Structure of the Ceramide Moiety of GM1 Ganglioside Determines Its Occurrence in Different Detergent-Resistant Membrane Domains in HL-60 Cells†

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ABSTRACT: To investigate the effect of the ceramide moiety of GM1 ganglioside on its association with detergent resistant membrane domains (DRMs) in human leukemia HL-60 cells, [^{3}H] labeled GM1 molecular species (GM1s) with ceramides consisting of C18 sphingosine acetylated or acylated with C_8, C_{12}, C_{14}, C_{16}, C_{18}, C_{22}, C_{24}, $C_{18:1}$, $C_{22:1}$, or $C_{24:1}$ fatty acids (FAs), or C20 sphingosine acetylated or acylated with C_8 or C_{18} FA were prepared and added to culture media. GM1s uptake by HL-60 cells was affected by the structure of their ceramides. Resistance to removal with trypsin and the stoichiometry of [^{125}I] cholera toxin (CT) binding indicated that the added GM1s were incorporated into the membranes of the cells used for the isolation of DRMs in a manner resembling endogenous gangliosides. The ceramide moieties of the GM1s determined their occurrence in DRMs and the dependence of their recovery in this membrane fraction on the amount of Triton X-100 (TX) used for extraction as well as on cholesterol depletion. The GM1s with sphingosine acylated with C_{14}, C_{16}, C_{18} C_{22}, or C_{24} FAs were similarly abundant in DRMs. GM1s acylated with $C_{18:1}$, $C_{22:1}$, or $C_{24:1}$ were less abundant than those acylated with saturated FA of the same length. GM1s acetylated or acylated with C_8 FA were detected in DRMs in the lowest proportion. Depletion of 73% of cell cholesterol with methyl-β-cyclodextrin significantly affected the recovery in DRMs of GM1s acetylated or acylated with C_8 or unsaturated FAs but not of GM1 acylated with C_{18}, C_{22}, or C_{24} FAs. After cross-linking with CT B subunit, all GM1s were recovered in DRMs in a similarly high proportion irrespective of their ceramide structure or cholesterol depletion. DRMs prepared with low TX concentration at the TX/cell protein ratio of 0.3:1 were separated by multistep sucrose density gradient centrifugation into two fractions. The GM1s with sphingosine acetylated or acylated with C_{18} or $C_{18:1}$ FAs occurred in these fractions in different proportions.

Gangliosides are sialic acid containing glycosphingolipids ubiquitously present in animal cell plasma membranes (*1*−*3*). Reports on changes in ganglioside content and composition during cell growth, differentiation, and oncogenesis (*4*) prompted research on their functions (*5*, *6*). Gangliosides seem to be involved in important physiological processes such as adhesion (*7*), apoptosis (*8*), cell motility (*9*), nerve regeneration (*10*), and signal transduction (*7*, *11*−*13*). Earlier studies on ganglioside distribution in plasma membranes indicated that they have a tendency to form clusters or microdomains (*14*), while more recent reports focus our attention on their presence in lipid rafts (*15*), membrane domains critical for key cellular activities (*16*−*24*). Glycosphingolipids are not necessary for raft formation (*25*); however, if present in plasma membranes, they can affect raft protein content and functions. Thus, a decrease in the glycosphingolipid level was reported to diminish raft association of the TAG-1 (*26*) and Src proteins (*27*) while exogenously added gangliosides seemed to displace GPI-anchored proteins from rafts in cells (*28*) and model membranes (*29*). Moreover, increased expression of GM1[1] ganglioside displaced PDGF receptor from rafts (*30*), while cross-linking of GM2 excluded ligand bound insulin receptor

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[1] Abbreviations: C18 sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadec-4-ene; C20 sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-eicos-4-ene; Cer, ceramide; CT, cholera toxin; CTB, cholera toxin B subunit; DRMs, detergent resistant membrane fraction; ESI-MS, electrospray ionization mass spectrometry; GLC, gas/liquid chromatography; GM1 ganglioside, Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer; GM1 ganglioside molecular species are abbreviated according to Palestini et al. (*71*) as follows: GM1 (18:1/2), GM1 ganglioside with *N*-acetylated C18 sphingosine; GM1(20:1/2), GM1 with N-acetylated C20 sphingosine; GM1(18:1/8), GM1 with C18 sphingosine N-acylated with caprylic acid; GM1(20:1/8), GM1 with C20 sphingosine N-acylated with caprylic acid; GM1(18:1/12), GM1 with C18 sphingosine N-acylated with lauric acid; GM1(18:1/14), GM1 with C18 sphingosine N-acylated with myristic acid; GM1(18:1/16), GM1 with C18 sphingosine N-acylated with palmitic acid; GM1(18:1/18), GM1 with C18 sphingosine N-acylated with stearic acid; GM1(20:1/18), GM1 with C20 sphingosine N-acylated with stearic acid; GM1(18:1/22), GM1 with C18 sphingosine N-acylated with behenic acid; GM1(18:1/24), GM1 ganglioside with C18 sphingosine N-acylated with lignoceric acid; GM1(18:1/18:1), GM1 with C18 sphingosine N-acylated with oleic acid; GM1(18:1/22:1), GM1 with C18 sphingosine N-acylated with erucic acid; GM1(18:1/24:1), GM1 with C18 sphingosine N-acylated with nervonic acid; GM1s, various GM1 molecular species; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; Na-5 mM Pi buffer, 5 mM sodium-phosphate buffer, pH 7.0; Na-2 mM Pi buffer, 2 mM sodium-phosphate buffer, pH 7.0; medium H, RPMI 1640 medium containing 10 mM HEPES buffer pH 7.3 and 5 μg/mL of insulin and transferrin; MβCD, methyl-β-cyclodextrin; PBS-G, PBS containing 0.1% gelatin.

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from them (*31*). The association of glycosphingolipids with rafts was proposed to result primarily from two types of interactions. In the first model, the major factor for raft formation would be the multiple hydrogen bonds because of glycosphingolipid oligosaccharide headgroups, as well as the amide bond and hydroxyl group of sphingosine. Cholesterol would be filling gaps in membrane packing resulting from the differences in cross sectional areas of the ceramide and oligosaccharide moieties of glycosphingolipids (*17*). In the second model, sphingosine and long saturated fatty acids of glycosphingolipids, together with cholesterol and sphingomyelin, would form liquid ordered phase domains (lo), tightly packed and thus relatively resistant to solubilization with detergents (*23*, *32*).

The purpose of our studies was to determine the effect of ceramide structure of GM1 ganglioside on its occurrence in the detergent resistant membrane fraction, DRMs, isolated from human leukemia HL-60 cells. These cells, used by others in extensive studies on ganglioside uptake (*33*), were chosen as they contain only trace amounts of endogenous GM1 (*34*). We prepared 14 GM1 molecular species, labeled with tritium in the terminal galactose and differing in the structure of their ceramide moieties, and characterized their uptake by HL-60 cells. We determined the effect of the length and unsaturation of the fatty acids as well as the type of sphingosine on the recovery of these GM1s in DRMs obtained under different experimental conditions. We studied the effects of the amount of detergent used for extraction, cholesterol depletion, and cross-linking with CTB on the occurrence of various GM1 molecular species in DRMs. Observations presented herein indicate that the ceramide moiety of GM1 ganglioside not only strongly affects its packing within the plasma membrane and hence resistance to Triton X-100 extraction but also influences, at least for some molecular species, their occurrence in membrane domains differing in buoyant density as well as solubility in the detergent.

MATERIALS AND METHODS

Materials. Reagents were purchased as indicated: fatty acids of over 99% purity, Fluka; *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, protease inhibitors, silicic acid, HPTLC and TLC plates, and columns and solvents for HPLC, Merck; cholesterol assay kit, Boehringer; Sep-Pak C_{18} cartridges, Waters; $Na^{125}I$, carrier free, [^{3}H]$NaBH_4$ of specific radioactivity 11−15 Ci/mmol, and EN^{3}HANCE spray, NEN Life Science Products; cholera toxin and its B subunit, galactose oxidase from the fungi Dactylium dendroides, Sigma-Fluor liquid scintillation mixture as well as the remaining reagents, Sigma-Aldrich (Pl).

GM1 Ganglioside. GM1 ganglioside was isolated from bovine brain and purified by repeated DEAE-Sephadex and silicic acid column chromatography (*35*). GM1(18:1/18) was prepared from this material by a reversed phase chromatography procedure based on the method of Gazotti et al. (*36*). Briefly, 20 mg of GM1 ganglioside dissolved in 500 μL of acetonitrile/water (1:1) was injected into a preparative HPLC reversed phase column (LiChrosorb RP-select B, 250 × 25 mm), which was eluted with acetonitrile/Na-5 mM Pi buffer (53:47) at 5 mL/min. The middle 70% of the first major peak, as well as about 60% of the second, detected at 195 nm and checked by HPTLC to contain GM1, were recovered, dried on a rotary evaporator, and freed of salts on two stacked Sep-Pak C_{18} cartridges as described (*37*). This material was further purified on a LiChrospher C8e, 250 × 10 mm reversed phase column. For the purification of GM1(18:1/18), the column was eluted with acetonitrile/Na-5 mM Pi buffer (65:35) at 3 mL/min, while for GM1(20:1/18) this column was eluted with methanol/Na-2 mM Pi (87:13) at the same flow rate, but the detector was set at 210 nm. About 500 nmol of GM1 was purified in a single run.

Lyso-GM1. Lyso-GM1 prepared as described by Schwarzmann and Sandhoff (*38*) was separated into C18- and C20-sphingosine containing species by reversed phase HPLC on a LiChrospher 100 RP-18, 250 × 10 mm column. The column was eluted at 3 mL/min with acetonitrile/Na-5 mM Pi buffer (40:60) changed stepwise, after collection of lyso-GM1(18:1), to (44:56) for the elution of lyso-GM1(20:1). About 1 μmol of lyso-GM1 was fractionated in a single run.

Acetylation and Acylation of Lyso-GM1. For acetylation, 1.5 μmol of lyso-GM1 in 1 mL of methanol containing 50 μL of pyridine and 50 μL of acetic anhydride was left overnight at RT. For N-acylation, 2 μmol aliquots of lyso-GM1 were treated with 3-fold excess of different fatty acid NHS-esters (*38*). The acylation mixture was dried under N_2, the residue dissolved in chloroform/methanol (1:1), and after the addition of chloroform to change this ratio to 85:15, the sample was applied to a column of silicic acid in chloroform packed in a Pasteur pipet to about 1 mL bed volume. Free fatty acids and NHS esters were washed away with 10 mL of chloroform/methanol (9:1), while the acylated GM1 was eluted with chloroform/methanol/water (5:5:1).

HPLC Purification of GM1 Molecular Species. GM1s with fatty acids shorter than C_{18} were purified on a LiChrospher 100 RP-18, 250 × 10 mm reversed phase column. It was eluted at 3 mL/min with acetonitrile/Na-5 mM Pi buffer, isocratically for GM1(18:1/2) and GM1(20:1/2) or by a gradient of acetonitrile/Na-5 mM Pi buffer changing from (40:60) to (70:30) for the remaining ones. GM1(18:1/18:1) was purified as described for GM1(18:1/18). GM1s acylated with fatty acids longer than C_{18} were purified on a LiChrospher C8e, 250 × 10 mm column eluted isocratically at 3 mL/min. For GM1(18:1/22) and GM1(18:1/24:1), the solvent consisted of methanol/Na-2 mM Pi (88: 12), for GM1(18:1/22:1) of methanol/Na-2 mM Pi (87:13), and for GM1(18:1/24) of methanol/Na-2 mM Pi (90:10). For the methanol containing solvent systems the detector was set at 210 nm.

Tritium Labeling of GM1 Ganglioside Molecular Species. All GM1 molecular species were labeled with tritium in the terminal galactose by the galactose oxidase method (*39*) scaled down to be used with about 1.5 μmol of gangliosides. At the completion of the oxidation reaction, the incubation mixture was freeze-dried, and the residue was suspended in chloroform/methanol (1:1) by ultrasonication in a cleaning bath. Triton X-100 present in the sample was separated from GM1 on a silicic acid column as described above after acylation. Oxidized GM1 was purified by preparative TLC on silica gel coated plates developed with chloroform/methanol/0.25% aqueous $CaCl_2$ (60:35:8), eluted from gel scrapings with chloroform/methanol/water (5:5:1), dried under N_2, dissolved in 10% methanol in water, and freed from contaminants on Sep-Pak C_{18} cartridges. For the

reduction with ^3H, about 1 μmol of oxidized GM1 dissolved in 700 μL of *n*-propanol was stirred overnight with a 3-fold excess of [^3H]$NaBH_4$ added in 300 μL of 0.1 N NaOH. The remaining [^3H]$NaBH_4$ was decomposed by the addition of 1 mL of 1% acetic acid in methanol (well-ventilated hood), the reaction mixture was dried under a stream of N_2, and the residue was suspended in 1 mL of 1% acetic acid in methanol and dried again. After repeating this step three times, the residue containing [^3H]GM1 was separated from salts on a Sep-Pak C_{18} light cartridge. Final purification of [^3H]GM1 was achieved by preparative TLC on analytical silica gel coated plates as described for oxidized GM1. The TLC areas containing [^3H]GM1 delineated after water spray were scraped from the plate while still slightly wet to avoid radioactive dust. [^3H]GM1 was eluted from the silica gel scrapings and further treated as described for oxidized GM1. Purified [^3H]GM1 diluted to 10 μCi/mL with ethanol containing 1% 0.2 M HEPES buffer pH 7.5 and 0.01% butylated hydroxytoluene was stored at − 20 °C.

Cell Culture. Human myelogenous leukemia HL-60 cells obtained from ATCC were cultured at 37 °C under 6% CO_2 in RPMI 1640 medium, containing 10% fetal calf serum (both from GIBCO/BRL Life Technologies), supplemented with 50 U/mL of penicillin and 50 μg/mL of streptomycin, to a density of 1−1.5 × 10^6/mL. When needed, cells were collected by centrifugation (1000*g*, 10 min), washed twice with PBS-G, and suspended in RPMI 1640 medium containing 10 mM HEPES pH 7.3 and 5 μg/mL of insulin and transferrin each (medium H).

Uptake of [^3H]GM1 by HL-60 Cells. All GM1 molecular species were added to cell cultures following the same protocol. [^3H]GM1, dried under N_2 followed by high vacuum in a desiccator for 15 min, was suspended by ultrasonication and heating at 40 °C in medium H at a concentration 3-fold higher than the final one used in the cell culture. Before addition to the cell culture, the [^3H]GM1 suspension was placed in the incubator for about 1 h. The cells suspended in medium H at a density of 4.5 × 10^6/mL were kept in the incubator for about 30 min before addition of [^3H]GM1 containing medium. Routinely, for ganglioside uptake studies, cells at a density of 3 × 10^6/mL were incubated in culture media containing 0.5 μM [^3H]GM1. The concentration of GM1 molecular species during incubations followed by the preparation of DRMs was 0.5 μM or lower, so their final uptake after 1 h did not exceed 10 fmol/10^3 cells.

Removal of [^3H]GM1 Taken Up by HL-60 Cells with BSA and Trypsin. To determine the resistance of [^3H]GM1s taken up by the cells to removal by BSA (*40*, *41*), after 1 h incubation with gangliosides, the cells were washed with PBS and suspended at a density of 2−10 × 10^6 cells/mL in medium H containing 0.1% gelatin. Aliquots of 100 μL were withdrawn, mixed with an equal volume of medium H containing 2% BSA or 0.1% gelatin, and incubated for 15 min at 37 °C. After washing three times through centrifugation with PBS-G, cell pellets were dissolved in 1 N NaOH before determination of their radioactivity by liquid scintillation counting.

To determine if the [^3H]GM1 molecules taken up by the cells are inserted into the plasma membrane or adsorbed on it as micelles (*40*, *41*), trypsin treatment was employed as described (*42*). Briefly, after incubation with [^3H]GM1 and washing with PBS, cells were suspended in medium H and aliquots containing 2 × 10^5 cells in 100 μL were mixed with an equal volume of 0.5% trypsin freshly dissolved in medium H. After 5 min at 37 °C the incubation media were diluted with 4 mL of cold PBS-G, cells were pelleted at 1500*g* for 10 min and dissolved in 1 N NaOH for the determination of their radioactivity by liquid scintillation counting.

Cross-Linking of [^3H]GM1 with CTB. To study the effect of cross-linking with CTB, cells (after incubation with [^3H]-GM1s) were washed twice with PBS-G and suspended at a density of 1 × 10^7/mL in medium H containing 0.1% gelatin. Routinely, CTB was added to the medium at a final concentration of 20 nM above the amount necessary to bind all GM1 molecules taken up by the cells assuming the GM1 to CTB binding stoichiometry as equal to 5:1. After 30 min incubation with CTB at 4 °C, cells were washed twice with PBS-G and used for the preparation of DRMs.

Preparation of DRMs. pH 11 Procedure. In most of the experiments, the DRM fraction was prepared by the procedure of Ostermeyer et al. (*25*). All solutions contained 2 mM Pefabloc SC as well as chymostatin, leupeptin, antipain, and pepstatin, each at 5 μg/mL. Cells, 6 × 10^7, were suspended in 1.7 mL of a solution consisting of 25 mM Tris, 150 mM NaCl, 5 mM EDTA, adjusted to pH 11.0 with Na_2CO_3 (TNE pH 11.0 buffer) and containing different amounts of Triton X-100. The cell suspension was pushed 10 times successively through 18G, 20G, and 25G × 1'' syringe needles and left on ice for 30 min. A 1.5 mL aliquot of the homogenate was withdrawn, mixed in an SW41 rotor tube with an equal volume of 86% sucrose in TNE pH 7.5 buffer, and overlayered with 6 mL of 38% sucrose followed by about 3 mL of 5% sucrose in the same buffer. For multistep sucrose gradient centrifugation, 1 mL of cell homogenate was mixed in an SW41 rotor tube with an equal volume of 70% sucrose in TNE pH 7.5 buffer. The resulting suspension was overlayered step by step with 10−30% sucrose gradient, consisting of 10 1-mL steps. After centrifugation for 16−18 h at 36 000 rpm in a Beckman SW41 rotor, 12 fractions of about 1 mL each were collected from the top of the tube. The radioactivity in the fractions as well as in the sediment, suspended in 1 mL of 0.25 M Na_2CO_3 and ultrasonicated in a cleaning bath to ensure homogeneity, was determined by liquid scintillation counting.

pH 7.5 Procedure. In some experiments, the DRM fraction was obtained by a procedure based on that of Brown and Rose (*43*). Cells were extracted in a Dounce homogenizer (30 strokes) with the same volume as in the pH 11.0 procedure of 1% Triton X-100 in 25 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5 (TNE pH 7.5 buffer) containing protease inhibitors and left on ice for 30 min. Cell debris and nuclei were removed (1500*g*, 15 min), while the supernatant mixed with 86% sucrose was centrifuged as described for the pH 11.0 procedure.

Cholesterol Determination and Depletion. For cholesterol depletion, cells, at a density of 3 × 10^6/mL, were incubated in medium H containing 0.1% BSA and 5 mM MβCD for 1 h at 37 °C. The cells were washed twice with PBS, suspended in medium H, and incubated under standard conditions with [^3H]GM1s. For the estimation of cholesterol, cell pellets or thoroughly dialyzed and freeze-dried sucrose gradient fractions were subjected to lipid extraction (*44*). The lipid extracts were dried under N_2 and suspended in 120 μL of 2-propanol, and their cholesterol content was determined by

the cholesterol oxidase method with the Boehringer kit. The volume of the reagents was reduced to permit microplate determinations.

Binding of Radioiodinated Cholera Toxin to Cells. CT was radioiodinated by the chloramine T procedure (*45*). Binding conditions were based on those reported by Miller-Podraza et al. (*46*). Briefly, after incubation with [^{3}H]GM1, the cells were washed with PBS-G and suspended in medium H containing 0.1% gelatin. Depending on the extent of [^{3}H]-GM1s uptake, from 3×10^4 to 1×10^6 cells were incubated in 200 μL of this medium with 10 nM radioiodinated CT for 45 min at 4 °C. Thereafter, cells were washed three times with PBS-G by centrifugation. If needed, to minimize high background because of the binding of [^{125}I] CT to plastic tubes, before last wash the cells were transferred with PBS-G to new tubes and collected by centrifugation. The amount of cell bound radioiodinated CT was determined in a γ-counter. Parallel incubations containing the same numbers of cells with or without radioiodinated CT were used to estimate the ratio of [^{3}H]GM1 present in the membranes and determined by liquid scintillation counting, to the amount of [^{125}I]CT bound to the cells. The extent of nonspecific binding was estimated in the presence of 200 nM unlabeled CT.

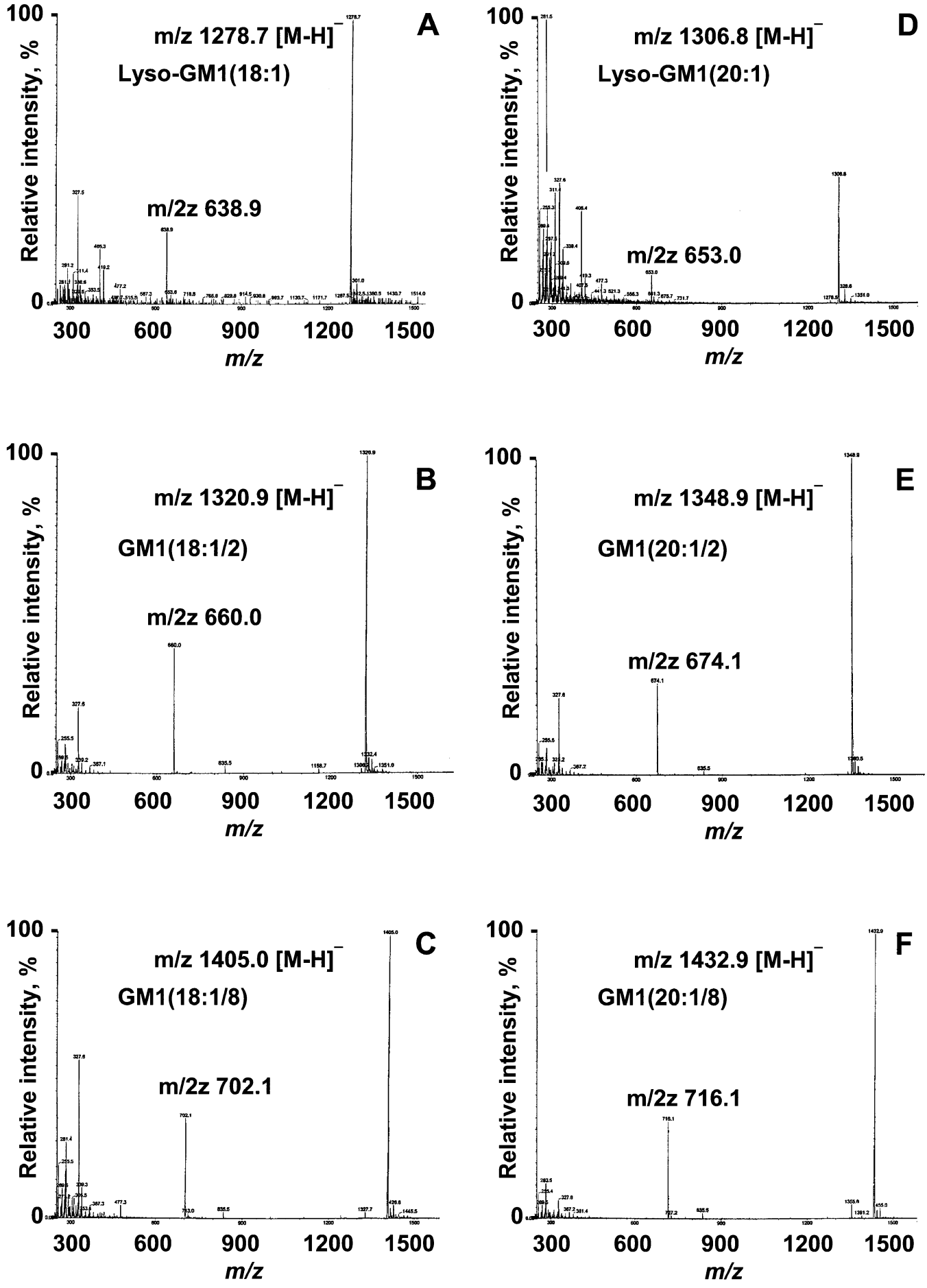
Other Procedures. ESI-MS analyses were performed on an Applied Biosystems API 365 apparatus with the ion spray voltage set at −4 kV. Gangliosides, dissolved in methanol at a concentration of 25 nmol/mL, were injected into the ion source at a rate of 3 μL/min. Negative ions were scanned between 250 and 2000, or for some preparations, between 350 and 2000 atomic mass units. Long chain bases in purified lyso-GM1 and GM1 isolated from bovine brain were analyzed by GLC as trimethylsilyl derivatives (*47*) on a 30 m long capillary HP-1 column run isothermally at 240 °C. Fatty acids were identified by GLC as methyl esters on a 60 m long sge BPX 70 (70% cyanopropyl) column. The initial column temperature of 95 °C was increased to 170 °C during the first 8 min, maintained at 170 °C for 2 min, further increased to 185 °C, and kept at this temperature until the end of the analysis. For the analyses of C_{22}, C_{24}, $C_{22:1}$, and $C_{24:1}$ fatty acids, the initial column temperature of 140 °C was increased at a rate of 15°/min to 210 °C, maintained at this temperature for 1 min, further increased to 215 °C, and kept at this temperature until the end of the analysis. Sialic acid was routinely estimated with resorcinol (*48*) and in purified [^{3}H]GM1 molecular species using the method of Skoza and Mohos (*49*). To characterize the radioactive material present in DRMs, an aliquot of the lipid extract prepared for the estimation of cholesterol was analyzed by HPTLC in a chloroform/methanol/0.25% aqueous $CaCl_2$ (60:35:8) solvent system. The distribution of radioactivity in separated spots was analyzed with a Phosphor Imager. Protein content was determined with bicinchoninic acid according to the protocol of Pierce Chemical Co. Statistical analyses were performed by Student's *t* test with the significance level at $p < 0.05$.

RESULTS

Partial Characterization of GM1 Molecular Species. HPLC-purified lyso-GM1(18:1) and lyso-GM1(20:1) migrated on HPTLC and emerged from the reversed phase analytical HPLC column as homogeneous material (not shown). Interestingly, GM1(18:1/2) as well as GM1(20:1/2) were eluted from this column ahead of the parent lyso-gangliosides, most probably reflecting intramolecular charge interactions between the carboxylate and the ammonium groups of sialic acid and sphingosine, respectively. The effect of ionization of the sialic acid could also be detected on mass spectra by the presence of peaks corresponding to doubly charged ions (Figure 1). The pseudomolecular ions detected by ESI-MS at *m*/*z* 1278.7 $[M - H]^-$ for lyso-GM1(18:1) and at *m*/*z* 1306.8 $[M - H]^-$ for lyso-GM1(20:1) confirmed their structure (Figure 1). In keeping with these data, GLC analysis revealed the presence of only C18 sphingosine for lyso-GM1(18:1) and C20 sphingosine for lyso-GM1(20:1). GM1 molecular species were prepared from lyso-gangliosides through N-acetylation, in over 90% yields, or by N-acylation in 71−80% yields, as determined by densitometric scanning of HPTLC separated, orcinol stained, reaction products (not shown). After HPLC purification, GM1 molecular species migrated on HPTLC and emerged from the analytical HPLC column as homogeneous material. GM1(18:1/2), GM1(20:1/2), GM1(18:1/8), GM1(20:1/8), GM1(20:1/18), GM1(18:1/22), GM1(18:1/24), GM1(18:1/22:1), and GM1(18:1/24:1) were analyzed by ESI-MS (Figure 1). The pseudomolecular ions of high relative abundance at *m*/*z* 1320.9 $[M - H]^-$ for GM1(18:1/2); at *m*/*z* 1348.9 $[M - H]^-$ for GM1(20:1/2); at *m*/*z* 1405.0 $[M - H]^-$ for GM1(18:1/8); at *m*/*z* 1432.9 $[M - H]^-$ for GM1(20:1/8); at *m*/*z* 1573.2 $[M - H]^-$ for GM1(20:1/18); at *m*/*z* 1602.0 $[M - H]^-$ for GM1(18:1/22); at *m*/*z* 1630.1 $[M - H]^-$ for GM1(18:1/24); at *m*/*z* 1600.1 $[M - H]^-$ for GM1(18:1/22:1); and at *m*/*z* 1628.2 $[M - H]^-$ for GM1-(18:1/24:1) confirmed their structures. GLC analysis of the acyl residues of the GM1 molecular species acylated with fatty acids higher than caprylic, except for GM1(18:1/18) and GM1(20:1/18), showed over 99% homogeneity. Bovine brain GM1(18:1/18) ganglioside, after the first HPLC column, contained stearic (90.3%) and palmitic acid (4.1%), as well as 4.9% of unidentified contaminants. After the second HPLC purification step, only stearic acid (98.3%) was identified in this preparation. The fatty acid composition of GM1(20:1/18) ganglioside fraction obtained after the first HPLC purification was not analyzed. After the second HPLC step, its fatty acid analysis revealed the presence of stearic acid at 98.8%.

[^{3}H]GM1 Molecular Species. The specific radioactivity of [^{3}H]GM1 molecular species, based on the sialic acid content, ranged from 2.0 to 3.8 Ci/mmol. When analyzed on HPTLC, they were at least 99% pure as determined by the Phosphor Imager. The purity of [^{3}H]GM1 was checked on HPTLC once a month. No degradation could be detected during one year of storage for most of the preparations used: lane 1 in Figure 2 shows HPTLC characteristics of [^{3}H]GM1(18:1/2) stored for this long at −20 °C. However, we did not determine the long-term storage stability of [^{3}H]-GM1(18:1/22:1) and [^{3}H]GM1(18:1/24:1) as they were used within three months since preparation.

Uptake of [^{3}H]GM1 Molecular Species by HL-60 Cells. As summarized in Table 1A,B, different GM1 molecular species are taken up by cells at a rate strongly dependent on the structure of their ceramide moieties: after 1 h incubation, the amount of GM1(20:1/8) taken up by cells was about 90-


A
m/z 1278.7 [M-H]⁻
Lyso-GM1(18:1)
m/2z 638.9
D
m/z 1306.8 [M-H]⁻
Lyso-GM1(20:1)
m/2z 653.0
B
m/z 1320.9 [M-H]⁻
GM1(18:1/2)
m/2z 660.0
E
m/z 1348.9 [M-H]⁻
GM1(20:1/2)
m/2z 674.1
C
m/z 1405.0 [M-H]⁻
GM1(18:1/8)
m/2z 702.1
F
m/z 1432.9 [M-H]⁻
GM1(20:1/8)
m/2z 716.1
Relative intensity, %
m/z
100
0
300
600
900
1200
1500

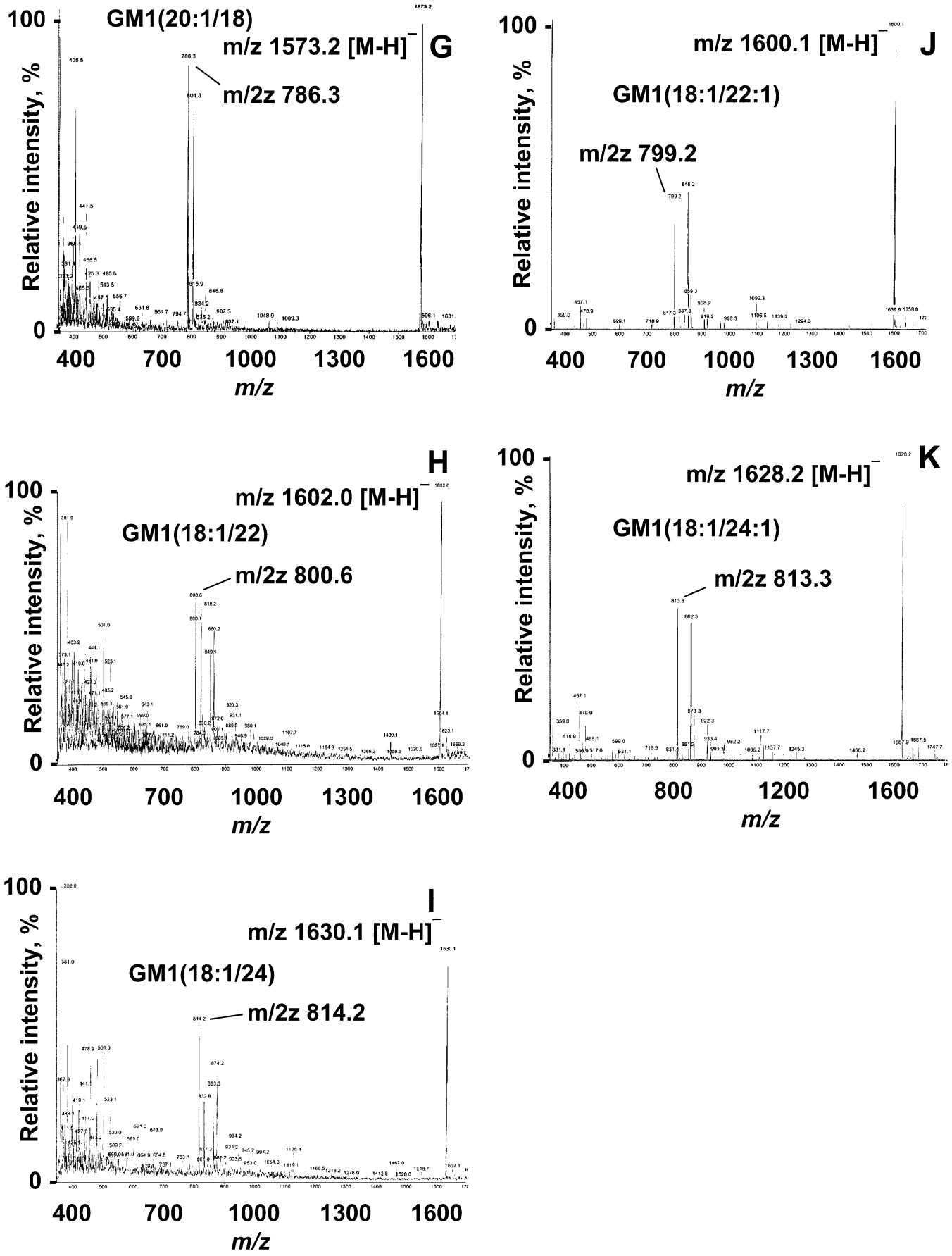


FIGURE 1: ESI-MS analysis of lyso-GM1s and some of GM1 molecular species used in this study. A, lyso-GM1(18:1); B, GM1(18:1/2); C, GM1(18:1/8)); D, lyso-GM1(20:1); E, GM1(20:1/2); F, GM1(20:1/8); G, GM1(20:1/18); H, GM1(18:1/22); I, GM1(18:1/24); J, GM1-(18:1/22:1); and K, GM1(18:1/24:1). The pseudomolecular ions at *m/z* and doubly charged ions at *m/2z* resulting from ionization of the carboxylic group of sialic acid are indicated.

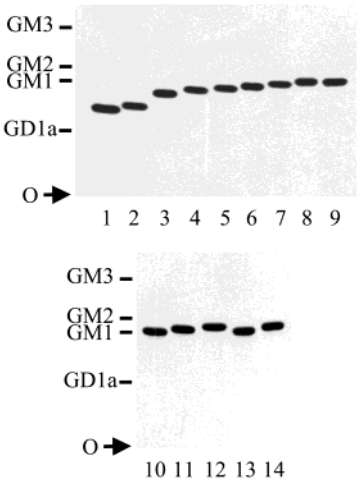


FIGURE 2: HPTLC analysis of different [^{3}H]GM1 molecular species. [^{3}H] labeled gangliosides were applied on two plates in lanes 1−14 as indicated: 1, [^{3}H]GM1(18:1/2); 2, [^{3}H]GM1(20:1/2); 3, [^{3}H]-GM1(18:1/8); 4, [^{3}H]GM1(20:1/8); 5, [^{3}H]GM1(18:1/12); 6, [^{3}H]-GM1(18:1/14); 7, [^{3}H]GM1(18:1/16); 8, [^{3}H]GM1(18:1/18); 9, [^{3}H]GM1(18:1/18:1); 10, [^{3}H]GM1(20:1/18); 11, [^{3}H]GM1(18:1/22); 12, [^{3}H]GM1(18:1/24); 13, [^{3}H]GM1(18:1/22:1); and 14, [^{3}H]-GM1(18:1/24:1). About 1×10^6 dpm of radioactive material was applied to each lane. Plates were developed in a solvent system of chloroform/methanol/0.25% aqueous $CaCl_2$ (60:35:8), dried, and sprayed with EN^{3}HANCE spray. The location of [^{3}H]GM1 molecular species was detected on X-ray film after a 2 h exposure at −80 °C. The mobility of ganglioside standards as well as the origin are indicated.

fold higher than that of GM1(18:1/24:1). The extent of the uptake depended not only on the length and saturation of the fatty acids but also on the structure of the sphingosine. However, the latter relation was a complex one involving also the structure of the fatty acids: during a 1 h incubation cells took up about eight times more of GM1(20:1/2) than GM1(18:1/2), while stearic acid containing GM1(20:1/18) was taken up less rapidly than GM1(18:1/18). All GM1s presented in Table 1A remained bound to the cells after incubation with 0.25% trypsin while some of them could be removed from the cells, in various proportions, during incubation with 1% BSA. The uptake of all GM1 molecular species presented in Table 1A resulted in the appearance of new [^{125}I]CT binding sites. After 1 h incubation, the molar ratio of [^{3}H]GM1 taken up by the cells to the amount of [^{125}I]CT bound ranged from 4.9:1 for GM1(18:1/16) to 5.4:1 for GM1(20:1/8). The GM1s acylated with longer than stearic fatty acids as well as GM1(20:1/18), combined in Table 1B, differed from the remaining ones in that they could be partially (up to 30%) released from the cells by trypsin and also, to a similar extent, by BSA. Importantly, the fraction of these GM1s remaining bound to the cells after initial incubation with BSA could not be further removed with trypsin. We assume that this fraction contained ganglioside molecules inserted into the membrane. This assumption is further strengthened by the stoichiometry of CT binding: after incubation with BSA, the ratio of [^{3}H]GM1 present in the cells to the bound [^{125}I]CT ranged from 5.6:1 for GM1-(18:1/24) to 6.2:1 for GM1(20:1/18).

DRMs Fraction. After sucrose gradient centrifugation, the DRMs fraction could be detected by measuring light scattering at 620 nm (not shown), and when a sufficient number of cells were used, it was visible as an opalescent band

Table 1: Effect of Ceramide Structure of GM1 Ganglioside on Its Uptake and Character of Association with HL-60 Cells

ceramide structure	[^{3}H]GM1 uptake (fmol/10^3 cells)	(A) [^{3}H]GM1 remaining bound to cells (% ± SD) after treatment with[a]	
		1% BSA	0.25% trypsin
18:1/2	5.6 ± 1.3	44.7 ± 12.7	94.3 ± 5.0
20:1/2	43.9 ± 10.2	21.0 ± 5.6	93.8 ± 5.8
18:1/8	50.5 ± 7.4	13.0 ± 4.1	94.0 ± 4.7
20:1/8	93.0 ± 11.5	10.1 ± 3.2	93.2 ± 4.1
18:1/12	64.6 ± 12.3	86.8 ± 4.8	93.5 ± 4.8
18:1/14	56.4 ± 7.0	96.8 ± 6.5	93.9 ± 5.2
18:1/16	6.4 ± 1.3	97.0 ± 3.2	94.0 ± 3.8
18:1/18	3.9 ± 0.6	98.9 ± 4.2	93.3 ± 5.4
18:1/18:1	9.9 ± 1.8	96.5 ± 4.9	93.9 ± 4.7

ceramide structure	[^{3}H]GM1 uptake (fmol/10^3 cells)	(B) [^{3}H]GM1 remaining bound to cells (% ± SD) after treatment with[b]		
		1% BSA	0.25% trypsin	
			without BSA	after BSA
20:1/18	1.9 ± 0.3	92.1 ± 5.0	90.9 ± 3.5	92.1 ± 4.9
18:1/22	1.2 ± 0.2	70.0 ± 12.5	67.8 ± 8.3	91.8 ± 4.0
18:1/24	1.5 ± 0.2	81.4 ± 11.8.	78.9 ± 9.7	92.8 ± 5.5
18:1/22:1	1.2 ± 0.1	73.5 ± 10.9	71.1 ± 8.8	92.0 ± 4.8
18:1/24:1	1.1 ± 0.1	70.0 ± 8.5	68.6 ± 8.3	93.1 ± 3.9

[a] Cells at a density of 3×10^6/mL were incubated for 1 h at 37 °C in medium H containing 0.5 μM [^{3}H]GM1s differing in the structure of their ceramides. Aliquots were withdrawn, washed with PBS, and used for the determination of radioactivity directly or after incubation for 15 min at 37 °C with 1% BSA or 5 min at 37 °C with 0.25% trypsin as specified under Materials and Methods. [b] Cells at a density of 3×10^6/mL were incubated for 1 h at 37 °C in medium H containing 0.5 μM [^{3}H]GM1s differing in the structure of their ceramides. Aliquots were withdrawn, washed with PBS, and used for the determination of radioactivity directly or after incubation for 15 min at 37 °C with 1% BSA or for 5 min at 37 °C with 0.25% trypsin. To determine the sensitivity to removal with trypsin of [^{3}H]GM1s remaining bound to cells after initial treatment with BSA, cells were treated with BSA first, washed once with PBS, and incubated for 5 min at 37 °C with 0.25% trypsin as specified under Materials and Methods.

floating above the 38% sucrose layer. Except for multistep sucrose gradient centrifugation, we included into the DRMs all the material recovered in fractions 2−4 (Figure 3). When multistep sucrose gradients were used, we considered as DRMs all fractions of a density lower than that of 30% sucrose (Figure 5). Unless indicated otherwise, for the preparation of the DRMs the cells were extracted with Triton X-100 in TNE pH 11.0 buffer.

Recovery of [^{3}H]GM1 in DRMs. Effect of Ceramide Structure. The structure of the ceramide moiety of different [^{3}H]GM1 molecular species strongly affected their recovery in the DRMs (Table 2). In the experiments where the extraction was performed with 1% Triton X-100, equivalent to the detergent: cell protein ratio of about 3:1, no more than 10% of [^{3}H]GM1 with ceramides containing fatty acids shorter than lauric could be detected in DRMs. Importantly, the fraction of these GM1s remaining bound to the cells after incubation with BSA, in experiments where this step was included, was also detected in DRMs in a similar low proportion as reported in Table 2 and increased to at least 76% after cross-linking with CTB.

For the remaining GM1 molecular species, acylation with fatty acids longer than myristic did not significantly increase their occurrence in the DRMs. Moreover, GM1(18:1/12) was significantly less abundant in DRMs only when compared

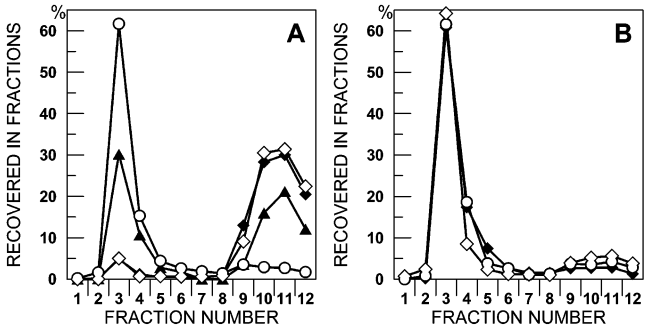


FIGURE 3: Distribution of different [^{3}H]GM1 molecular species and cholesterol in sucrose density gradient fractions. Cells were incubated for 1 h in medium H containing [^{3}H]GM1(18:1/2), open diamonds; [^{3}H]GM1(18:1/8), closed diamonds; or [^{3}H]GM1(18:1/18), open circles and washed with PBS-G. (A) About 6×10^7 cells were extracted with 1.7 mL of TNE buffer containing 1% Triton X-100 and subjected to sucrose gradient centrifugation (SW41 rotor, 36 000 rpm, 17 h). After centrifugation, gradient fractions, about 1 mL each, were collected from the top of the tube. Radioactivity of the fractions as well as cholesterol, closed triangles, and protein content (not shown) were determined. (B) Before extraction with Triton X-100 the cells were incubated with CTB, washed with PBS-G, and further treated as in panel A. Overlapping points for [^{3}H]-GM1(18:1/2) and [^{3}H]GM1(18:1/8) are shown as open diamonds.

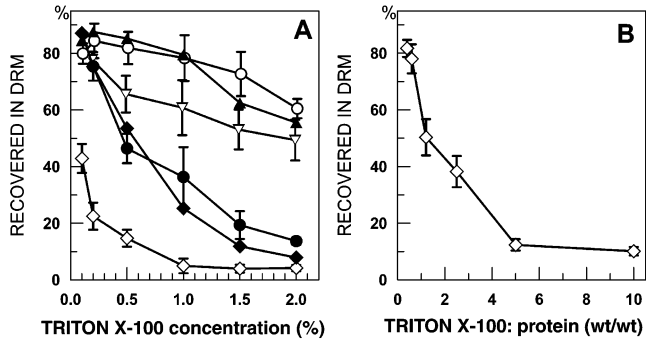


FIGURE 4: (A) The effect of ceramide structure of [^{3}H]GM1 molecular species on their recovery in DRMs prepared after extraction with different Triton X-100 concentrations. After incubation with [^{3}H]GM1(18:1/2), open diamonds; [^{3}H]GM1(18:1/12), open triangles; [^{3}H]GM1(18:1/18), open circles; [^{3}H]GM1(20:1/18), closed triangles; [^{3}H]GM1(18:1/18:1), closed circles; or [^{3}H]-GM1(18:1/24:1), closed diamonds, cells were washed with PBS and used for preparation of DRMs. In experiments with [^{3}H]GM1-(20:1/18) and [^{3}H]GM1(18:1/24:1), after incubation with [^{3}H] gangliosides, cells were treated for 15 min at 37 °C with 1% BSA in medium H, washed, and used for the preparation of DRMs. Routinely, about 6×10^7 cells were extracted with 1.7 mL of TNE pH 11.0 buffer containing the indicated concentration of Triton X-100. The DRMs were obtained after sucrose gradient centrifugation, and their radioactivity was determined by liquid scintillation counting. Values for GM1(20:1/18) and GM1(18:1/24:1) are shown without ±SD to avoid overlapping. (B) Effect of Triton X-100: cell protein ratio on the recovery of [^{3}H]GM1(18:1/18:1) in DRMs. After incubation with [^{3}H]GM1(18/18:1), a decreasing number of cells was extracted with 1.7 mL of 0.2% Triton X-100. DRMs were obtained after sucrose gradient centrifugation, and their radioactivity was determined by liquid scintillation counting.

with GM1(18:1/22), GM1(18:1/24), and GM1(20:1/18). Substitution of C20 sphingosine for C18 sphingosine did not significantly affect the recovery of the GM1s in DRMs. This observation was valid not only for GM1s acetylated or acylated with caprylic acid but also when comparing GM1-(20:1/18) with GM1(18:1/18).

The effect of unsaturation of the fatty acids was studied for GM1(18:1/18:1), GM1(18:1/22:1), and GM1(18:1/24:1).

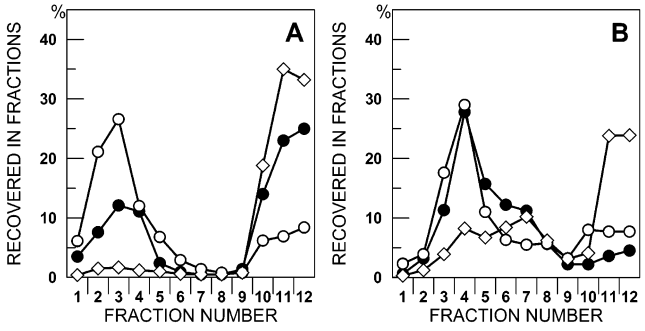


FIGURE 5: Effect of ceramide structure and Triton X-100 concentration on the recovery of GM1 molecular species in DRM fractions separated by sucrose multistep gradient centrifugation. After incubation with [^{3}H]GM1(18:1/2), open diamonds; [^{3}H]GM1(18:1/18:1), closed circles; or [^{3}H]GM1(18:1/18), open circles, cells were washed with PBS and used for preparation of DRMs. (A) Cells were extracted with TNE pH 11.0 buffer containing 1% Triton X-100. (B) Cells were extracted with TNE pH 11.0 buffer containing 0.1% Triton X-100. Two mL of cell homogenate in 35% sucrose were overlayered with 30−10% sucrose gradient, consisting of 10 1-mL steps. After centrifugation for 16−18 h at 36 000 rpm, 12 fractions of about 1 mL were collected from the top of the tube, and their radioactivity was determined by liquid scintillation counting. Similar distribution of radioactivity was detected in three independent experiments.

Table 2: Effects of Ceramide Structure, Extraction Conditions, and Treatment with CTB on the Recovery of Different [^{3}H]GM1 Ganglioside Molecular Species in DRMs Prepared from HL-60 Cells[a]

ceramide structure	TNE pH 7.5	TNE pH 11.0	TNE pH 11.0, CTB before extraction
18:1/2	8.8 ± 0.9	5.0 ± 2.6	74.3 ± 2.6
20:1/2	9.2 ± 2.4	8.2 ± 1.0	79.8 ± 3.0
18:1/8	5.2 ± 1.2	3.4 ± 1.0	74.9 ± 5.1
20:1/8	4.9 ± 1.1	4.8 ± 1.3	77.3 ± 7.2
18:1/12	72.9 ± 3.5	60.8 ± 9.7	80.2 ± 4.8
18:1/14	79.5 ± 6.9	72.9 ± 6.7	78.9 ± 9.7
18:1/16	78.6 ± 10.5	73.0 ± 4.9	80.6 ± 8.5
18:1/18	79.8 ± 2.1	78.3 ± 8.1	79.5 ± 2.2
20:1/18[b]	N. D.	79.5 ± 5.1	N. D.
18:1/22[b]	N. D.	81.8 ± 4.4	N. D.
18:1/24[b]	N. D.	79.3 ± 9.8	N. D.
18:1/18:1	22.7 ± 2.7	36.3 ± 10.6	76.6 ± 3.2
18:1/22:1[b]	N. D.	21.4 ± 2.5	83.1 ± 7.0
18:1/24:1[b]	N. D.	25.3 ± 2.7	86.4 ± 6.5

[a] After incubation with [^{3}H]GM1, cells were washed, and where indicated, [b]suspended in medium H containing 1% BSA and incubated for 15 min at 37 °C. Where shown, cells were incubated with CTB for 30 min at 4 °C. For the preparation of DRMs, cells were washed with PBS and extracted with 1% Triton X-100 in TNE buffer of pH 7.5 or 11.0. The ratio of Triton X-100 to the cell protein was maintained at about 3.0:1. The DRMs were collected after sucrose gradient centrifugation, and their radioactivity was determined. Values are means (±SD) from three independent experiments. N. D., not determined. Radioactivity in DRMs in relation to all gradient fractions, % ± SD.

Less than half of these GM1s was recovered in the DRMs as compared to their saturated acids containing analogues. Lengthening of the hydrocarbon chain of the unsaturated fatty acids in the GM1 molecules did not increase their occurrence in DRMs. The pH of the extraction buffer slightly affected the recovery of GM1s in DRMs; however, these differences were not statistically significant.

Effect of Extraction Conditions. Detergent Concentration and Detergent: Cell Protein Ratio. Lowering the Triton X-100 concentration in the TNE pH 11.0 buffer to 0.2%,

Table 3: Effect of Decreasing Triton X-100 Concentration on the Recovery of Different [^{3}H]GM1 Ganglioside Molecular Species, Cholesterol, and Protein in DRMs Prepared from HL-60 Cells[a,f]

ceramide structure	concentration of Triton X-100 used for extraction		
	0.2%	0.1%	0.05%
18:1/2	25.5 ± 4.8	42.9 ± 5.1	50.2 ± 3.9
20:1/2	35.8± 3.9	50.1 ± 9.3	53.9 ± 12.1
18:1/8	21.0 ± 4.2	45.3 ± 11.2	49.5 ± 7.2
20:1/8	22.0 ± 3.3	50.3 ± 2.5	55.1 ± 8.3
18:1/12	77.8 ± 2.8	N. D.	N. D.
18:1/14	77.5 ± 7.6	N. D.	N. D.
18:1/16	78.5 ± 6.4	N. D.	N. D.
18:1/18	84.4 ± 6.1	79.9 ± 3.6	61.7 ± 3.9
20:1/18[b]	87.6 ± 7.5	84.5 ± 8.2	N. D.
18:1/18:1	75.4 ± 5.0	N. D.	N. D.
18:1/22:1[b]	71.3 ± 2.8	82.2 ± 4.3	N. D.
18:1/24:1[b]	75.3 ± 4.6	85.5 ± 3.9	N. D.
cholesterol	73.1 ± 2.8[c]	76.1 ± 5.1[d]	74.7 ± 5.0[e]
protein	2.1 ± 0.5	5.1 ± 1.3	6.6 ± 0.8

[a] Cells were incubated for 1 h with different [^{3}H]GM1 ganglioside molecular species, and where indicated, [b]treated with 1% BSA in medium H for 15 min at 37 °C. The DRMs were obtained after extraction with 0.2, 0.1, or 0.05% Triton X-100 in TNE buffer at pH 11.0 followed by sucrose gradient centrifugation. Values are means (±SD) from three independent experiments. Cholesterol content in DRMs was [c]25.5 (±5.0) nmol/10^7 cells, [d]26.7 (±5.3) nmol/10^7 cells, and [e]21.2 (±2.6) nmol/10^7 cells. N. D., not determined. [f]Recovered in DRMs in relation to all gradient fractions, % ± SD.

Table 4: Effects of Extraction Conditions and Cross-Linking with CTB on the Recovery of Different [^{3}H]GM1 Ganglioside Molecular Species in DRMs Prepared from HL-60 Cells after Cholesterol Depletion with MβCD[a]

ceramide structure	Concentration of Triton X-100 used for extraction			
	1%	1% (CTB)	0.2%	0.1%
18:1/2	N. D.	83.0 ± 7.0	13.5 ± 1.3	29.9 ± 4.0
18:1/18	66.7 ± 7.1	82.3 ± 7.2	80.2 ± 4.2	79.2 ± 6.7
20:1/18[b]	72.1 ± 4.9	85.0 ± 8.3	N. D.	N. D.
18:1/22[b]	75.2 ± 6.9	N. D.	N. D.	N. D.
18:1/24[b]	75.6 ± 4.7	N. D.	N. D.	N. D.
18:1/18:1	12.7 ± 3.4	77.0 ± 2.6	57.8 ± 3.0	N. D.
18:1/22:1[b]	9.7 ± 3.3	80.7 ± 5.8	62.8 ± 3.4	N. D.
18:1/24:1[b]	14.3 ± 3.7	86.2 ± 4.7	66.0 ± 5.6	N. D.

[a] Cells were incubated for 1 h at 37 °C in medium H containing 5 mM MβCD and 0.1% BSA, washed twice with PBS-G, suspended in medium H, and incubated for 1 h with different [^{3}H]GM1 molecular species. Where indicated, [b]cells were treated with 1% BSA in medium H for 15 min at 37 °C. Where shown, cells were incubated at 4 °C for 30 min with CTB. For preparation of the DRMs, cells were washed and homogenized in the presence of indicated Triton X-100 concentration followed by sucrose gradient centrifugation. The DRMs were collected, and their radioactivity was determined. Values are means (±SD) from three independent experiments. N. D., not determined. Radioactivity in DRMs in relation to all gradient fractions, % ± SD.

equivalent to the detergent/protein ratio of about 0.6:1, increased the content of all [^{3}H]GM1 molecular species in DRMs (Table 3). However, the extent of this effect differed depending on the structure of the ceramides. Thus, under these conditions the recovery of GM1(18:1/8) in DRMs was almost 7-fold higher than obtained with 1% detergent, for GM1s containing unsaturated fatty acids it went up about 3-fold, but for GM1(18:1/18) and GM1(20:1/18) it increased by less than 10%, and this change was not statistically significant (Tables 2 and 3). Reduction of detergent concentration in TNE pH 11.0 buffer to 0.1% increased significantly the recovery in DRMs of GM1 molecular species with sphingosines acetylated or acylated with caprylic acid. Further lowering of the detergent concentration to 0.05% did not significantly increase the recovery of these GM1 molecular species, and in the case of GM1(18:1/18) less of it was found in the DRMs than under the conditions when more detergent was used. Decreasing the amount of Triton X-100 in the extraction buffer increased the recovery in DRMs not only of GM1 ganglioside but also of cholesterol, from 42.0 ± 3.8% for 1% Triton to 73.1 ± 2.8% for 0.2% Triton and to 76.1 ± 5.1% for 0.1% Triton extracted cells, corresponding to 14.6 (±2.1) nmol/10^7 cells, 25.5 (±5.0) nmol/10^7 cells, and 26.7 ± (5.3) nmol/10^7 cells, respectively. Also, the protein content in the DRMs increased, from 1.6 ± 0.5%, to 2.1 ± 0.5%, and to 5.1 ± 1.3% for 1, 0.2, and 0.1% detergent concentrations, respectively. This caused a change in the enrichment factors for GM1 molecular species calculated as a ratio of radioactivity (dpm) per protein (μg) in DRMs to radioactivity per protein in all gradient fractions. For instance, for GM1(18:1/18) this factor, amounting to about 48.9, was highest when the cells were extracted with 1% Triton X-100; for GM1(18:1/18:1) it was 35.9 for cells extracted with 0.2% Triton X-100, while for GM1 acetylated and acylated with caprylic acid it did not exceed 17.0 being highest for GM1(20:1/2) and cells extracted with 0.2% detergent.

The effect of Triton X-100 concentration on the recovery of GM1 ganglioside in DRMs prepared from a constant number of cells was studied for GM1(18:1/12), GM1(18:1/18), GM1(20:1/18), GM1(18:1/18:1), and GM1(18:1/24:1) (Figure 4A). Since it was most pronounced for the GM1s with unsaturated fatty acids, we used GM1(18:1/18:1) in an experiment where a variable number of cells was extracted with a constant Triton X-100 concentration of 0.2% (Figure 4B). In this way, it was shown directly that the ratio of the amount of the detergent to the number of cells affects the recovery of this GM1 molecular species in the DRM fraction.

Effect of Cholesterol Depletion. Extraction of cells with 5 mM MβCD removed over 70% of cell cholesterol (73% ± 6.0) lowering its content from 40.3 (±4.8) nmol/10^7 cells to 11.0 (±2.3) nmol/10^7 cells. Cholesterol content in DRMs prepared with 1% Triton X-100 decreased from 14.6 (±2.1) nmol/10^7 cells before MβCD to 4.7 (±0.9) nmol/10^7 cells after MβCD treatment. The effect of cholesterol depletion was studied for eight different GM1 molecular species (Table 4). The cholesterol depleted cells, after incubation with [^{3}H]-GM1s (followed, where indicated, by BSA treatment), were extracted with different concentrations of Triton X-100, equivalent to the detergent/cell protein ratio of 3:1, 0.6:1, and 0.3:1. The effect of cholesterol depletion was not the same for all GM1 molecular species. It significantly decreased the recovery in the DRMs of GM1(18:1/2) for cells extracted with 0.2 as well as 0.1% Triton X-100. Likewise, significantly less GM1(18:1/18:1), GM1(18:1/22:1), and GM1(18:1/24:1) was found in the DRMs obtained after extraction with 1 or 0.2% detergent. However, for GM1-(18:1/18), GM1(18:1/22), GM1(18:1/24), and GM1(20:1/18), not only with the cells extracted with the lower detergent concentrations but even when 1% Triton X-100 was used, the effect of cholesterol depletion was not statistically significant.

Effect of Cross-Linking with CTB. The effect of incubation with CTB on the recovery of different GM1 molecular

species in the DRMs depended on the structure of their ceramides (Tables 2 and 4). Thus, for GM1s containing myristic, palmitic, and stearic acid, incubation with CTB slightly increased their amount in the DRMs, but this effect was not statistically significant. For the remaining GM1 molecular species checked, the cross-linking caused very strong effects, the highest for those GM1s whose occurrence in DRMs was without CTB the lowest (Table 2 and Figure 3). For GM1(18:1/2), GM1(18:1/18:1), GM1(18:1/22:1), and GM1(18:1/24:1) this effect of CTB occurred irrespective of cholesterol depletion. Routinely, CTB was used at a concentration of 20 nM, but in the experiments when small amounts of the gangliosides were taken up by the cells, it was effective even at concentrations below 1 nM.

Fractionation of DRMs on Multistep Sucrose Density Gradient. The total amount of radioactivity estimated in DRMs (i.e., in fractions 1−9, all of sucrose concentration lower than 30%) did not significantly differ from the values reported in Tables 2 and 3. The distribution of radioactivity in different gradient fractions depended on the structure of the ceramide moiety of the GM1 ganglioside taken up by the cells and on the extraction conditions (Figure 5). When cells were extracted with 1% Triton X-100 at the detergent to cell protein ratio of 3:1 in experiments with GM1(18:1/18) and GM1(18:1/18:1), the peak of DRMs-associated radioactivity was detected in fraction 3 containing about 14.4% sucrose. Decreasing Triton X-100 concentration to 0.1% at the detergent to cell protein ratio of 0.3:1 resulted in a shift of this peak to fraction 4 containing about 16.6% sucrose. Moreover, under these conditions, in experiments with GM1(18:1/2) an additional peak in fraction 7, containing 23.4% sucrose, was detected. For GM1(18:1/18:1) the second peak was not separated, but the percentage of radioactivity in fractions 5−7 was significantly higher than for GM1(18:1/18) (Figure 5B). Not only the distribution of different GM1 ganglioside species but also the protein content in DRM fractions separated by multistep sucrose density gradient centrifugation depended on the amount of Triton X-100 used. Lowering the amount of this detergent resulted in a slight increase in the protein content in the lower buoyancy fractions 1−4 and also in the appearance of a second protein peak in fractions 5−8 (not shown).

DISCUSSION

The structural diversity of membrane lipids is an important factor determining the plasma membrane lateral heterogeneity (*50*). Glycosphingolipids, including gangliosides, are not only structurally diverse but also display a propensity to cluster, forming domains in cellular as well as artificial membranes (*12*, *51*). These domains, detectable in earlier studies because of their resistance to detergent extraction (*52*, *53*), can be isolated upon sucrose density gradient centrifugation in the DRM fraction (*43*). Since gangliosides are complex molecules, it was interesting to find out if their enrichment in different membrane domains would depend not only on their oligosaccharide moieties as already reported (*54*, *55*) but also on the structure of their ceramides. Toward this goal we prepared 14 [^{3}H]GM1 molecular species and studied their uptake and occurrence in DRM fraction in HL-60 cells. Exogenous gangliosides added to culture media bind to the cells not only through insertion into the membrane, thus mimicking endogenous glycosphingolipids, but also can adhere to cells as micelles. It is generally accepted that ganglioside molecules bound to cells with their ceramides inserted into the membrane cannot be released with trypsin (*40*, *41*). Since our objective was to study the behavior of ganglioside molecules intercalating the membrane, it was necessary to determine the extent of uptake and character of association with HL-60 cells of the [^{3}H]GM1s used in the present study.

In agreement with the earlier report of Fishman et al. (*56*), we show that different GM1 molecular species are taken up by cells at a rate dependent on the structure of their ceramides. As summarized in Table 1A,B, the ceramide structure affects the extent of the uptake as well as the character of association of the GM1s with cell membranes. Since gangliosides added to culture media occur in solution as micelles while they are inserted into the cell membranes as monomers (*40*, *41*), it can be expected that micelle stability should greatly affect ganglioside uptake. Moreover, as demonstrated in this report, the ceramide structure of a ganglioside inserted into the membrane affects its packing with other membrane constituents that, if not strong enough, could enable removal of the ganglioside during incubation with BSA. We have not attempted to determine the critical micelle concentrations (cmc) for different GM1s used in this report; however, the data presented in Table 1A,B seem to support such considerations: GM1(18:1/2) is taken up by cells less rapidly than GM1(20:1/2), but less of it can be removed with BSA. In keeping, GM1s acylated with C_{22}, C_{24}, $C_{22:1}$, or $C_{24:1}$ fatty acids are not only taken up by cells very slowly, but their micelles seem more stable or bound to the cells strongly enough to partly withstand washing with PBS. Consequently, when working with these GM1s as well as GM1(20:1/18), we routinely treated the cells with BSA. Obviously, the above reasoning would be correct only if the cmc values for GM1(18:1/2) and GM1(18:1/8) were lower than for their C20-sphingosine containing analogues, a situation at variance with reports on gangliosides acylated with naturally occurring fatty acids where the presence of C20 sphingosine lowers the cmc (*57*). Irrespective of this uncertainty, we assume that about 90% of the GM1 molecules in the cells used for the preparation of DRMs have their ceramides inserted into the membrane just as endogenous glycosphingolipids. This conclusion is supported not only by the observations that [^{3}H]GM1s could not be removed by trypsin but also by the stoichiometry of CT binding (i.e., the ratio of the number of ganglioside molecules present in the cells to the number of [^{125}I]CT molecules binding to them). These ratios determined in our studies were close to the theoretical value of 5:1 as established earlier for unmodified GM1 ganglioside (*58*), as well as for cholesterol based GM1 ganglioside analogue (*59*).

The structure of the ceramide in GM1 ganglioside not only affects its uptake from the medium by HL-60 cells, but also, which was the major subject of our studies, their occurrence in DRMs (Tables 2 and 3). As demonstrated, the length and saturation of the fatty acids of the GM1s determine their occurrence in DRMs (Table 2). Since all GM1 molecular species used in this study can form the same number of hydrogen bonds, the ability of the ceramide to pack with other membrane constituents, with the formation for instance of the lo phase (*20*), seems a decisive factor conferring to ganglioside molecules their relative insolubility during

detergent extraction. These observations do not contest the well-established role of hydrogen bonds in maintaining membrane stability (*60*), substantiated for glycosphingolipids by the observations of Schroeder et al. (*61*) that cerebrosides in phosphatidylcholine membranes have a stronger propensity than sphingomyelin to resist extraction with Triton X-100. Moreover, the ability of the oligosaccharide chain of glycosphingolipids to form numerous hydrogen bonds seems an important factor maintaining detergent insolubility of glycosphingolipid enriched membrane domains after cholesterol depletion.

It is becoming increasingly clear that DRMs isolated by density gradient centrifugation represent a heterogeneous fraction reflecting the presence of various membrane domains. This conclusion is based on the observations that extraction conditions such as the use of different detergents (*62*−*64*) or different ratio of cells to the amount of the detergent used for the extraction influenced DRMs protein content and composition (*65*). Also, gangliosides are not evenly distributed among membrane domains since lipid rafts with different ganglioside and protein content have been found in migrating T cells (*55*). Moreover, very importantly for our studies, glycosignaling domains containing most of GM3 ganglioside but little cholesterol have been detected in murine melanoma cells (*66*). As shown in our experiments, cholesterol depletion does not have a significant effect on the occurrence in DRMs of GM1s acylated with long, saturated fatty acids. Therefore, it seems likely that glycosignaling domains organized by glycosphingolipids and not critically dependent on cholesterol, as proposed by Iwabuchi et al. (*66*), could be of general occurrence and importance for ganglioside functions (*11*, *12*).

Since the recovery of various GM1s in DRMs was differently affected by cholesterol depletion as well as the amount of detergent used, it seemed possible that the structure of their ceramides could be important not only for the strength of the packing with other membrane constituents in the same domains but also for their partition among various membrane domains. We attempted to check this possibility with GM1(18:1/18), GM1(18:1/18:1), and GM1-(18:1/2). Decreasing the amount of Triton X-100 used for the extraction not only resulted in a higher recovery of GM1-(18:1/18:1) and particularly GM1(18:1/2) in the first DRM fractions of lower buoyant density but permitted the detection of denser DRM fractions (Figure 5). Interestingly, even though the plasma membrane is a fluid structure, the GM1 ganglioside molecular species used in our studies demonstrated a characteristic distribution, based on their ceramides, between these two types of fractions. As a working hypothesis, we assume that these DRM fractions result from the presence of different membrane domains.

It was originally described by Fishman et al. that acetylated GM1 ganglioside is a very efficient receptor for CT (*56*), and more recently it was shown that caveolae- and caveolae-like domains are involved in CT endocytosis leading to adenylate cyclase activation (*67*). However, as reported here, only a small fraction of acetylated GM1 molecular species could be recovered in the DRMs after extraction with 1% Triton X-100. This apparent discrepancy is resolved by the experiments demonstrating that after cross-linking with CTB, all GM1 molecular species are found in DRMs in a similar, high proportion. An increased occurrence of GM1 ganglioside in caveolae after cross-linking with CTB was reported by Parton (*68*), also indicating that CTB should be used with caution since as a pentavalent ligand it affects membrane domain location of its receptors. In agreement with our observations, the occurrence in DRMs of GM1 cross-linked with CT even after cholesterol depletion of human intestinal T 84 cells was reported by Wolf et al. (*69*). We demonstrate that in HL-60 cells this effect does not depend on the ceramide structure of the GM1 molecular species used. It is difficult to asses if the partitioning of cross-linked GM1 molecular species into DRMs is a mechanism common to all lipids. Recently, it was demonstrated that the binding of anti-fluorescein antibodies to fluorescein conjugated phospholipid analogues in membrane monolayers resulted in their partitioning into lipid rafts. This effect, however, was not detected for lipids with unsaturated fatty acids (*31*), while in our experiments, after incubation with CTB, GM1(18:1/18:1), GM1(18:1/22:1), and GM1(18:1/24:1) were found in DRM in a similar proportion as GM1(18:1/18).

Even though our understanding of DRMs is incomplete, it is clear that their characteristics can vary depending on the properties of cells as well as the type and amount of detergents used. For instance, in differentiating neurons lower proportion of radioiodinated CT was associated with DRMs than could be expected from our observations (*70*). Moreover, again at variance with our results, cholesterol depletion decreased the recovery of the toxin in the detergent insoluble cell fraction (*70*). The basis of these discrepancies is at present unclear; however, it should be considered that neurons contain much higher amounts of gangliosides than HL-60 cells (*71*). Thus, the possible relation between the amount of gangliosides in the membrane and the dependence on cholesterol of glycosphingolipid enriched domains stability awaits further investigations.

The experiments described in this report represent the first systematic study aimed at defining the importance of the ceramide moiety of GM1 ganglioside for its association with different membrane domains. Our observations based on density gradient centrifugation should be strengthened by other techniques like, for instance, atomic force microscopy. We are also aware that statistical analysis, based on results obtained from three independent experiments, can only distinguish gross differences.

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REFERENCES

1. Klenk, E. (1942) *Hoppe-Seyler's Zeit. Physiol. Chem. 273*, 76−86.
2. Hakomori, S. (1981) *Annu. Rev. Biochem. 50*, 733−764.
3. Wiegandt, H. (1985) in *New Comprehensive Biochemistry* (Wiegandt, H., Ed.) Vol. 10, pp 199−260, Elsevier, Amsterdam.
4. Hakomori, S. (1998) *Acta Anat. 161*, 79−90.
5. Hakomori, S., and Igarashi, Y. (1995) *Behav. Brain Res. 66*, 99−104.
6. Huwiler, A., Kolter, T., Pfeilschifter, J., and Sandhoff, K. (2000) *Biochim. Biophys. Acta 1485*, 63−99.
7. Hakomori, S. (2000) *Glycoconjugate J. 17*, 143−151.
8. Bhunia, A. K., Schwarzmann, G., and Chatterjee, S. (2002) *J. Biol. Chem. 277*, 16396−16402.

9. Ono, M., Handa, K., Sonnino, S., Withers, D. A., Nagai, H., and Hakomori, S. (2001) *Biochemistry 40*, 6414−6421.
10. Vyas, A., Patel, H. V., Fromholt, S. E., Heffer-Lauc, M., Vyas, K. A., Dang, J., Schachner, M., and Schnaar, R. L. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 8412−8417.
11. Hakomori, S., Handa, K., Iwabuchi, K., Yamamura, S., and Prinetti, A. (1998) *Glycobiology 8*, xi−xix.
12. Hakomori, S. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 225−232.
13. Miljan, E. A., Meuillet, E. J., Mania-Farnell, B., George, D., Yamamoto, H., Simon, H.-G., and Bremer, E. G. (2002) *J. Biol. Chem. 277*, 10108−10113.
14. Spiegel, S., Kassis, S., Wilchek, M., and Fishman, P. H. (1984) *J. Cell Biol. 99*, 1575−1581.
15. Kasahara, K., and Sanai, Y. (1999) *Biophys. Chem. 82*, 121−127.
16. Simons, K., and van Meer, G. (1988) *Biochemistry 27*, 6197−66202.
17. Simons, K., and Ikonen, E. (1997) *Nature 387*, 569−572.
18. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol. 14*, 111−136.
19. Hooper, N. M. (1999) *Mol. Membr. Biol. 16*, 145−156.
20. Brown, D. A., and London, E. (2000) *J. Biol. Chem. 275*, 17221−17224.
21. Simons, K., and Toomre, D. (2000) *Nature Rev. Mol. Cell Biol. 1*, 31−39.
22. Galbiati, F., Razani, B., and Lisanti, M. P. (2001) *Cell 106*, 403−411.
23. Brown, D. A. (2001) *Int. J. Med. Microbiol. 291*, 433−437.
24. Maxfield, F. R. (2002) *Curr. Opin. Cell Biol. 14*, 483−487.
25. Ostermeyer, A. G., Beckrich, B. T, Ivarson, K. A., Grove, K. E., and Brown, D. A. (1999) *J. Biol. Chem. 274*, 34459−34466.
26. Kasahara, K., Watanabe, Y., Takeuchi, K., Kaneko, H., Oohira, A., Yamamoto, T., and Sanai, Y. (2000) *J. Biol. Chem. 275*, 34701−34709.
27. Inokuchi, J., Uemura, S., Kabayama, K., and Igarashi, Y. (2000) *Glycoconjugate J. 17*, 239−245.
28. Simons, M., Friedrichson, T., Schultz, J. B, Pitto, M., Masserini, M., and Kurzhalia, T. (1999) *Mol. Biol. Cell 10*, 3187−3193.
29. Dietrich, C., Volovyk, Z. N., Thompson, N., and Jakobson, K. (2001) *Proc. Natl. Acad. Sci. U.S.A 98*, 10642−10647.
30. Mitsuda, T., Furukawa, K., Fukumoto, S., Miyazaki, H., Urano, T., and Furukawa, K. (2002) *J. Biol. Chem. 277*, 11239−1246.
31. Vainio, S., Heino, S., Mansson, J.-E., Fredman, P., Kuismanen, E., Vaarala, O., and Ikonen, E. (2002) *EMBO Rep. 3*, 95−100.
32. Brown, D. A., and London, E. (1998) *J. Membr. Biol. 164*, 103−114.
33. Nakamura, M., Tsunoda, A., Furukawa, Y., Sakai, T., and Saito, M. (1997) *FEBS Lett. 400*, 350−354.
34. Stroud, M. R., Handa, K., Salyan, M. E., Ito, K., Levery, S. B., Hakomori, S., Reinhold, B. B., and Reinhold, W. N. (1996) *Biochemistry 35*, 758−769.
35. Pacuszka, T., Duffard, R. O., Nishimura, R. N., Brady, R. O., and Fishman, P. H. (1978) *J. Biol. Chem. 253*, 5839−5846.
36. Gazotti, G., Sonnino, S., Ghidoni, R., and Tettamanti, G. (1984) *J. Neurosci. Res. 12*, 179−192.
37. Schnaar, R. L. (1994) *Methods Enzymol. 230*, 348−370.
38. Schwarzmann, G., and Sandhoff, K. (1987) *Methods Enzymol. 138*, 319−341.
39. Sonnino, S., Chigorno, V., and Tettamanti, G. (2000) *Methods Enzymol. 311*, 639−656.
40. Saqr, H. E., Pearl, D. K., and Yates, A. J. (1993) *J. Neurochem. 61*, 395−411.
41. Schwarzmann, G. (2001) *Semin. Cell Dev. Biol. 12*, 163−171.
42. Pacuszka, T., Panasiewicz, M., Hoser, G., and Kawalec, M. (1998) *Acta Biochim. Polon. 45*, 403−415.
43. Brown, D. A., and Rose, J. K. (1992) *Cell 68*, 533−544.
44. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
45. Hunter, W. M., and Greenwood, F. C. (1962) *Nature 194*, 495−496.
46. Miller-Podraza, H., Bradley, R. M., and Fishman, P. H. (1982) *Biochemistry 21*, 3260−3265.
47. Riboni, L., Acquotti, D., Casellato, R., Ghidoni, R., Montagnolo, G., Benevento, A., and Sonnino, S. (1992) *Eur. J. Biochem. 203*, 107−113.
48. Svennerholm, L., and Fredman, P. (1980) *Biochim. Biophys. Acta 617*, 97−109.
49. Skoza, L., and Mohos, S. (1976) *Biochem. J. 159*, 457−462.
50. Rietveld, A., and Simons, K. (1998) *Biochim. Biophys. Acta 1376*, 467−479.
51. Yuan, C., Furlong, J., Burgos, P., and Johnson, L. J. (2002) *Biophys. J. 82*, 2526−2535.
52. Hagman, J., and Fishman, P. H. (1982) *Biochim. Biophys. Acta*, *720*, 181−187.
53. Okada, Y., Mugnai, G., Bremer, E. G., and Hakomori, S. (1984) *Exp. Cell Res. 155*, 448−456.
54. Vyas, K. A., Patel, H. V., Vyas, A. A., and Schnaar, R. L. (2001) *Biol. Chem. 382*, 241−250.
55. Gomez-Mouton, C., Abad, J. L., Mira, E., Lacalle, R. A., Gallardo, E., Jimenez-Baranda, S., Illa, I., Bernad, A., Manes, S., and Martinez-A, C. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 9642−9647.
56. Fishman, P. H., Pacuszka, T., Hom, B., and Moss, J. (1980) *J. Biol. Chem. 255*, 7657−7664.
57. Fishman, P. H., Moss, J., and Osborne, J. C., Jr. (1978) *Biochemistry 17*, 711−716.
58. Pacuszka, T., and Fishman, P. H. (1991) *Biochim. Biophys. Acta 1083*, 153−160.
59. Patra, S. K., Alonso, A., and Goni, F. M. (1998) *Biochim. Biophys. Acta 1373*, 112−118.
60. Schroeder, R., London, E., and Brown, D. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12130−12134.
61. Roper, K., Corbeil, D., and Huttner, W. B. (2000) *Nature Cell Biol. 2*, 582−592.
62. Madore, N., Smith, K. L, Graham, C. H., Jen, A., Brady, K., Hall, S., and Morris, R. (1999) *EMBO J. 18*, 6917−6926.
63. Taylor, C., Coetzee, T., and Pfeiffer, S. E. (2002) *J. Neurochem. 81*, 993−1004.
64. Milan, J., Puertollano, R., Fan, L., and Alonso, M. A. (1997) *Biochem. Biophys. Res. Commun. 233*, 707−712.
65. Iwabuchi, K., Handa, K., and Hakomori, S. (1998) *J. Biol. Chem. 273*, 33766−33773.
66. Orlandi, P., and Fishman, P. H. (1998) *J. Cell Biol. 141*, 905−915.
67. Parton, R. G. (1994) *J. Histol. Cytol. 42*, 155−166.
68. Wolf, A. A., Fujinaga, Y., and Lencer, W. I. (2002) *J. Biol. Chem. 277*, 16249−16256.
69. Shogomori, H., and Futerman, A. H. (2001) *J. Biol. Chem. 276*, 9182−9188.
70. Yu, R. K., and Saito, M. (1989) in *Neurobiology of Glycoconjugates* (Margolis, R. U., and Margolis, R. K., Eds.) pp 1−42, Plenum Press, New York.
71. Palestini, P., Alietta, M., Sonnino, S., Tettamanti, G., Thompson, T. E., and Tillack, T. W. (1995) *Biochim. Biophys. Acta 1235*, 221−230.

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