Monosialogangliosides of Human Myelogenous Leukemia HL60 Cells and Normal Human Leukocytes. 1. Separation of E-Selectin Binding from Nonbinding Gangliosides, and Absence of Sialosyl-Le^x Having Tetraosyl to Octaosyl Core[†]

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ABSTRACT: Previous studies suggested that sialosyl-Le^x (SLe^x) is a ligand expressed in human neutrophils and myelogenous leukemia HL60 cells which binds to E-selectin and possibly P-selectin. However, clear data on structures of carbohydrate epitopes in these cells were lacking. A systematic study was therefore initiated, employing a large quantity of HL60 cells (≥1200 mL packed) and human leukocytes (≈100 mL packed). Gangliosides were extracted, followed by extensive fractionation and examination of the E- and P-selectin binding ability of each fraction. The following results were of particular interest: (i) Only monosialogangliosides having a polylactosamine core with >10 monosaccharide units (or >4 *N*-acetyllactosamine units) showed E-selectin binding under static conditions with thin-layer chromatography overlay technique employing ^{32}P-labeled E-selectin-expressing CHO cells. (ii) Sulfate groups were not detectable in the binding fractions, and di- and trisialoganglioside fractions did not show E-selectin binding under these conditions. (iii) None of the fractions showed P-selectin binding under a similar assay system using ^{32}P-labeled P-selectin-expressing CHO cells. (iv) Major gangliosides of HL60 cells were structures **I**−**XI** (shown in Table 1 of text), none of which showed E-selectin binding under the above conditions. (v) SLe^x gangliosides having tetraosyl to octaosyl ceramide core, which are the major gangliosides of epithelial tumors (shown in Table 2), were completely absent from HL60 cells and neutrophils. Isolation and chemical characterization of ganglioside structures **I**−**XI** are described in this paper.

Recruitment of leukocytes to inflammatory lesions following infection or wounding is mediated through endothelial expression of P- or E-selectin, whose N-terminal domain is generally believed to recognize some yet-unidentified carbohydrate epitope. Based on antibody reactivity of neutrophils, sialosyl-Le^x (SLe^x)[1] is thought to be expressed on normal neutrophils in the form of O-linked, N-linked, or lipid-linked carbohydrate chains, and to be the epitope recognized by selectins (see below and Discussion).

Although many selectin epitope studies have been published during the past five years, they were mostly based on inhibition by or adherence to some suspected structure (see Discussion). There has been almost no unambiguous characterization of the real carbohydrate target structure of selectins present in normal human neutrophils or monocytic leukemia HL60 cells, because of the extreme difficulty of isolating and characterizing the essential epitope expressed in these cells. Tiemeyer et al. (1991) claimed that GSLs with VIM-2 epitope structure (Macher et al., 1988) (same as Str **VII** in Table 1) are the E-selectin binding site. GSL with the same structure was previously isolated from a large quantity of myelogenous leukemia cells (Fukuda et al., 1984). However, Lowe et al. (1991) failed to observe E-selectin-dependent adhesion of VIM-2-positive, SLe^x-negative CHO cells and were therefore unable to confirm this role of VIM-2. "Carbohydrate library" patterns of HL60, U937, RAMOS, and GOS7 cells were compared in another study (Patel et al., 1994). Approximately 3% of total radiolabeled oligosaccharides released from plasma membrane glycoproteins of U937 cells bound to E-selectin column. The bound structure was suggested to contain sialosyl-dimeric Le^x based on enzymatic and chemical degradation patterns and matrix-

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[1] Abbreviations: amu atomic mass units; Cer, ceramide; CID, collision-induced dissociation; C/M, chloroform/methanol; EC, endothelial cell; ES-MS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fr fraction(s); GSL, glycosphingolipid; Hex, hexose; HexNAc, *N*-acetylhexosamine; IHW, isopropyl alcohol/hexane/water; LacNAc or LacN, *N*-acetyllactosamine (Galβ1→4GlcNAc); mAb, monoclonal antibody; NeuAc or Neu5Ac, *N*-acetylneuraminic acid; SLe^x, sialosyl-Le^x; PLA, polyactosamine; Sph, sphingosine; Str, structure(s); TLC, thin-layer chromatography. Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Lipids 12,* 455−463, 1977); however, the suffix -OseCer is shortened to -Cer.

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assisted laser resorption mass spectrometry, but no unambiguous structural data were presented.

Using large quantities of HL60 cells (in total ≥1200 mL packed) and human neutrophils (≈100 mL packed), we systematically investigated the structures binding to E-selectin expressed on CHO cells. Major gangliosides present on HL60 cells were characterized (this paper), and properties of E-selectin nonbinding gangliosides were compared to those of E-selectin binding gangliosides (accompanying paper: Stroud et al., 1996).

MATERIALS AND METHODS

Cells and Binding Assay. HL60 cells were obtained originally from American Type Culture Collection (ATCC) and grown in RPMI supplemented with 10% FCS. Cells were maintained in 5% CO_2 at 37 °C, expanded for two cycles in roller bottles to collect large amounts of cells, and harvested by centrifugation. HL60 cells cultured in this manner showed a level of E-selectin binding activity similar to that of cells cultured continuously in a CO_2 incubator; *i.e*., large-scale culture in roller bottles in this way did not cause significant loss of E-selectin binding activity. Altogether, 1200 mL of packed HL60 cells were divided into ≈400 mL packed aliquots, each of which was extracted as described in the following section. Normal (nonleukemic) human leukocytes (mostly neutrophils) were kindly provided by Mr. Masakazu Adachi (Japan Immunoresearch Laboratories, Takasaki City, Japan), who collected them using an *ex vivo* circulatory system with a specific adhesion column. Frozen neutrophils were subjected to the same extraction procedure used for HL60 cells (see below).

CHO cell transfectants with E- and P-selectin cDNA were established as described previously (Handa et al., 1995). Briefly, E-selectin cDNA in pCDM-8 was obtained from R and D Systems, Minneapolis MN. P-selectin cDNA was cloned from HEL cells (ATCC) based on the published sequence (Johnston et al., 1989) and ligated in pRC/CMV (In Vitrogen, San Diego CA). CHO DG44 cells, kindly donated by Dr. L. A. Chasin (Columbia University, New York), were cotransfected with E-selectin/pCDM-8 or P-selectin/pRC/CMV with pSV2/dhfr (ATCC) as described previously (Ito et al., 1994). The transfected genes were purified by stepwise selection for resistance to increasing concentrations of methotrexate (up to 3 and 5 μM for P- and E-selectin expressors, respectively) (Kaufman, 1991). P- and E-selectin-expressing clones were isolated by cytofluorometry using anti-P-selectin mAb P1A and anti-E-selectin mAb E1A. These mAbs were established through immunization of BALB/c mice with NS-1 cells expressing P- or E-selectin (Handa K, unpublished).

Preparation of Ganglioside Fractions. (A) Glycolipid Extraction. Approximately 100 mL of packed human neutrophils or 400 mL of packed HL60 cells was extracted by homogenization in a Waring blender with 10 volumes of the lower phase of IHW (55:25:20). The extract was filtered through a Whatman No. 1 filter and the residue re-extracted as above. The extraction/filtration procedure was repeated once more, and the combined filtrates were concentrated under reduced pressure at 40 °C using a Brinkmann rotary evaporator. The concentrated extract was subjected to Folch partitioning by dissolving the residue in 3 L of C/M (2:1) containing 500 mL of water. After vigorous shaking, the extract was allowed to separate until two translucent phases appeared (≈8 h). The upper phase was removed and the lower phase re-extracted by the addition of C/M/1% KCl (1:10:10) to the original volume. The liquid-extraction procedure was repeated two times, and the combined upper phases were concentrated by rotary evaporation, reconstituted in water, and dialyzed exhaustively against deionized water using Spectropor 3 dialysis tubing (MW cutoff = 3500).

(B) Anion-Exchange Chromatography. After dialysis, the upper-phase extract was evaporated to dryness as above and dissolved in 50 mL of C/M/water (30:60:8) by a combination of warming (37 °C) and sonication. Insoluble material was removed by centrifugation at 1000*g* for 10 min and re-extracted by sonication in an additional 50 mL of the same solvent. Following centrifugation as above, the combined supernatants were loaded onto a DEAE-Sephadex column (300 mL bed volume; acetate form) and washed with 2 L of C/M/water (30:60:8) to remove all neutral lipids. The column was equilibrated with 500 mL of methanol and the monosialoganglioside fraction eluted with 2 L of 0.05 M NH_4OAc in methanol. Subsequent removal of di-, tri-, and polysialosylgangliosides was achieved by eluting batchwise with 0.15, 0.45, and 1.0 M NH_4OAc, respectively. The eluted ganglioside fractions were dried by rotary evaporation, dialyzed against water, and dried as above.

Purification of Monosialogangliosides from HL60 Cells. (A) High Performance Liquid Chromatography. The monosialoganglioside fraction was solubilized in 10 mL of IHW and transferred from the evporation flask to a 15 mL tube. The sample was completely dried under N_2 using an N-EVAP (Organomation Inc.) and reconstituted in 2 mL of IHW by sonication. The sample was injected onto a preparative Iatrobead column (6RS-8010; 0.8 × 60 cm; Iatron Laboratories Inc., Kanda/Tokyo, Japan) pre-equilibrated with IHW (55:40:5) and subjected to a linear gradient from IHW 55:40:5 to 55:25:20 with a flow rate of 1 mL/min. Four milliliter fractions were collected over 400 min. Each fraction was spotted onto an HPTLC plate, developed in an appropriate solvent system (described below), visualized by spraying with 0.5% orcinol in 2 N sulfuric acid, and pooled according to migration. Pooled fractions containing more than one band by TLC were dried under N_2, resolubilized in 1 mL of IHW, and injected onto a semipreparative Iatrobead column (0.4 × 60 cm). A linear gradient from IHW 55:40:5 to 55:25:20 over 200 min with a flow rate of 0.5 mL/min was used. One millimeter fractions were collected and pooled according to HPTLC migration. Fractions containing a single band by HPTLC were labeled according to order of migration in C/M/0.5% $CaCl_2$ (50:55:19); *i.e*., the fastest migrating band was labeled #1 and the slowest #20 (see Figure 1A). Fractions containing multiple bands were further purified by preparative HPTLC (described below).

(B) High Performance TLC. Monosialoganglioside fractions that were not resolved into single bands by HPLC were separated by preparative HPTLC. Fractions within bands 1−7 were resolved in a solvent system of C/M/0.5% $CaCl_2$ (50:40:10). Fractions within bands 8−14 were resolved in C/M/0.5% $CaCl_2$ (50:55:19). Fr 12 and 13 were further resolved (into Fr 12-1 through 12-5 and 13-1 through 13-3, respectively) using a solvent system of 1-propanol/water/concentrated NH_4OH (6:3.2:1). Separation of Fr 12 into components is shown, as an example, in Figure 1, right panel. Preparative TLC was performed by streaking ≈50 μL of sample across a 10 × 20 cm HPTLC silica gel plate (silica gel 60; EM Science, Gibbstown, NJ), drying, and developing

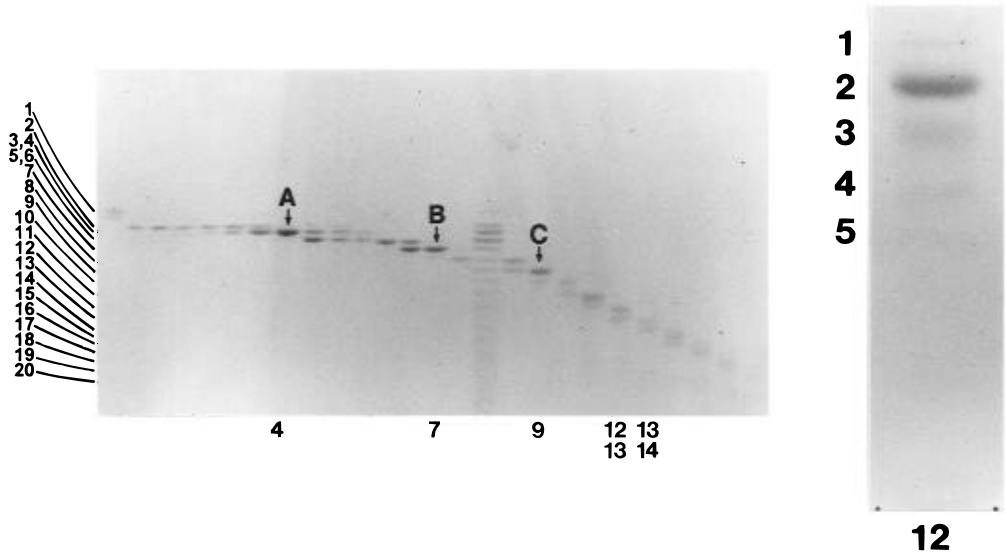


FIGURE 1: HPTLC profile of HL60 cell monosialoganglioside fraction separated by HPLC. Left panel: The monosialoganglioside fraction was prepared from 400 mL of packed HL60 cells as described in the text. The fraction was dissolved in 2 mL of IHW 55:25:20 v/v, sonicated, and injected onto an Iatrobead column (6RS-8010, 0.8 × 60 cm) pre-equilibrated with IHW 55:40:5. Gradient elution from this solvent to IHW 55:25:20 was performed over 400 min at a flow rate of 1 mL/min. Four milliliter fractions were collected, and a 5 μL sample from each fraction was spotted on an HPTLC silica gel plate (EM Science, Gibbstown, NJ). HPTLC was developed with C/M/0.5% $CaCl_2$ (50:55:19), and bands were revealed by orcinol−sulfuric acid reaction. Fraction numbers on the left side of the figure are described in the text and correspond to those in Table 1. A, B, and C represent TLC migration positions of (respectively) three types of SLe^x GSL, *i.e.*, structures **XII**, **XIII**, and **XIV** in Table 2. The real structures present in the bands corresponding to A, B, and C were identified as Str **III**, **IV**, and **VII**−**VIII** in Table 1. Right panel: Example of separation of an apparently homogeneous ganglioside fraction (Fr 12) into components by HPTLC using 1-propanol/water/concentrated NH_4OH (6:3.2:1). Fr 12 was resolved into five bands as shown. Major band 12-2 was identified as having the same structure as Str **X** (Table 1), but with Cer having a shorter fatty acid. Band 12-3 was E-selectin binding and was analyzed as described in the accompanying paper (Stroud et al., 1996).

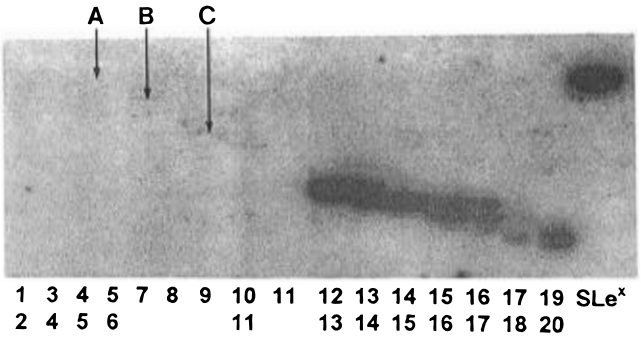


FIGURE 2: TLC of polar monosialoganglioside fractions of HL60 cells separated by HPLC in IHW solvent system as described in the text. Bands were revealed by TLC blotting with E-selectin-expressing CHO cells metabolically labeled with ^{32}P (Swank-Hill et al., 1987). Lanes contain fractions as shown in Figure 1, left panel. The right-hand lane is SLe^x Cer hexasaccharide. A, B, and C as in Figure 1. All E-selectin binding fractions were slow-migrating GSLs containing long-chain PLAs. No band eluted corresponding to a SLe^x-containing GSL (see above), although these are found abundantly in eluates from human carcinoma tissues (Fukushi et al., 1984; Fukushima et al., 1984). Major reactivity with E-selectin was observed in very polar fractions, beginning with fraction 12.

in the appropriate solvent system. Plates were dried, and bands were visualized by spraying with 0.03% primulin in 80% acetone. Bands were marked with a pencil under UV light. Marked bands were scraped from the plate using a razor blade, and gangliosides were extracted from the silica by sonicating for 20 min in IHW (55:25:20; 2 mL per band). The silica was removed by centrifuging at 1000*g* for 10 min and re-extracted as above, and the combined supernatants were dried under N_2. Samples were cleaned up using 1 cm^3 C-18 Sep-Pak cartridges (Waters, Milford, MA) by first dissolving the sample in 1 mL of PBS and then applying it to a column re-equilibrated with PBS after sequentially washing with 5 mL of methanol and 5 mL of water. Once the sample was retained, the column was washed with 10 mL of water, followed by 10 mL of 50% methanol, and eluted in 10 mL of 100% methanol. The sample was dried under N_2, dissolved in 1 mL of IHW (55:25:20), and injected onto an Iatrobead column (0.4 × 60 cm) as above using a linear gradient from IHW 55:40:5 to 55:25:20 for 100 min at a flow rate of 1 mL/min. One milliliter fractions were collected and visualized by HPTLC using the orcinol−sulfuric acid reaction. Orcinol-positive fractions were pooled and dried under N_2 prior to structural analysis.

E- or P-Selectin Binding Assay. Metabolically ^{32}P-labeled CHO cells expressing E- or P-selectin were prepared as described in the preceding section and used for binding assays. Briefly, selectin binding to GSLs separated by HPTLC was assayed by binding of ^{32}P-labeled CHO cells as above using a specific chamber as described previously (Swank-Hill et al., 1987) (see Figure 2 legend). GSL fractions separated by HPLC as described later were analyzed by HPTLC developed in various polar solvents (see Figures 1 and 2 legends).

Functional Analysis for Sulfate. The cationic dye Azure A was used to check for the presence of sulfate groups as described previously (Iida et al., 1989; Schnaar & Needham, 1994). Sodium chlorate, which blocks the transfer of sulfate from PAPS to an appropriate acceptor substrate (Keller et al., 1989; Guimond et al., 1993), was used to ascertain the role of sulfate in HL60 cell adhesion to E-selectin.

^{1}H-NMR Spectroscopy. Samples were deuterium-exchanged by repeated lyophilization from D_2O (99.96 atom %; Cambridge Isotope Laboratories, Woburn, MA) and then dissolved in 0.4 mL of DMSO-d_6 (99.96 atom %; Aldrich, Milwaukee, WI) containing 2% D_2O (Dabrowski et al., 1980)

and 0.1% tetramethylsilane as chemical shift reference. 500 MHz ^{1}H-NMR spectra were acquired at 308 and 328 ± 2 K with a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer and pulse programmer, operating in the Fourier transform mode with quadrature detection, a spectral width of 5000 Hz over 16K data points, and a 2 s relaxation delay (Levery et al., 1986).

Methylation. Two procedures were used, the first as described by Hellerqvist (1990), which is a modification of an earlier method employing sodium hydride in dimethyl sulfoxide (DMSO) and methyl iodide (Hakomori, 1964). Briefly, vacuum-desiccated GSL samples were dissolved in 300 μL of DMSO. An equal volume of methyl sulfinylcarbanium (MSC) reagent was added and allowed to stand at room temperature overnight. It is essential to react with the MSC reagent overnight in order to achieve complete methylation of PLA lipids. Methyl iodide (volume equal to MSC reagent) was then added and sonicated in a cold room (4 °C) until the solution became clear. If it was not clear after 60 min, half the previous volume of DMSO was added. Countercurrent chloroform−water extraction was used to recover the permethylated GSL.

The second procedure was performed according to Ciucanu and Kerek (1984). Vacuum-desiccated GSL samples were dissolved in 100 μL of a suspension of NaOH in DMSO (prepared by vortexing DMSO and powdered NaOH). After 1 h at room temperature, 35 μL of methyl iodide was added and the suspension sat for 1 h at room temperature with occasional vortexing (Linsley et al., 1994). The methylated product was extracted into chloroform and back-washed with water until neutral. Methylation was repeated as necessary to ensure complete derivatization.

Negative-Ion ($^-$ion) and Positive-Ion ($^+$ion) FABMS Analyses. FABMS was performed on a JEOL (Tokyo, Japan) HX-110/DA-5000 double focusing mass spectrometer/data system. A linear upward scan was employed with acceleration, 10 kV; resolution, 3000; xenon beam, 6 kV; filter, 100 Hz. In $^-$ion mode the matrix was triethanolamine; calibration standard, NaI in glycerol. In $^+$ion mode the matrix was 3-nitrobenzyl alcohol/15-5 crown ether; calibration standard, CsI/KI. Identification of pseudomolecular ion species in $^+$ion mode was confirmed by addition of NaOAc to the FAB matrix, which produced a mass shift of 22 amu for each $[M + H]^+$ ion.

ES-MS Analysis. The instrument used in this study was a Finnigan-MAT TSQ-700 (Finnigan-MAT Corp., San Jose, CA) equipped with an electrospray ion source. Methylated samples were dissolved in methanol/water solutions (6:4 v/v) containing 0.25 mM NaOH and analyzed by syringe pump flow injected at a rate of 0.75 μL/min directly into the electrospray chamber through a stainless steel hypodermic needle. The voltage difference between the needle tip and the source electrode was −3.5 kV. For CID studies, multiply charged precursor ions were selectively transmitted by the first mass analyzer and directed into the collision cell containing argon at roughly 2 mTorr with acceleration voltages of 30−40 V, hence kinetic energies of 60−80 eV.

RESULTS

General Pattern of Monosialogangliosides in HL60 Cells, and Their Binding to E-Selectin and P-Selectin. The HPLC elution pattern of monosialogangliosides as determined by HPTLC is shown in Figure 1, left panel. An example of further separation into subfractions is shown in Figure 1, right panel. Gangliosides capable of binding to E-selectin, as revealed by staining with ^{32}P-labeled E-selectin-expressing CHO cells, were exclusively monosialogangliosides, not di- or trisialogangliosides. Among the monosialoganglioside fractions, 1−11 showed no binding whatsoever. Only monosialogangliosides with slow HPTLC mobility showed E-selectin binding (Figure 2). Structures of gangliosides present in Fr 1 through 12-2 were determined by ^{1}H-NMR and $^-$ion FABMS of the native form or $^+$ion FABMS after permethylation. Structures of these gangliosides are summarized in Table 1. The major component of Fr 12 (*i.e.*, 12-2; see Figure 1, right panel) did not bind. The fastest-migrating binding fraction was 12-3. Fr 13 and 14 and a series of slower-migrating (Fr 15−20), E-selectin binding components were clearly observed (Figure 2). None of these gangliosides showed P-selectin binding in an assay using ^{32}P-labeled P-selectin-expressing CHO cells under the same conditions as for the E-selectin binding assay. E-selectin binding components present in Fr 12, 13, and 14 were characterized as described in the accompanying paper (Stroud et al., 1996). Because quantities of Fr 15−20 were extremely small, it was difficult to isolate and chemically identify them even under our experimental conditions; *i.e* extracts from >1200 mL of packed HL60 cells did not yield sufficient quantities.

The E-selectin binding pattern of gangliosides in HL60 cells was compared to that of gangliosides from normal human neutrophils (Figure 3). The patterns were essentially identical; like HL60 cells, human neutrophils also have slow-migrating gangliosides with at least a 10-sugar core as the minimum binding requirement (see Discussion).

Sulfate groups were undetectable in the E-selectin binding fractions, since there was no staining with Azure A on TLC. The E- and P-selectin binding properties of HL60 cells were only marginally reduced (relative to control cells) when cells were cultured in the presence of sodium chlorate, an inhibitor of sulfation.

Gangliosides with SLe^x determinant (IV^{3}NeuAcIII^{3}FucnLc$_4$Cer) (Table 2, Str **XII**), VI^{3}NeuAcV^{3}FucnLc$_6$Cer (Str **XIII**), and VI^{3}NeuAcV^{3}FucIII^{3}FucnLc$_6$Cer (Str **XIV**)], which are abundantly present in various human solid tumors and elute at the same position under the same HPLC conditions as the fractions marked A, B, and C in Figure 1, were completely absent in the eluant from HL60 gangliosides and presumably absent from normal neutrophils.

^{1}H-NMR Spectroscopy of Gangliosides. (A) Fr 2, 3, 4, and 5. The 1-D ^{1}H-NMR spectrum of gangliosides present in Fr 2 and 3 in DMSO-d_6/2% D_2O was almost identical to that reported by Levery et al. (1988) for IV^{3}NeuAcnLc$_4$Cer from human blood cell membranes (data not shown). The spectra of gangliosides present in Fr 4 and 5 under the same conditions were identical to that for IV^{6}NeuAcnLc$_4$Cer. The spectrum for Fr 5 is shown in Figure 4A. α2→3 and α2→6 sialosylation affected various signal changes in IVGal and IIIGlcNAc. In particular, the α2→6 linkage affected the upfield shift of GlcNAc NAc($\Delta\delta$ 0.015 ppm) and the shift of H3 ($\Delta\delta$ 0.50 ppm) (data not shown). ^{1}H-NMR spectra of gangliosides in these fractions were characterized by the presence of (a) complex proton resonances consisting of β-anomeric signals at 4.694 and 4.228 ppm (β-GlcNAc III-I and β-Gal IV-1, respectively), indicating the terminal trisaccharide group NeuAcα2→6Galβ1→4GlcNAcβ1→R; (b)

Table 1: Structures of Monosialogangliosides Present in Fr 1−11 Isolated from HL60 Cells[a]

Fr	Str		Cer ion	EB*
1	I	NeuAcα3Galβ4Glcβ Cer		–
2	II	NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ Cer	646/648	–
3		same as above but with different Cer	536	–
4	III	NeuAcα6Galβ4GlcNAcβ3Galβ4Glcβ Cer	646/648	–
5		same as above but with different Cer	536	–
6	IV	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer	643	–
7		same as above but with different Cer	536	–
8a	V	Galβ4GlcNAcβ6 Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer (NeuAcα3)	658/660	–
8b	VI	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer (NeuAcα3)	658/660	–
9a	VII	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer (NeuAcα3, Fucα3)	658/660/ 546/548	–
9b	VIII	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer (NeuAcα3, Fucα3)	658/660/ 546/548	–
9c		same as Str. V (Fr. 8a) but with different Cer	546/548	–
10a		same as Str. VII (Fr. 9a) but with different Cer	546/548	–
10b		same as Str. VIII (Fr. 9b) but with different Cer	546/548	–
10c		same as Str. V (Fr. 8a) but with different Cer	546/548	–
11a	IX	Galβ4GlcNAcβ6 Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer (NeuAcα3)	546/548	–
11b	X	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer (NeuAcα3, Fucα3) (same as ACFH-18 antigen)	658/660	–
11c	XI	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer (NeuAcα3, Fucα3)	548	–
12-2a		same as Str. X (Fr. 11b), but with different Cer	548	–
12-2b		same as Str. XI (Fr. 11c), but with different Cer	548	–

[a] (*) EB, E-selectin binding determined under static conditions by TLC overlay technique using ^{32}P-labeled E-selectin-expressing CHO cells.

β-anomeric proton resonances for the core →3Galβ1→4Glcβ1→1Cer found at 4.169 and 4.263 ppm (βGlc I-I and βGal II-I, respectively); (c) an α-NeuAc H-3$_{eq}$ doublet of doublets at 2.624 ppm (B-3$_{eq}$), and a pair of 3-proton NAc singlets at 1.833 and 1.879 ppm (β-GlcNAc III-NAc and α-NeuAc B-NAc, respectively); (d) a Cer consisting of "normal" Sph (4-sphingenine) and saturated, non-hydroxy fatty acids, indicated by a complex of resonances including Sph signals at 3.977, 3.886, 5.355, 5.539, and 1.937 ppm (R-1b, R-3, R-4, R-5, and R-6, respectively) and a fatty acid signal at 2.029 ppm (nFA-2).

(B) Fr 6 and 7. The ^{1}H-NMR spectrum of major gangliosides present in Fr 6 and 7 in DMSO-d_6 is almost identical to that reported previously (Levery et al., 1988) for IV^{3}NeuAcnLc$_6$Cer from human neutrophil membranes. The spectrum for Fr 6 is shown in Figure 4B. The following key saccharide resonances can be observed in the spectrum: (a) the β-anomeric proton resonances for the core →3Galβ1→4Glcβ1→1Cer are found at 4.169 and 4.263 ppm (β-Glc I-1 and β-Gal II-1, respectively); (b) a set of β-anomeric proton resonances for the interpolated *N*-acetyllactosamine group (→3Galβ1→4GlcNAcβ1→) can be observed at 4.657 and 4.263 ppm (β-GlcNAc III-1 and β-Gal IV-1, respectively); (c) the terminal trisaccharide group NeuAcα2→3Galβ1→4GlcNAcβ1→ is now delineated (Koerner et al., 1983; Levery et al., 1988) by a complex of resonances consisting of β-anomeric proton signals at 4.640 and 4.198 ppm (β-GlcNAc V-1 and β-Gal VI-1, respectively), an α-NeuAc H-3$_{eq}$ doublet of doublets at 2.751 ppm (A-3$_{eq}$), a coincident pair of 3-proton NAc singlets at 1.820 (β-GlcNAc III-NAc and V-NAc), a third NAc singlet occurring at 1.889 ppm (α-NeuAc A-NAc), along with additional reporter resonances for the subterminal β-Gal at 3.696 and 3.970 ppm (VI-3 and VI-4, respectively). Resonances from Cer were observed at virtually identical positions as for Fr 5, except that additional signals at 5.322 (*cis*-vinyl) and 1.980 ppm (*cis*-allyl) indicated some degree of unsaturation of the fatty acid moiety.

(C) Fr 8. The 1-D ^{1}H-NMR spectrum of this fraction (Figure 4C) was acquired at a higher temperature than those previously discussed (328 vs 308 K). Nevertheless, the spectrum can be usefully compared with the published data

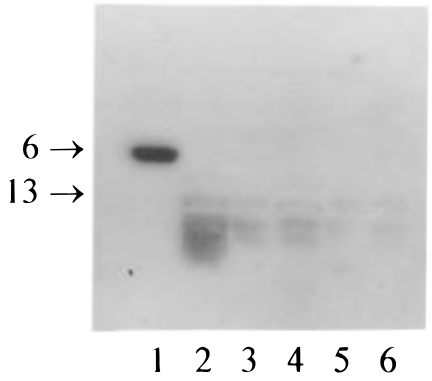


FIGURE 3: Comparison of E-selectin-binding monosialoganglioside fractions extracted from human neutrophils and HL60 cells. ≈100 mL of human neutrophils were extracted, and the monosialoganglioside fraction was prepared as described in the text. This fraction was compared with a corresponding fraction prepared from HL60 cells by HPTLC followed by blotting with ^{32}P-labeled E-selectin-expressing CHO cells (Swank-Hill et al., 1987). Lane 1, SLe^x Cer hexasaccharide. Lane 2, total Folch's upper-layer GSLs from HL60 cells. Lane 3, purified monosialoganglioside fraction from lane 2. Lane 4, purified monosialoganglioside fraction from Folch's upper-layer GSLs from human neutrophils. Quantity of ganglioside mixture used for lanes 3 and 4 was based on approximately equal numbers of HL60 cells and neutrophils. Lanes 5 and 6: same as lanes 3 and 4 but diluted 2×. Left margin: "6" and "13" represent positions of SLe^x (6 sugar residues, *i.e.*, Str **XII**) and myeloglycan (13 sugar residues; *e.g.*, Str **XVII**, see accompanying paper (Stroud et al., 1996)), respectively.

on neolacto-series gangliosides (Levery et al., 1988), provided one allows for the slight positive temperature-shift coefficient in DMSO-d_6/2% D_2O found for the majority of observable resonances.[2] For neolacto-series GSLs, β-Glc and β-Gal H-1 generally shift downfield from 0 to 0.015 ppm on going from 308 to 328 K, while β-GlcNAc H-1 shift downfield by approximately 0.015 ppm for the same temperature change. Other resonances, such as NAc singlets and α-NeuAc H-3$_{eq}$, shift only slightly. By these criteria, the spectrum of Fr 8 is a close match with that published for VI^{3}NeuAcIV^{6}Galβ4GlcNAcnLc$_6$Cer from human blood cells (Levery et al., 1988), except for one anomeric resonance buried under the large triplet peak at 4.233 ppm.[3] The salient features of this spectrum can be summarized as follows. (a) The α2→3 sialosylated linear type 2 chain hexasaccharide core is represented by a series of β-anomeric proton resonances observed at 4.170, 4.273, 4.678, 4.308, and 4.646 ppm (β-Glc I-1, β-Gal II-1, β-GlcNAc III-1, β-Gal IV-1, and β-GlcNAc V-1,[4] with the resonance for β-Gal VI-1 at this temperature expected at approximately 4.22 ppm, and therefore buried by the impurity peak); in this case the chemical shift of the resonance at 4.308 (4.300 ± 0.003 ppm at 308 K) is virtually diagnostic for attachment of a GlcNAcβ1→6 branch at β-Gal IV of a nLc$_6$Cer core. (b) In agreement with this is the observation of two more β-anomeric proton signals at 4.430 and 4.213 ppm for the branching *N*-acetyllactosamine residues (β-GlcNAc V′-1 and β-Gal VI′-1, respectively)—at 308 K these are found at 4.416 and 4.198 ppm, respectively, shifts indicative of a lack of terminal substitution of β-Gal VI′. (c) Additional resonances supporting the →4GlcNAcβ1→ 3(→4GlcNAcβ1→6)-Galβ1→4GlcNAcβ1→3 branching structure are a suite of β-GlcNAc NAc signals at 1.821, 1.838, and 1.844 ppm. (d) The presence of NeuAcα2→3 (on β-Gal VI) is further indicated by a complex of resonances including signals at 2.754 (α-NeuAc H-3$_{eq}$ doublet of doublets), 3.983 (β-Gal VI-3 doublet of doublets), and 1.886 ppm (α-NeuAc NAc singlet). The Cer appears similar to that of Fr 6, having R-1b, R-3, R-4, R-5, R-6, nFA-2, *cis*-vinyl, and *cis*-allyl signals occurring at their usual chemical shifts and amplitudes, allowing for the temperature difference (note that R-1b is observed at 3.954 ppm, shifted upfield from its position at 308 K).

(D) Fr 9 and 11. The ^{1}H-NMR spectra of these gangliosides showed patterns very similar to that of "ACFH-18 antigen". The spectrum of Fr 11 is described here as an example. This spectrum displays, at least in its major features, a clear similarity to that obtained from a tumor-associated fucoganglioside antigen reactive with the antibody ACFH-18 (see Figure 4D). Characteristic resonances in common with the ACFH-18 antigen and other NeuAcα2→3 terminated, Fucα1→3 substituted poly(*N*-acetyllactosamine) GSL structures (Levery et al., 1986; Nudelman et al., 1988) include the following: (i) the α-anomeric signal at 4.875 ppm, generally diagnostic for an Le^x-type Fucα1→3 linked to a type 2 chain GlcNAcβ1→3 residue; (ii) a broadened and distorted quartet at 4.594 ppm, assignable to H-5 of the same Fucα1→3 substituent; (iii) a doublet at 1.015 ppm, assignable to the Fucα1→3 methyl group (H-6); (iv) a doublet of doublets at 2.756 ppm for H-3$_{eq}$ of terminal NeuAcα2→3 (the corresponding signal for terminal NeuAcα2→6 would be expected at ca. 2.62−2.63 ppm; Levery et al., 1988); (v) a singlet at 1.889 ppm for the NAc methyl group of NeuAcα2→3 (the corresponding signal for terminal NeuAcα2→6 would be expected at ca. 1.878 ppm; Levery et al., 1988); (vi) a β-anomeric signal at 4.174 ppm, assignable to the Glcβ1→ residue linked to Cer; and (vii) a number of other β-anomeric signals in typical ranges for the alternating Galβ1→4 and GlcNAcβ1→3 residues of a poly-(*N*-acetyllactosamine) chain.

FABMS of Monosialogangliosides from HL60 Cells. *(A) Fr 2, 3, 4, and 5*. The native forms of Fr 2, 5, and a mixture of 3 and 4 were analyzed by $^-$ion FABMS (spectra not shown). Pseudomolecular ions ([M − H]$^-$, nominal *m*/*z* 1516, 1626, 1628) were consistent with the sugar composition NeuAc·Hex$_3$·HexNAc plus Cers consisting of Sph/fatty acid combinations d18:1/16:0, d18:1/24:1, and d18:1/24:0. Cer ions were found at *m*/*z* 536, 646, and 648, respectively. Cer-containing fragments established the linear sequence NeuAc·Hex·HexNAc·Hex·Hex (Figure 5A).

(B) Fr 6 and 7. Fr 6 and 7 as native GSL were analyzed by $^-$ion FABMS (spectra not shown). Pseudomolecular ions ([M − H]$^-$, nominal *m*/*z* 1993 and 1881, respectively) were consistent with the sugar composition NeuAc·Hex$_4$·HexNAc$_2$ plus Cers consisting of Sph/fatty acid combinations d18:1/24:0 and d18:1/16:0, respectively. Cer-containing fragments demonstrated the linear sequence NeuAc·Hex·HexNAc·Hex·HexNAc·Hex·Hex (Figure 5B).

[2] Negative temperature shift coefficients have been observed for a few structural reporter resonances, most notably for H-5 of α-Gal in globo-series GSLs, H-5 of α-Fuc in Le^x trisaccharide, and R-1b of Cer (Levery et al., 1986, 1988), as well as for a number of protons found in repetitive A GSL structures (Clausen et al., 1985).

[3] Due to an editorial error, the numbering of residues in the spectra of Figure 5 in Levery et al. (1988) is not the same as that in the structure drawn at the top of the figure. In addition, a number of arrows connecting residues in Table I(B) were omitted.

[4] Recent 2-D ^{1}H-NMR analysis of VI^3 NeuA*cis*onLc$_8$Cer and VI^3-NeuAcVI′^{3}NeuA*cis*o-nLc$_8$Cer (including TOCSY and NOESY studies; S. B. Levery, unpublished) indicates that the assignments for β-GlcNAc III-1 and V-1 published previously (Levery et al., 1988) must be reversed, as indicated here for the former compound.

Table 2: SLe^x Gangliosides Not Found in HL60 Cells

Str.	
XII	Galβ4GlcNAcβ3Galβ4Glcβ Cer 3 3 NeuAcα Fucα
XIII	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer 3 3 NeuAcα Fucα
XIV	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer 3 3 3 NeuAcα Fucα Fucα
XV	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer 3 3 NeuAcα Fucα
XVI	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer 3 3 NeuAcα Fucα

The permethylated Fr 7 analyzed in $^+$ion FABMS supports the saccharide sequence observed in $^-$ion FABMS. A_1 type fragments (Dell, 1987; Egge and Peter-Katalinic, 1987) were found at *m/z* 825 and 1274, representing NeuAc·Hex·HexNAc and NeuAc·Hex_2·$HexNAc_2$, respectively.

(C) Fr 8. Permethylated Fr 8 was analyzed by $^+$ion FABMS. A set of pseudomolecular ions (MH^+, nominal *m/z* 2806, 2808) were consistent with the composition NeuAc·Hex_5·$HexNAc_3$ plus Cers consisting of Sph/fatty acid combinations d18:1/24:1 and d18:1/24:0, respectively. A_1 fragments (Dell, 1987; Egge and Peter-Katalinic, 1987) observed at *m/z* 464 (Hex·HexNAc), 825 (NeuAc·Hex·HexNAc), and 1723 (NeuAc·Hex_3·$HexNAc_3$) established the asymmetric branch structure shown in Figure 5C. Additional fragments at *m/z* 1029 and 1274, representing NeuAc·Hex_2·HexNAc and NeuAc·Hex_2·$HexNAc_2$, respectively, demonstrated the presence of the linear sequence NeuAc-O-Hex-O-HexNAc-O-Hex-O-HexNAc- in Fr 8 (Figure 5D). The presence of sodium was observed at *m/z* 2828 and 2830, a shift of 22 amu from the molecular ion. Also observed were *m/z* 2874 and 2876, respectively, representing neutral loss of CH_3OH from the $[M + H]^+$ ion.

(D) Fr 9. Following permethylation, Fr 9 was analyzed by $^+$ion FABMS. Predominant pseudomolecular ions (MH^+, nominal *m/z* 2694, 2696) were consistent with the composition NeuAc·Hex_5·$HexNAc_3$ plus Cers consisting of d18:1 Sph in combination with 16:1 and 16:0 fatty acids. Corresponding Cer ions were found at *m/z* 546 and 548, respectively. A_1 fragments observed at *m/z* 464 (Hex·HexNAc), 825 (NeuNAc·Hex·HexNAc), 1478 (NeuAc·Hex_3·$HexNAc_3$), and 1723 (NeuAc·Hex_3·$HexNAc_3$) established the asymmetric branch sugar cone similar to that observed in Fr 8 (Figure 5C). Although the sugar compositions are similar for Fr 8 and 9, the Cers differ, which would explain the difference in TLC migration. Sodium adducts, $[M + Na]^+$, were observed at *m/z* 2717 and 2719, respectively. $[MH - CH_3OH]^+$ was observed at *m/z* 2663 and 2665.

The presence of a deoxyHex residue was observed in the ion sequence *m/z* 825, 1448, and 1897 (Figure 5E). The abundant ion at *m/z* 1242 represents the loss of deoxyHexOH from the fragment *m/z* 1448, a type of neutral loss that occurs preferentially from the 3-position of HexNAc. The absence of *m/z* 999, representing NeuAc·Hex·[deoxyHex]·HexNAc, and the observed ion sequence place the deoxyHex on the second HexNAc from the nonreducing end. An isomeric structure was also observed by the presence of sequence *m/z* 825, 1274, and 1897. The neutral loss of deoxyHexOH from *m/z* 1897 was found at *m/z* 1691. The presence of *m/z* 1274 (NeuAc·Hex_2·$HexNAc_2$) and the absence of *m/z* 999 support the assignment of deoxyHex on the third HexNAc from the nonreducing end. Although these fragments indicate the distinct presence of a deoxyHex residue, the molecular ion is unclear. However, pseudomolecular ions corresponding with the Cer ions *m/z* 546, 548, 658, and 660 would yield MH^+ (nominal) at *m/z* 2868, 2870, 2980, and 2982, respectively.

(E) Fr 10. Permethylated Fr 10 was analyzed by $^+$ion FABMS. A predominant set of pseudomolecular ions (MH^+, nominal *m/z* 2868, 2870) are consistent with the composition NeuAc·deoxyHex·Hex_5·$HexNAc_3$ plus Cers consisting of Sph/fatty acid combinations d18:1/16:1 and d18:1/16:0, respectively. Key A_1-type fragments and Cer ions that established the presence of isomeric monofucosylated structures in Fr 9 were observed to be the major components in Fr 10 (Figure 5F). The abundant ion at *m/z* 1242 probably represents the loss of deoxyHexOH from the fragment *m/z* 1448, a type of neutral loss that occurs preferentially from the 3-position of HexNAc (Egge and Peter-Katalinic, 1987). Corresponding sodium adducts, $[M + Na]^+$, and $[MH - CH_3OH]^+$ ions were also observed.

Less abundant pseudomolecular ions (*m/z* 2694, 2696) are consistent with the sugar composition and Cer combinations of the major component found in Fr 9. Supporting ions *m/z* 464, 825, and 1723 that established the asymmetric branch sugar core observed in Fr 8 and 9 were also observed in Fr 10. As before, $[M + Na]^+$ and $[MH - CH_3OH]^+$ ions were also observed.

(F) Fr 11. Following permethylation, Fr 11 was analyzed by $^+$ions FABMS. A predominant set of pseudomolecular ions (MH^+, nominal *m/z* 3143, 3145) are consistent with the sugar composition NeuAc·Hex_6·$HexNAc_4$ plus Cers consisting of d18:1 Sph in combination with 16:1 and 16:0 fatty acids. Cer ions were found at *m/z* 546 and 548, respectively. A_1 fragments observed at *m/z* 464 (Hex·HexNac), 825 (NeuAc·Hex·HexNAc), 1723 (NeuAc·Hex_3·$HexNAc_3$), and 2172 (NeuAc·Hex_4·$HexNAc_4$) established the asymmetric branch structure shown in Figure 5G. $[M + Na]^+$ and [MH

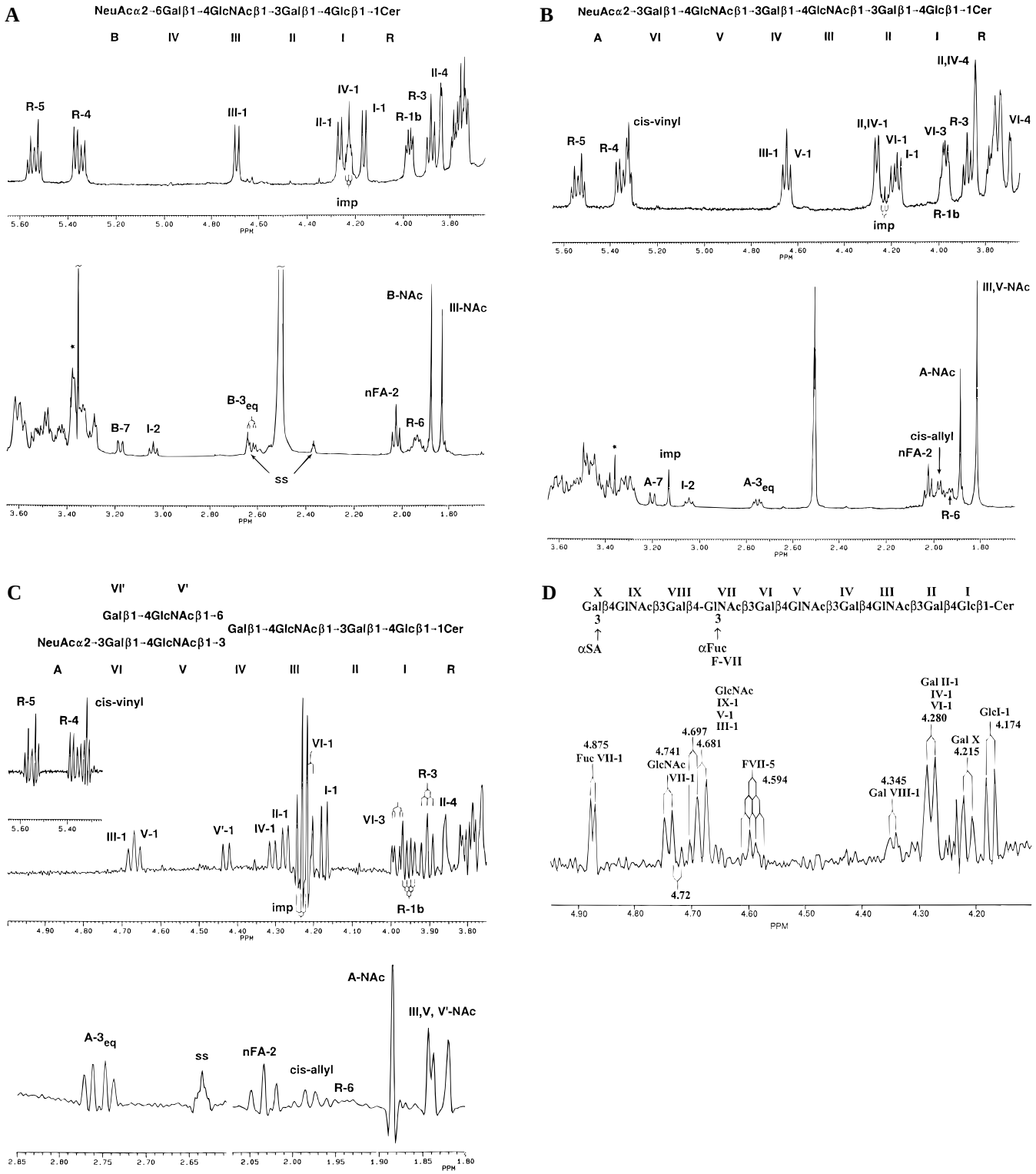
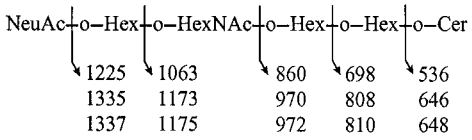


FIGURE 4: 500 MHz ^{1}H-NMR spectra of gangliosides present in various fractions isolated from HL60 cells in DMSO-d_6/2% D_2O. Panel A: Fr 5, 308 ± 2 K. Lower section, $^1/_4$ × Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure. R refers to protons of Sph core only. nFA refers to protons of non-hydroxylated fatty acids; imp, impurity triplet at 4.228 ppm overlaps with IV-1 signal; ss, spinning sidebands from residual DMSO centered at 2.5 ppm; *, preirradiation of residual HOD signal at 3.35 ppm. Panel B: Fr 6, 308 ± 2 K. Lower section, $^1/_4$ × Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure. R refers to protons of Sph core only. nFA refers to protons of non-hydroxylated fatty acids; *cis*-vinyl and *cis*-allyl refer to vinyl and adjacent allyl protons of unsaturated fatty acids; imp, impurity peaks; *, preirradiation of residual HOD signal at 3.35 ppm. Panel C: Selected regions of Fr 8, 328 ± 2 K. Lower section from 2.07 to 1.80 ppm, $^1/_4$ × Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure. R refers to protons of Sph core only. nFA refers to protons of non-hydroxylated fatty acids; *cis*-vinyl and *cis*-allyl refer to vinyl and adjacent allyl protons of unsaturated fatty acids; imp, impurity peak; ss, spinning sideband from residual DMSO centered at 2.5 ppm. Panel D: Selected downfield regions of Fr 11, 328 ± 2 K, from 4.10 to 4.90 ppm. Roman numerals refer to sugar residues in the structure shown at top. Arabic numerals refer to protons of pyranose ring in each sugar residue.

− $CH_3OH]^+$ ions were observed at *m*/*z* 3165/3167 and 3111/3113, respectively.

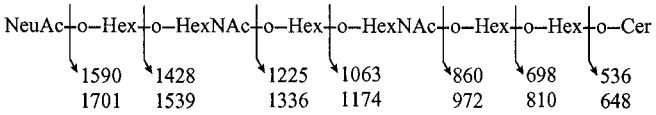
A less abundant set of pseudomolecular ions (*m*/*z* 3429 and 3431) are consistent with the composition NeuAc·Hex_6·

A



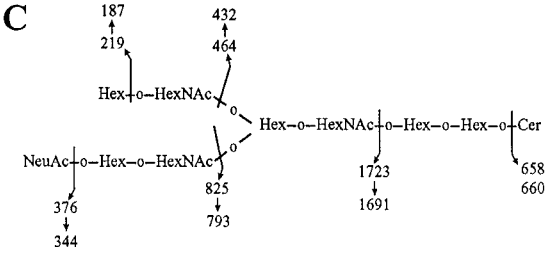
Sph	FA	MW	Fraction
d18:1	16:0	1517	3/4, 5
d18:1	24:1	1627	2
d18:1	24:0	1629	3/4, 2

B

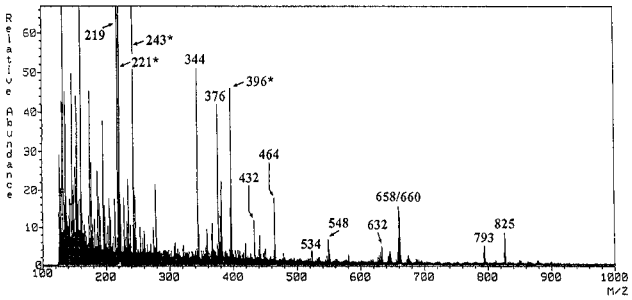


Sph	FA	MW	Fraction
d18:1	16:0	1882	7
d18:1	24:0	1994	6

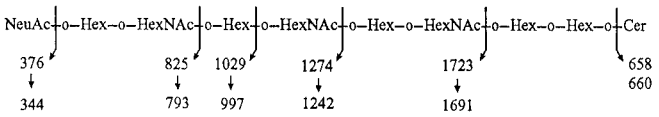
C

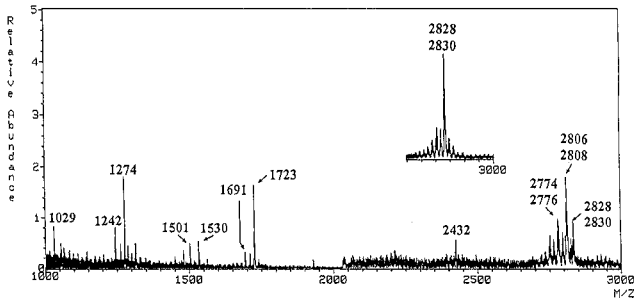


Cer^+	Sph	FA	MW
658	d18:1	24:1	2805
660	d18:1	24:0	2807

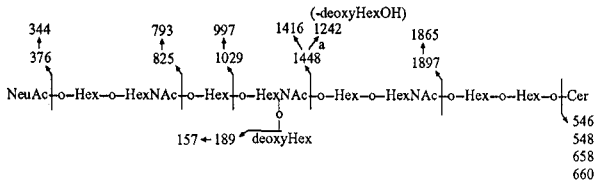


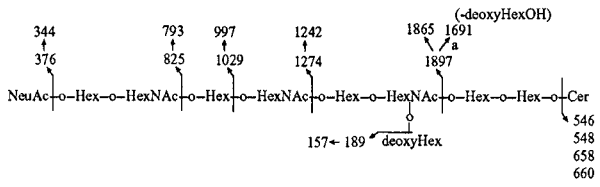
D



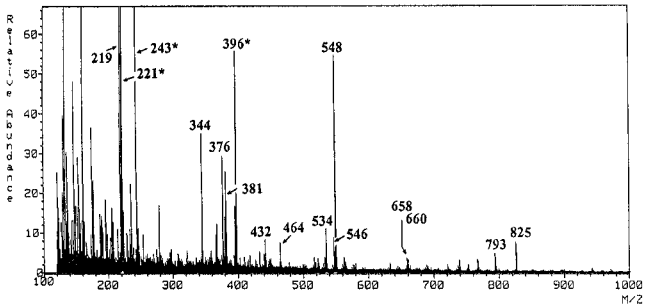


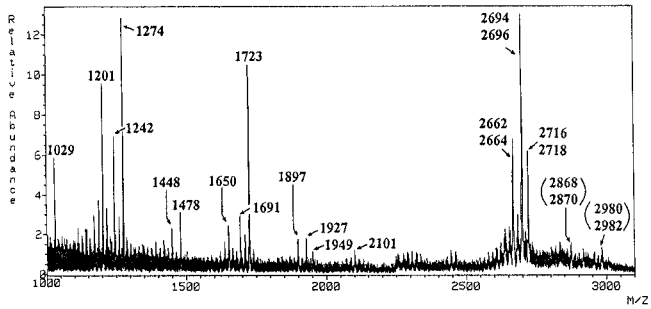
E





Cer^+	Sph	FA	MW
546	d18:1	16:1	2867
548	d18:1	16:0	2869
658	d18:1	24:1	2979
660	d18:1	24:0	2981





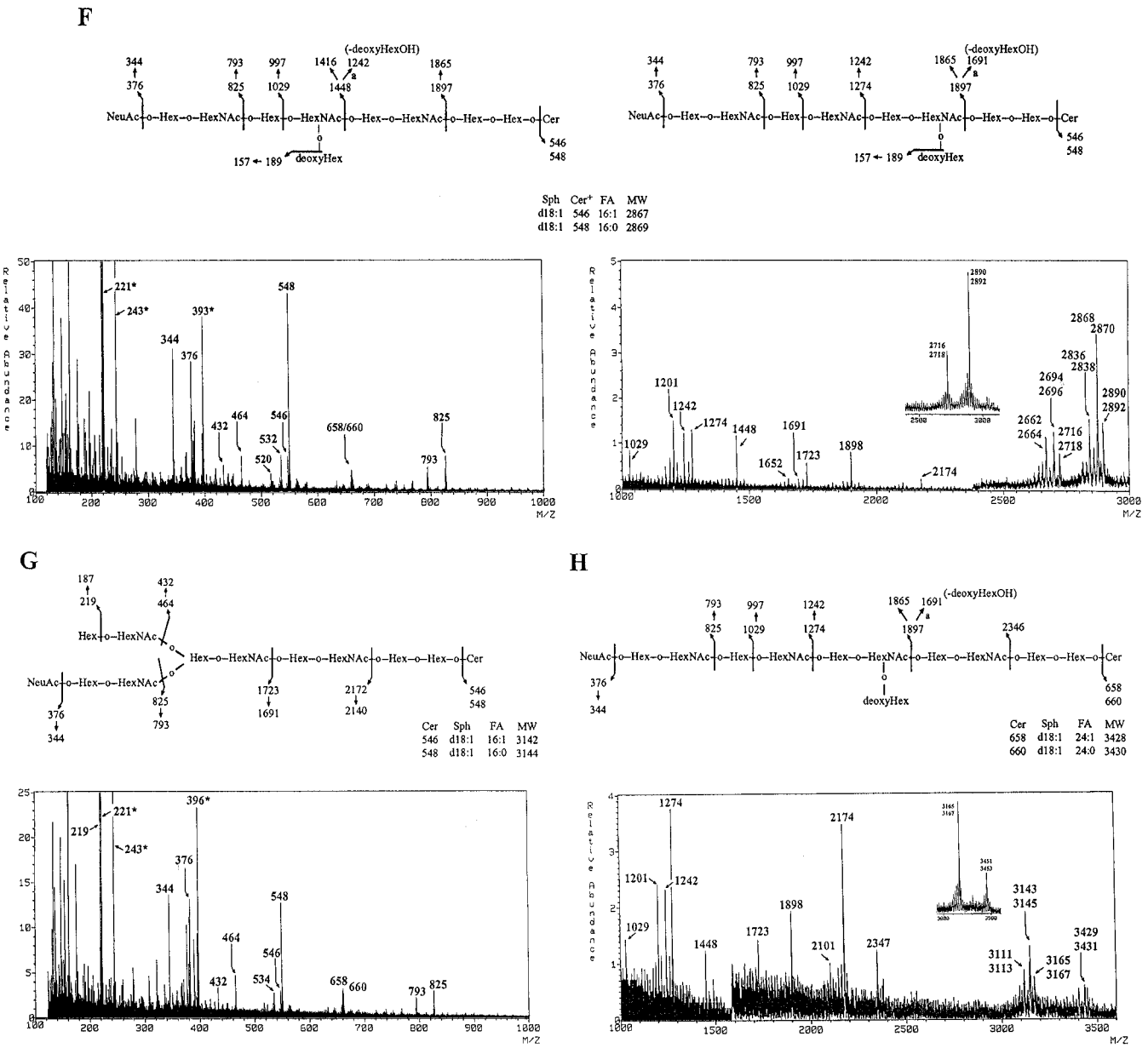


FIGURE 5: Fragmentation patterns and $^{+}$ion and $^{-}$ion FABMS spectra of various gangliosides isolated from HL60 cells. Panels A and B: Fragmentation schemes for $^{-}$ion FABMS of native fractions (A) 2, 3/4, and 5; (B) 6 and 7. Panels C and D: $^{+}$Ion FABMS spectrum of permethylated Fr 8 is shown above (mass range 100−3600 amu; scan slope 1′20″). Proposed fragmentation schemes are presented in panels C and D. Neutral loss of MeOH yields secondary fragments. Panel E: $^{+}$Ion FABMS spectrum of permethylated Fr 9 is shown above (conditions same as Fr 8). Proposed fragmentation schemes are presented in panel E. Neutral loss of MeOH yields secondary fragments; (a) neutral loss of 3-linked substituent. Panel F: $^{+}$Ion FABMS spectrum of permethylated Fr 10 is shown above (conditions same as Fr 8). Proposed fragmentation schemes are presented in panel F. Neutral loss of MeOH yields secondary fragments; (a) neutral loss of 3-linked substituent. Panels G and H: $^{+}$Ion FABMS spectrum of permethylated Fr 11 is shown above (conditions same as Fr 8). Proposed fragmentation schemes are presented in panels G and H. Neutral loss of MeOH yields secondary fragments; (a) neutral loss of 3-linked substituent. * indicates matrix or contaminant ions.

$HexNAc_4$ plus Cers consisting of d18:1 Sph in combination with 24:1 and 24:0 fatty acids (Figure 5H). Cer ions were found at *m*/*z* 658 and 660, respectively. Of particular importance are the ions at *m*/*z* 825, 1274, and 1897, along with the absence of *m*/*z* 999 representing NeuAc·Hex·[deoxyHex]·HexNAc, which place the deoxyHex on the third HexNAc from the nonreducing end. A less abundant ion at *m*/*z* 1448 may represent a minor component in which the deoxyHex is placed on the second HexNAc from the nonreducing end. $[M + Na]^+$ and $[MH - CH_3OH]^+$ ions were observed at *m*/*z* 3451/3453 and 3397/3399, respectively.

ES-MS Analysis of Monosialogangliosides from HL60 Cells. (A) Fr 12-2. Direct ES-MS injection of permethylated gangliosides from Fr 12-2 provided the spectrum shown in Figure 6A. Threee major ions dominated the spectrum by the adduction of 2, 3, and 4 sodium ions to a single component, *m*/*z* 1683.5^{2+}, 1129.8^{3+}, and 853.3^{4+}, respectively. These data indicate a molecular weight of 3321.7. The triply charged ion was selected for CID, which yielded major fragments on the reducing side of each HexNAc residue and the sequence Neu5Ac-LacN-(deoxyHex)LacN-LacN-LacN-Hex-Hex-Cer from both the reducing and nonreducing terminus (Figure 6B). The facile HexNAc rupture positioned the fucosyl moiety on the penultimate lactosamine residue, and the suquence ions satisfy the observed molecular weight and composition data ($Neu5Ac_1$, Fuc_1, $HexNAc_4$, Hex_6). From established GSL fragmentation patterns (Re-

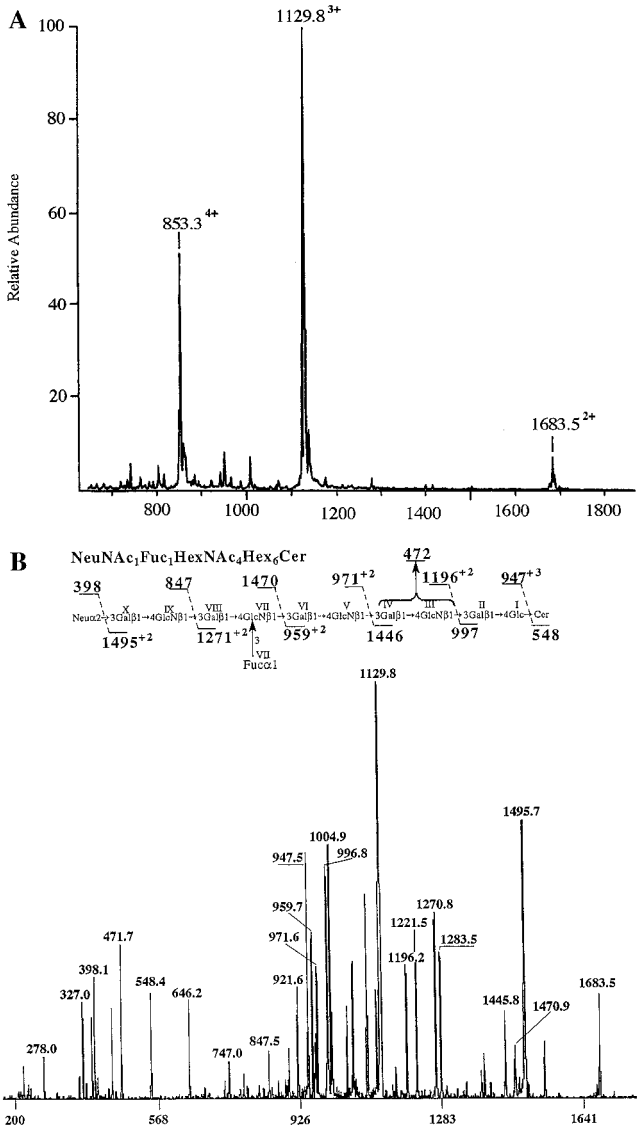


FIGURE 6: Panel A: ES-MS spectrum of gangliosides present in Fr 12-2 isolated from HL60 cells. Panel B: CID spectrum *m/z* 1129.8 $[M + 3Na]^{3+}$. Inset: Proposed structure and fragmentation scheme.

inhold et al., 1994) and myeloglycan motifs (Stroud et al., 1995), the spectrum was consistent with the fucosylated structure presented in the inset scheme of Figure 6B. The fragments *m/z* 548 and 278.1 indicate the Cer to be comprised of a sphingenine moiety and by difference a palmitoyl *N*-acyl residue.

DISCUSSION

Basis of Previous Assignments of Carbohydrate Epitope for E-Selectin and P-Selectin. Immediately after the overall sequence of selectins was clarified through cDNA cloning, and the presence of a C-type lectin domain at the N-terminal domain of both P- and E-selectin was demonstrated (*e.g.*, Bevilacqua et al., 1989; Johnston et al., 1989), many workers undertook an intensive search for the carbohydrate epitopes recognized by these selectins. SLe^x has been considered to be a plausible ligand of E- and possibly P-selectin based on the following observations: (i) Transfection of Lewis fucosyltransferase cDNA to Chinese hamster ovary (CHO) cells expressing sialosyl type 2 chain (NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→R) resulted in acquisition of the ability to adhere to TNFα-activated endothelial cells (ECs) (Lowe et al., 1990). (ii) HL60 cells, previously shown to react with mAb FH6 (see Discussion of accompanying paper, Stroud et al., 1996, regarding specificity of this mAb), are capable of binding to TNFα- or IL-1-activated ECs, and this binding can be inhibited by liposomes containing SLe^x-bearing GSLs but not by liposomes containing sialosylparagloboside, sialosyl*nor*hexaosyl-Cer, or Le^x-glycosyl-Cer. mAbs SNH3 and SNH4 (see Discussion of accompanying paper regarding specificities) inhibited E-selectin-dependent HL60 cell adhesion (Phillips et al., 1990). Many other subsequent studies utilized anti-SLe^x mAbs, or oligosaccharides or GSLs containing SLe^x structure or its analogues. Further studies indicated that selectin-dependent binding, particularly in tumor cells, is also mediated by SLe^a (a positional isomer of SLe^x) (Berg et al., 1991; Takada et al., 1991; Handa et al., 1991). However, SLe^a, which has a lacto-series type 1 chain structure (Galβ1→3GlcNAcβ1→3Galβ1→R), is completely absent from human neutrophils and HL60 cells (Ito et al., 1994) and is presumably not involved in the "rolling" and recruitment of neutrophils associated with inflammatory responses. The failure of P-selectin to bind to any ganglioside, including those from slow-migrating fractions, suggests that the P-selectin and E-selectin binding epitopes are physiologically independent from each other. Liposomes containing SLe^x epitope (*e.g.*, Str **XII**, **XIII**, and **XIV** in Table 2) were able to bind directly to P-selectin expressed on activated platelets (Handa et al., 1991). Recent studies with transfection of cDNA encoding PSGL-1 core protein into SLe^x-expressing, P-selectin non-binding tumor cells clearly indicate a requirement for a PSGL-1-like presenting molecule in addition to specific SLe^x glycosylation (Handa et al., 1995). Thus, the requirements for P-selectin binding are entirely different from those for E-selectin binding.

Structural Features of Monosialogangliosides in HL60 Cells. The major gangliosides of HL60 cells that show E-selectin binding are monosialo species. With the exception of Str **III**, all gangliosides are terminally 2→3 sialosylated. Di- and trisialogangliosides are extremely minor components in these cells and do not bind to E-selectin. No sulfated GSL was detected in the E-selectin binding fraction. If sulfated GSLs were present, they would be present in the "di- or trisialoganglioside" fraction. None of the HL60 gangliosides, under the static conditions employed, bind to P-selectin. The major monosialogangliosides were Str **I**−**IV** and common unbranched lacto-series type 2 chain structures with various Cer compositions. No type 1 chain structures were detected. Two types of branched lacto-series type 2 chain gangliosides (Str **V** and **IX**) have the common β1→6 linked *N*-acetyllactosamine side chain. These gangliosides have been found as the major gangliosides of normal blood cell membranes (Watanabe et al., 1979). Both of the branched structures are minor components. This is in striking contrast to placental gangliosides, which have multiply branched type 2 chain core structures as major components and unbranched linear chain as minor components (Levery et al., 1989).

Four types of gangliosides having an 8-sugar core were isolated from Fr 8−10. These are $VI^3NeuAcIV^6Gal\beta1\rightarrow4GlcNAcnLc_6Cer$ (Str **V**), $VIII^3NeuAcnLc_8Cer$ (Str **VI**), $VIII^3NeuAcV^3FucnLc_8Cer$ (Str **VII**), and $VIII^3NeuAcIII^3FucnLc_8Cer$ (Str **VIII**). Str **V** is the same as the previously-described "G-8" (Watanabe et al., 1979). Str **VII** is a

ganglioside having the "VIM-2" epitope (Macher et al., 1988). Str **VIII** has not been found previously.

Three types of E-selectin nonbinding gangliosides having a 10-sugar core have been detected. VIII^{3}NeuAcVI^6-Galβ1→4GlcNAcnLc$_8$Cer (Str **IX**), X^{3}NeuAcVII^{3}FucnLc$_{10}$-Cer (Str **X**), and X^{3}NeuAcV^{3}FucnLc$_{10}$Cer (Str **XI**) were detected in Fr 11. Str **X** is the same as the previously-described ACFH-18 antigen (Nudelman et al., 1988) and was found also in Fr 12-2a. Str **XI** was not previously reported. These structures are all E-selectin nonbinding under static conditions. Structural features of the E-selectin binding gangliosides present in Fr 12-3, 13-1, 13-2, and 14 are described in the accompanying paper (Stroud et al., 1996).

The major fucosylation site in HL60 gangliosides was found on the internal GlcNAc (second or third GlcNAc from the nonreducing end), not at the penultimate GlcNAc of either the 8- or 10-monosaccharide core. Thus, Str **VII** and **X**, having the epitope "VIM-2" (Fukuda et al., 1986; Macher et al., 1988), were relatively abundant in HL60 cells. This is in striking contrast to fucogangliosides of common human solid cancers which have abundant SLe^x structures (Str **XII**, **XIII**, and **XIV**). Fucosyl substitution at the penultimate GlcNAc of 2→3 sialylated gangliosides with an 8- or 10-monosaccharide core, but without internal fucosylation (SLe^x without internal fucosylation), *i.e.*, Str **XV** and **XVI** (Table 2), was completely absent in HL60 cells. GSLs having SLe^x at the nonreducing terminus comprised an extremely minor component and always had one or two α1→3 fucosyl residues at the internal GlcNAc (see accompanying paper, Stroud et al., 1996).

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