion Assay—The adhesion assay was performed as described previously (15). Briefly, plates (96-well) were coated with FN (5 μg/ml), type I collagen (20 μg/ml), type IV collagen (20 μg/ml), laminin (20 μg/ml), or BSA (20 μg/ml) at 37°C for 1 h and then blocked with BSA (20 μg/ml) at 37°C for 1 h. Cells (3 × 10⁴) were allowed to attach to the coated plates at 37°C for 24 h in a serum-free E-RDF medium and then incubated with N-SMase (1 unit/ml) at 37°C for the indicated times. The plates were inverted and centrifuged at 500 rpm for 3 min to remove non-adherent cells. Cells were fixed with methanol at room temperature for 10 min and stained with 1% (w/v) crystal violet at room temperature for 10 min. After washing five times with PBS, the cells were lysed with 1% (w/v) sodium deoxycholate. The absorbance of each well was measured at 595 nm (with reference to absorbance at 405 nm) by a micro-plate reader. The cell number and the absorbance showed linear correlation between 1 and 8 × 10⁴.

To examine the effect of sphingolipid inhibitors on cell adhesion, the cells (1 × 10⁵) were seeded onto a 96-well plate in serum-free E-RDF medium and incubated at 37°C for 72 h in the presence of indicated concentrations of inhibitors. Cells were then subjected to cell adhesion assay as described above.

Lipid Analysis—Cells (1.5–2 × 10⁴) were harvested and total lipids were extracted with a mixture of chloroform/methanol/water (1:2:0.8, v/v). Lipid equivalents to 3–4 × 10⁵ cells were applied per lane on TLC. The solvent systems used in this study were chloroform/methanol (95:5, v/v) for cholesterol, chloroform/methanol/acetic acid/water (25:15:4:2, v/v) for phospholipid, and chloroform/methanol/12 M HCl (5:4:1, v/v) for GSL analysis. Cholesterol, phospholipids, and GSLs were detected with Zatkins, Zitter, and ornithin reagents, respectively. The amounts of each lipid were quantitated at 560 nm for cholesterol, at 650 nm for phosphatidylcholine, and at 540 nm for GSLs, using a TLC densitometer, CS-9000 (Shimadzu, Japan).

To analyze lipids in substrate attachment matrix (SAM), the cells (2 × 10⁴ cells) were detached with PBS containing 3.8 mM EDTA (16). Lipids of the substrates were extracted sequentially with methanol and chloroform/methanol (1:2, v/v). Lipids extracted from SAM of 4 × 10⁵ cells were applied per lane on TLC.

Establishment of Stable Transfectants with GcT-1 cDNA—We have isolated GcT-1 cDNA by expression cloning and designated it as pCG1 (13). The cDNA encoding GcT-1 was cloned into a mammalian expression vector, pcDNA I (Invitrogen). Stable transfectants were established by cotransfection of pCG1 and pSV2neo (17) into GM-95 cells. The cells were selected with 100 μg/ml G418 and 600 μg/ml G418, and those having the highest level of GcT-1 RNA were selected.

Immunocytochemical Analysis—Cells were grown on glass coverslips in minimum Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C (10). Cells were removed with PBS containing 3.8 mM EDTA as described previously (16), and substrates remaining on the coverslips were fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, 0.2% picric acid in PBS, pH 7.2, at room temperature for 10 min. After washing three times with PBS, the substrates were incubated with primary antibodies (M2590 and VJ41) at 37°C for 2 h. The substrates were washed three times with PBS, then blocked with 3% BSA containing 0.25% Triton X-100 for 30 min, and then reacted with fluorescein isothiocyanate-conjugated goat anti-mouse IgM antibody at 37°C for 2 h.

RESULTS

Characterization of Lipids in GM-95, Glycosphingolipid-deficient Cell Line—We first examined lipid compositions of GM-95 and MEB-4 cells (Table I). MEB-4 cells had two major GSLs (glucosylceramide and Gm₃ ganglioside) and SM, whereas GM-95 cells had no GSLs as previously reported (7). The cells had SM as a sole complex sphingolipid. Interestingly, the SM content of GM-95 cells increased over 1.5-fold when compared with that of MEB-4 cells. Consequently, the total amounts of complex sphingolipids in MEB-4 and GM-95 cells were almost equal. Among other phospholipids, the phosphatidylcholine content of GM-95 cells decreased by less than 17% compared with that of MEB-4 cells. Previous studies have shown that SM is mainly synthesized by phosphatidylinositol ceramide cholinephosphotransferase that catalyzes a base-exchange reaction between ceramide and phosphatidylinositol (18). The reduction of the phosphatidylinositol content in GM-95 cells possibly resulted from consumption of the lipid for SM synthesis by the exchange enzyme. The content of other lipids such as cholesterol and phosphatidylethanolamine was not altered in either cell line.

Requirement of Sphingolipids for Cell to Substratum Adhesion—Participation of GSLs in cell to substratum adhesion is still controversial. There are many studies suggesting that GSLs play an important role in cell to substratum adhesion (5, 6). Several others, however, indicate that sugar chains of GSLs do not affect the adhesion (10, 19). To answer this question, we compared the adhesion ability of MEB-4 and GM-95 cells with several ECM proteins and examined the effects of N-SMase on the attachment (Figs. 1 and 2). GM-95 cells adhered to all ECM proteins examined (Fig. 1). Attachment of GM-95 cells to FN, however, was 60–70% of that of MEB-4 cells. The surface expression of a5 integrin, an FN receptor, was at the same level in both GM-95 and MEB-4 cells when examined by fluorescence-activated cell sorter analysis with anti-a5 integrin mAb (data not shown). These results support the previous reports that GSLs modulate cell to substratum adhesion (20, 21). N-SMase treatment of GM-95 cells caused the rounding up of GM-95 cells (Fig. 2A) and reduced the adhesion to ECM proteins examined. The treatment, however, affected neither the morphology nor the adhesion of MEB-4 cells (Fig. 2, A and B). The effects were dependent on incubation time and concentration of N-SMase (Fig. 2, C and D). N-SMase treatment did not significantly affect the viability of either cell line (data not shown). Furthermore, the adhesion of GM-95 cells was restored by removal of N-SMase, meaning that any effect was reversible (Fig. 3). In addition, phospholipase C treatment had no apparent influence on cell adhesion in either cell line (data not shown). Therefore, the decreased adhesion resulted from removal of SM by N-SMase treatment.

We further investigated the alteration of the lipid composition by N-SMase treatment. After N-SMase treatment, SM was neither chemically detectable (Fig. 4) nor immunostained with VJ41, an anti-SM mAb (data not shown), indicating loss of all

### Table I

| Lipid | MEB-4 | GM-95 |
|-------|-------|-------|
|        | nmol/10⁶ cells | %     | nmol/10⁶ cells | %     |
| PE     | 4.36 | 37.2 | 4.54 | 40.4 |
| PS + PI | 0.81 | 6.9 | 0.91 | 8.1 |
| PC     | 5.60 | 47.7 | 4.67 | 41.6 |
| SM     | 0.68 | 5.8 | 1.11 | 9.9 |
| GlcCer | 0.16 | 1.4 | 0 | 0 |
| Gm₃    | 0.12 | 1.0 | 0 | 0 |
| Total  | 11.73 | 100 | 11.23 | 100 |

*Values are indicated as nmol of each lipid per 10⁶ cells.*

**Lipid Composition of Melanoma Cells**

| Lipid | MEB-4 | GM-95 |
|-------|-------|-------|
|        | nmol/10⁶ cells | %     | nmol/10⁶ cells | %     |
| PE     | 4.36 | 37.2 | 4.54 | 40.4 |
| PS + PI | 0.81 | 6.9 | 0.91 | 8.1 |
| PC     | 5.60 | 47.7 | 4.67 | 41.6 |
| SM     | 0.68 | 5.8 | 1.11 | 9.9 |
| GlcCer | 0.16 | 1.4 | 0 | 0 |
| Gm₃    | 0.12 | 1.0 | 0 | 0 |
| Total  | 11.73 | 100 | 11.23 | 100 |
complex sphingolipids from GM-95 cells, whereas the GSL content of MEB-4 cells did not change by this enzyme treatment. The treatment did not significantly alter the relative content of the other phospholipids.

Effect of Sphingolipid Inhibitors on Cell to Substratum Adhesion—When cells were stimulated with various kinds of agonists, sphingolipid-metabolic intermediates such as ceramide, sphingosine, and sphingosine 1-phosphate were shown to be produced from SM by cellular SMase. The metabolites exert diverse biological responses (1–3). Among them, sphingosine was shown to stimulate cell to substratum adhesion (22). Therefore, there is a possibility that these intermediates produced by N-SMase are involved in the loss of cell to substratum adhesion. To exclude this possibility, we used two different metabolic inhibitors for the synthesis of ceramide, which is a common precursor of all complex sphingolipids, including GSLs and SM. Fumonisin B1 inhibits dihydroceramide, responsible for dihydroceramide synthesis (23), ISP-1 inhibits serine palmitoyltransferase by which 3-ketosphinganine is formed (24). Thus, these inhibitors were expected to be useful reagents to clarify involvement of the intermediates in cell to substratum adhesion. In particular, ISP-1 could exclude any possibility that metabolic intermediates affect adhesion because the inhibitor blocks the initial step of sphingolipid synthesis. By treatment with ISP-1 (5 μM), the adhesion ability of MEB-4 and GM-95 cells was reduced to 70 and 40% of that of the control, respectively (Fig. 5), whereas fumonisin B1 (100 μM) inhibited the adhesion of GM-95 cells significantly (60% of that of the control) but not that of MEB-4 cells. To confirm the effect of these inhibitors on sphingolipid biosynthesis, we examined the complex sphingolipid contents of both cells after the inhibitor treatments (Table II). ISP-1 and fumonisin B1 reduced the total amount of complex sphingolipids in both cells to less than 20 and 30% of that of the control, respectively. In contrast to SM, the content of GSLs, particularly glucosylceramide, in MEB-4 cells was drastically decreased after ISP-1 and fumonisin B1 treatments (1.6 and 8.2% of the control, respectively).

Graphs and Figures:

**Fig. 1.** Adhesion of MEB-4 and GM-95 cells to ECM proteins. Adhesion assay was performed as described under “Experimental Procedures.” Solid and shaded columns represent MEB-4 and GM-95 cells, respectively. The bars indicate standard deviation. Adhesive ratios of GM-95 are shown relative to MEB-4 cells.

**Fig. 2.** N-SMase treatment of the melanoma cells. Adhesion assay was performed as described under “Experimental Procedures.” A, photographs (phase contrast) of melanoma cells with N-SMase treatment. Cells were cultured on FN-coated dishes and then treated with or without N-SMase (1 unit/ml) at 37 °C for 60 min. Bar, 100 μm. B, effect of N-SMase on cell adhesion to ECM proteins. Cells were cultured on dishes coated with ECM proteins and then treated with or without N-SMase (1 unit/ml) at 37 °C for 1.5 h. Solid and shaded columns represent MEB-4 and GM-95 cells, respectively. The bars indicate standard deviation. Values are shown as ratios relative to control data (without N-SMase treatment). Abbreviations are the same as listed in the legend to Fig. 1. C, time-dependent effect of N-SMase. Cells were cultured on FN-coated dishes and then treated with or without N-SMase (1 unit/ml) at 37 °C for the indicated times. Values are shown as ratios relative to time 0. Open and closed circles represent MEB-4 without and with N-SMase treatment, respectively. Open and closed squares represent GM-95 without and with N-SMase treatment, respectively. D, dose-dependent effect of N-SMase. Cells were cultured on FN-coated dishes and then treated with the indicated concentration of N-SMase at 37 °C for 1.5 h. Solid and shaded columns represent MEB-4 and GM-95 cells, respectively. The bars indicate standard deviation. Values are shown as ratios relative to control data (without N-SMase treatment).
respectively). Although the complex sphingolipid contents of MEB-4 and GM-95 cells were reduced similarly by the treatment, the effect on the adhesion was more dominant in GM-95 cells than that in MEB-4 cells. Because all GSLs could not be eliminated from MEB-4 cells after the treatment, the remaining GSLs may mediate cell to substratum adhesion more effectively than SM. This finding suggests that sugar chains bound to ceramide may contribute to the cell to substratum adhesion more potently than phosphocholine bound to it. From the above observations, we concluded that the reduction of all sphingolipids, but not the accumulation of metabolic intermediates, caused the loss of cell to substratum adhesion.

Prevention of N-SMase Effect by Transfection of GlcT-1 cDNA into GM-95 Cells—We have isolated a cDNA encoding GlcT-1 and established stable transfectants by introducing the cDNA into GM-95 cells (13). The transfectants (CG1 through CG5) expressed GSLs at different levels (Table III). Irrespective of the expression level of GSLs, these cells became resistant to the effects of N-SMase on the cell adhesion to substratum to an extent similar to that of MEB-4 cells (Fig. 6). By N-SMase treatment, SM in all transfectants was undetectable (data not shown). These findings supported a conclusion that the presence of either GSLs or SM is sufficient for the cell to substratum adhesion.

Distribution of Complex Sphingolipids in the SAM—GSLs were shown to be present in SAM (11, 12, 16). Here, we found that SM was also enriched in the focal contact of both MEB-4 and GM-95 cells (Table IV). In both cells, SM content of SAM was more than double that of whole cells. However, sphingomyelin was unexpectedly not detectable by immunoblotting with E-RDF medium and then additionally incubated in the same medium at 37 °C for 24 h. Adhesion assay was performed as described under “Experimental Procedures.” Solid and shaded columns represent parental cells (MEB-4) and mutant cells (GM-95), respectively. Values are shown as ratios relative to control data (without metabolic inhibitors). The bars indicate standard deviation.

The effect on the adhesion was more dominant in GM-95 cells than that in MEB-4 cells. Because all GSLs could not be eliminated from MEB-4 cells after the treatment, the remaining GSLs may mediate cell to substratum adhesion more effectively than SM. This finding suggests that sugar chains bound to ceramide may contribute to the cell to substratum adhesion more potently than phosphocholine bound to it. From the above observations, we concluded that the reduction of all sphingolipids, but not the accumulation of metabolic intermediates, caused the loss of cell to substratum adhesion.

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![Graph](image)

**Fig. 3. Reversibility of the effect of N-SMase.** Cells were cultured on FN-coated dishes and then treated with or without N-SMase (1 unit/ml) at 37 °C for 1.5 h. The effect on the adhesion was more dominant in GM-95 cells than that in MEB-4 cells. Because all GSLs could not be eliminated from MEB-4 cells after the treatment, the remaining GSLs may mediate cell to substratum adhesion more effectively than SM. This finding suggests that sugar chains bound to ceramide may contribute to the cell to substratum adhesion more potently than phosphocholine bound to it. From the above observations, we concluded that the reduction of all sphingolipids, but not the accumulation of metabolic intermediates, caused the loss of cell to substratum adhesion.

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![Graph](image)

**Fig. 4. Thin layer chromatography of phospholipids extracted from cells after N-SMase treatment.** Incubation was performed in the same manner as that in Fig. 2A. Lipid extraction was performed as described under “Experimental Procedures.” Lane 1, MEB-4 (untreated); lane 2, MEB-4 (N-SMase treatment); lane 3, GM-95 (untreated); lane 4, GM-95 (N-SMase treatment). PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylinositol; Ori., origin.

![Graph](image)

**Fig. 5. Effect of sphingolipid inhibitors on cell adhesion.** FN was coated on the wells. The treatment of inhibitors was performed as described under “Experimental Procedures.” The cells were then subjected to the cell adhesion assay. Solid and shaded columns represent parental cells (MEB-4) and mutant cells (GM-95), respectively. Values are shown as ratios relative to control data (without metabolic inhibitors). The bars indicate standard deviation.

**Table II**

| Cell   | Treatment | GlcCer | GM₃  | SM   | Total |
|--------|-----------|--------|------|------|-------|
|        |           |        |      |      |       |
| MEB-4  | None      | 0.16   | 0.12 | 0.68 | 0.96  |
|        | ISP-1 (5 μM) | <0.01 | 0.02 | 0.12 | 0.14  |
|        | FB1 (100 μM) | 0.01 | 0.04 | 0.21 | 0.26  |
| GM-95  | None      | 0      | 0    | 1.11 | 1.11  |
|        | ISP-1 (5 μM) | 0  | 0.20 | 0.20 |       |
|        | FB1 (100 μM) | 0  | 0.33 | 0.33 |       |

*Cells were treated with inhibitors at 37 °C for 3 days.

**Table III**

| Cell   | GlcCer | GM₃  | SM   | Total |
|--------|--------|------|------|-------|
|        |        |      |      |       |
| MEB-4  | 0.16   | 0.12 | 0.68 | 0.96  |
| GM-95  | 0      | 0    | 1.11 | 1.11  |
| CG1    | 0.18   | 0.18 | 0.51 | 0.87  |
| CG2    | 0.11   | 0.13 | 0.68 | 0.92  |
| CG3    | 0.15   | 0.11 | 0.67 | 0.92  |
| CG4    | 0.03   | 0.04 | 1.05 | 1.12  |
| CG5    | 0.16   | 0.12 | 0.74 | 1.02  |
| DNA1   | 0      | 0.91 | 0.91 |
| DNA2   | 0      | 0.95 | 0.95 |
| DNA3   | 0      | 0.83 | 0.83 |

*Values are indicated as nmol of each sphingolipid per 10⁶ cells.
anti-GM3 ganglioside monoclonal antibody M2590, these cells expressed GlcT-1 cDNA into GM-95 cells. Judging by the reactivity with the representative independent clones that were established by transfection of the treatment of sphingolipid inhibitors that blocked biosynthesis of GSLs and SM in cell to substratum adhesion. N-SMase treatment caused detachment of GM-95 cells from ECM proteins. 2) The adhesion of both cells was decreased by N-SMase treatment caused detachment of GM-95 cells from ECM proteins. Since GM-95 cells possessed SM as the sole complex sphingolipid, the mutant allowed us to test the role of complex sphingolipids in cell to substratum adhesion. N-SMase treatment caused detachment of GM-95 cells from ECM proteins. Under this condition, GSLs stayed at a normal level in the cells, whereas complete removal of SM by N-SMase treatment caused detachment of GM-95 cells from ECM proteins. 2) The adhesion of both cells was decreased by the treatment of sphingolipid inhibitors that blocked biosynthesis of GSLs and SM. Thus, SM and GSLs are not responsible for the phenomenon, based on the following observations: 1) Although the intermediates were produced after N-SMase treatment, MEB-4 cells still attached to ECM proteins. Under this condition, GSLs stayed at a normal level in the cells, whereas complete removal of SM by N-SMase treatment caused detachment of GM-95 cells from ECM proteins. 2) The adhesion of both cells was decreased by the treatment of sphingolipid inhibitors that blocked biosynthesis of GSLs and SM. Thus, SM and GSLs are not responsible for the phenomenon, based on the following observations: 1) Although the intermediates were produced after N-SMase treatment, MEB-4 cells still attached to ECM proteins. Under this condition, GSLs stayed at a normal level in the cells, whereas complete removal of SM by N-SMase treatment caused detachment of GM-95 cells from ECM proteins.

**TABLE IV**

| Lipid   | MEB-4        | GM-95        |
|---------|--------------|--------------|
|         | Cell | SAM | Cell | SAM |
| PE      | 37.2 | 26.6 | 40.4 | 24.7 |
| PS + PI | 6.9  | 8.7  | 8.1  | 3.7  |
| PC      | 47.7 | 45.5 | 41.6 | 51.1 |
| SM      | 5.8  | 16.7 | 9.9  | 20.5 |
| GlcCer  | 1.4  | 1.0  | 0    | 0    |
| Gm3     | 1.0  | 1.5  | 0    | 0    |
| Total   | 100  | 100  | 100  | 100  |

*Values are indicated as nmol per 10^6 cells.

In the present study, we demonstrated that when cells lose all complex sphingolipids, they lose the ability to adhere to ECM proteins. Since GM-95 cells possessed SM as the sole complex sphingolipid, the mutant allowed us to test the role of GSLs and SM in cell to substratum adhesion. N-SMase treatment caused detachment of GM-95 cells from ECM proteins, but the same treatment affected neither the morphology nor the attachment of MEB-4 cells that contained GSLs. Transfection of GlcT-1 cDNA into GM-95 cells restores the expression of GlcT-1 cDNA into GM-95 cells. Judging by the reactivity with the anti-GM3 ganglioside monoclonal antibody M2590, these cells expressed different amounts of Gm3 ganglioside. DNA1–DNA3 represent negative control clones that were transfected with the vector alone. M. F. I., mean fluorescent intensity by flow cytometric analysis.

**FIG. 6.** Effect of N-SMase treatment on cell to substratum adhesion of stable transfectants with GlcT-1 cDNA. FN was coated on the wells. N-SMase (1 unit/ml) treatment was performed at 37 °C for 1.5 h. Values are shown as ratios relative to control data (without N-SMase treatment). The bars indicate standard deviation. CG1–CG5 represent independent clones that were established by transfection of GlcT-1 cDNA into GM-95 cells. Judging by the reactivity with the anti-GM3 ganglioside monoclonal antibody M2590, these cells expressed different amounts of Gm3 ganglioside. DNA1–DNA3 represent negative control clones that were transfected with the vector alone. M. F. I., mean fluorescent intensity by flow cytometric analysis.

**FIG. 7.** Immunofluorescent staining of complex sphingolipids in the SAM. Immunofluorescent staining was performed as described under “Experimental Procedures.” The monoclonal antibodies used in this analysis, M2590 and VJ 41, are for detection of Gm3 ganglioside and SM, respectively. Bar, 25 µm.
brane. In fact, a fluorescent derivative of SM incorporated into the plasma membrane was quickly internalized after N-SMase treatment (data not shown).

We have also examined the lipid composition of GM-95 and MEB-4 cells in detail. The SM content of GM-95 cells was significantly higher than that of MEB-4 cells. Consequently, the total amount of complex sphingolipids was identical between MEB-4 and GM-95 cells, suggesting that the cellular content of complex sphingolipids is maintained at a certain level. Presumably, factors responsible for ceramide synthesis regulate the total content of complex sphingolipids. In conclusion, we have proven that complex sphingolipids are essential for cell to substratum adhesion. Since cell to substratum adhesion is important for cell growth and differentiation (32, 33), it is possible that complex sphingolipids also play important roles in such cellular processes.

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