Monosialogangliosides of Human Myelogenous Leukemia HL60 Cells and Normal Human Leukocytes. 2. Characterization of E-Selectin Binding Fractions, and Structural Requirements for Physiological Binding to E-Selectin†

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ABSTRACT: E-selectin binding gangliosides were isolated from myelogenous leukemia HL60 cells, and the E-selectin binding pattern was compared with that of human neutrophils as described in the preceding paper in this issue. The binding fractions were identified as monosialogangliosides having a series of unbranched polylactosamine cores. Structures of fractions 12-3, 13-1, 13-2, and 14, which showed clear binding to E-selectin under the conditions described in the preceding paper, were characterized by functional group analysis by application of monoclonal antibodies, ^{1}H-NMR, FAB-MS, and electrospray mass spectrometry with collision-induced dissociation of permethylated fractions. Fractions 12-3, 13-1, and 13-2 were characterized by the presence of a major ganglioside with the following structure: NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)-GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcβCer. Fractions 12-3 and 13-2 contained, in addition, small quantities (10−15%) of extended SLe^x with internally fucosylated structures: NeuAcα2→3Galβ1→4-(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(±Fucα1→3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcβCer. Fraction 13-1, showing stronger E-selectin binding activity than 12-3 and 13-2, contained only a trace quantity (<1%) of SLe^x. Fraction 14, which also showed clear binding to E-selectin, was characterized by the presence of the following structures, in addition to two internally monofucosylated structures (**XX** and **XXI**, Table 2, text): NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcβCer; and NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)-GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcβCer. SLe^x determinant was completely absent. Thus, the E-selectin binding epitope in HL60 cells is carried by unbranched terminally α2→3 sialylated polylactosamine having at least 10 monosaccharide units (4 *N*-acetyllactosamine units) with internal multiple fucosylation at GlcNAc. These structures are hereby collectively called "myeloglycan". Monosialogangliosides from normal human neutrophils showed an essentially identical pattern of gangliosides with selectin binding property. Myeloglycan, rather than SLe^x, provides a major physiological epitope in E-selectin-dependent binding of leukocytes and HL60 cells.

Monosialogangliosides, isolated from HL60 cells, with unbranched polylactosamine (PLA)[1] structures having >10 monosaccharide units (Fr 12, 13, and 14) show clear E-selectin binding (see Stroud et al., 1996, hereafter referred to as "preceding paper"). The presence of a series of longer-chain, slower-migrating, E-selectin binding monosialogangliosides was also observed. Fr 12 was further divided into five subfractions by HPTLC (see preceding paper). Two of these subfractions were separated: major nonbinding Fr 12-2 (characterized in preceding paper) and binding Fr 12-3. Subfractions 13-1 and 13-2 (separated from Fr 13 by HPTLC under the same conditions as Fr 12) bound strongly to E-selectin. Fr 14 could not be separated into subfractions by existing methods (see preceding paper).

Here, we describe functional group analysis of Fr 12, 13, and 14 by specific mAbs before and after desialylation, ^{1}H-NMR analysis, and $^+$ion FABMS and electrospray ES-MS with CID of permethylated fractions. Our results clearly indicate that neither SLe^x nor sulfated structures (*e.g.*, sulfated Le^x) are responsible for E-selectin-dependent physiological binding of leukocytes and HL60 cells. Rather, long-chain unbranched PLAs with α2→3 sialylation at the terminal Gal

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[1] Abbreviations: same as in preceding paper in this issue (Stroud et al., 1996).

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Table 1: Functional Group Analysis of GSL Fractions by mAbs and ^{1}H-NMR

		mAb				
	binding to E-selectin-expressing cells	1B2[a] (LacNAc)	SH1[b] (Le^x)	PL82G2[c]	FH6[d] (SLex)	α 1Fuc1→3GlcNAc (4.875 ppm)
test fractions						
Fr 12-2	−	−	−	−	+	+
desialylated Fr 12-2	−	++	−	+	−	
Fr 13-1	++	−	−	−	+	++
desialylated Fr 13-1	−	++	−	++	−	
Fr 13-2	++	−	−	−	+	ND[e]
desialylated Fr 13-2	−	++	−	++	−	
Fr 14	+	−	−	ND[e]	+	++
desialylated Fr 14	−	++	−		−	
reference compds						
Le^x (IV^{3}FucnLc4)		−	++	−	−	
SLe^x (VI^{3}NeuAcV^{3}FucIII^{3}FucnLc6)	++	−	−	−	+	
desialylated SLE^x	−	−	++	++	−	
sialylparagloboside (IV3NeuAcIII3FucnLc6)	−	−	−	−	−	
desialylated sialylparagloboside	−	++	−	−	−	

[a] Young et al., 1981. [b] Singhal et al., 1990. [c] K. Handa, M. R. Stroud, and S. Hakomori, unpublished data. [d] Fukushi et al., 1984b. [e] ND, not determined.

and multiple fucosylation at defined internal locations, collectively called "myeloglycan", are the physiological epitopes for E-selectin-dependent adhesion.

MATERIALS AND METHODS

Determination of Terminal Structure by Immunoblotting with or without Desialylation. In order to determine whether the GSL in question has SLe^x structure or NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→structure, GSLs were desialylated in 1% acetic acid (100 °C, 1 h) followed by TLC immunostaining with anti-Le^x mAbs (*e.g.*, SH1, FH2, anti-SSEA-1) (Fukushi et al., 1984b; Singhal et al., 1990), or with mAb 1B2 (which does not react with Le^x but does react with unsubstituted LacNAc terminus Galβ1→4GlcNAcβ1→3Galβ1→R) (Young et al., 1981). The immunostaining procedure was necessary because all mAbs previously considered to be directed to SLe^x structure actually cross-react strongly with various related structures (see Results/Reactivities of Various Gangliosides with mAbs...).

Functional Analysis by mAbs Directed to Internally Located α1→3GlcNAc and to SLe^x before and after Desialylation. The reactivity of each fraction was tested before and after desialylation using a novel mAb PL82G2 which defines internally located Fucα1→3GlcNAc but does not recognize α1→3 fucosylation at the penultimate GlcNAc, *i.e.*, Le^x structure (K. Handa, M. R. Stroud, and S. Hakomori, unpublished). Each fraction was also tested for reactivity with various antibodies described previously as being directed to SLe^x, namely, FH6 (Fukushi et al., 1984b), CSLEX (Fukushima et al., 1984), and SNH3 and -4 (A. K. Singhal, E. D. Nudelman, and S. Hakomori, unpublished).

Reactivities of various mAbs with purified GSLs were tested by antibody binding to GSLs coated on the surface of plastic wells (Kannagi et al., 1983), or by TLC immunostaining (Magnani et al., 1980; Abe et al., 1983).

Preparation of *CHO Cells Expressing P- and E-Selectin*. Preparation of these cells, assay of GSL binding to these cells, and isolation and characterization of gangliosides were performed as described in the preceding paper.

RESULTS

Functional Group Analysis of E-Selectin Binding vs Nonbinding Fractions. Preliminary functional group analysis of E-selectin nonbinding vs binding fractions, *i.e.*, Fr 12-2 vs 13-1, 13-2, and 14, was made by immunostaining with mAbs directed to defined epitope structures before and after desialylation, and by ^{1}H-NMR. As shown in Table 1, the four fractions reacted equally well with mAb FH6, which was previously claimed to be directed to SLe^x. Reactivity of the four fractions was equally abolished following desialylation. The same trends of reactivity were observed for anti-SLe^x mAbs CSLEX and SNH3 (data not shown). These results pose a basic question on the specificities of these mAbs (see Discussion). The gangliosides present in the four fractions showed no reactivity with mAb 1B2, which reacts exclusively with Galβ1→4GlcNAc, but became strongly reactive following desialylation. Since 1B2 does not react with Le^x, the reactivity patterns as above exclude the possible presence of SLe^x at the terminus of the ganglioside present as the major component in the four fractions. This was further confirmed by the observation that gangliosides present in the four fractions did not react, either before or after desialylation, with mAb SH1, which reacts exclusively with Le^x structure. The quantity of SLe^x-containing gangliosides (Str **XVIII** and **XIX**) present in Fr 13-1 and 13-2 was presumably too small to be detected by immunoblotting with SH1 after desialylation. Reactivity of the gangliosides present in the four fractions with novel mAb PL82G2 was observed only after desialylation. This mAb does not react with Le^x; rather, it reacts with α1→3 fucosylated internal GlcNAc (not penultimate GlcNAc) (K. Handa, M. Stroud, T. White, and S. Hakomori, unpublished data). The only clear difference between nonbinding 12-2 and binding 13-1 and 13-2 was in the intensity of ^{1}H-NMR spectra at 4.875 ppm, representing Fucα1→3, which was 2−3 times higher in the binding fractions [Table 1; Figure 4D (preceding paper) vs Figure 1A (this paper)]. Together, these preliminary functional analytical data indicate that binding vs non-binding requirement may be due not to the presence of SLe^x, but

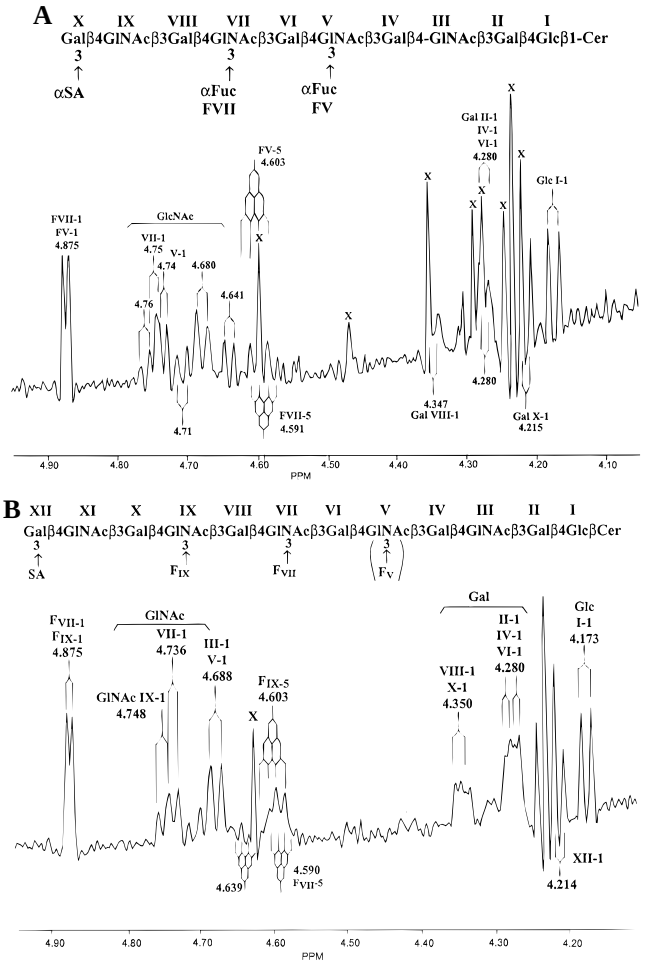


FIGURE 1: 500 MHz ^{1}H-NMR spectra of gangliosides present in selected fractions isolated from HL60 cells in DMSO-d_6/2% D_2O. Panel A: Selected downfield regions of Fr 13-1, 328 ± 2 K, from 4.10 to 4.90 ppm. Roman numerals refer to sugar residues in the structure shown at top. Arabic numerals refer to protons of pyranose ring in each sugar residue. Panel B: Selected downfield regions of Fr 14. Conditions and conventions as in panel A. X, impurity.

rather of terminally sialosylated, internally polyfucosylated structures. Gangliosides with SLe^x structure may be absent in these fractions, or present as extremely minor components undetectable by functional group analysis as performed in this study (see Discussion). This possibility was supported by further characterization of each fraction as described below.

Structures of gangliosides present in Fr 12-2, 12-3, 13-1, and 13-2 were determined by ES-MS because only trace quantities of these gangliosides were available. Structures of gangliosides in Fr 14 were determined by $^+$ion FABMS after permethylation, since sufficient quantity was available.

^{1}H-NMR Spectroscopy of Gangliosides. Fr 13-1 and 14. ^{1}H-NMR spectra of E-selectin-binding gangliosides in Fr 13-1 and 14 are shown in Figure 1, panels A and B, respectively. Although not of high quality, these spectra reveal a number of distinctive structural features. In comparison with the spectrum of Fr 11 (an ACFH-18 structure) (preceding paper, Figure 4D), Fr 13-1 and 14 exhibit several similar features characteristic of NeuAcα2→3 terminated, Fucα1→3 substituted poly(*N*-acetyllactosamine) structures, but with some significant differences. There is again observed a resonance at 4.875 ppm diagnostic for H-1 of Fucα1→3 linked to GlcNAcβ1→3 of type 2 poly(*N*-acetyllactosamine) structures (Levery et al., 1986; Nudelman et al., 1988), but the amplitude of this resonance appears to be greater than twice that of various GlcNAc H-1 resonances (at 4.680, 4.740, etc.) in both Fr 13-1 and 14. In contrast, the amplitude of the 4.875 ppm resonance in Fr 11 is roughly the same as those of the resonances for GlcNAc H-1. The additional Fucα1→3 also gives rise to an extra H-5 resonance at 4.591 ppm and to a similar H-5 resonance, as seen in the Fr 11 spectrum at 4.603 ppm (preceding paper, Figure 4D), partially overlapping each other in the spectrum of 13-1 (Figure 1A), along with a concomitant increase in the amplitude of the H-6 signal at 1.014 ppm. Similar H-5 quadrant resonances at 4.603 and 4.490 ppm, and a relatively weak resonance at 4.639 ppm, are seen in the spectrum of Fr 14 (Figure 1B), with a concomitant increase in the amplitude of the H-6 signal at 1.014 ppm. It is expected that the presence of an extra Fucα1→3 somewhere in the chain will be manifested by both short- and long-range substitution effects on other sugar resonances and that these effects will provide clues to the position of the additional residue. This will be discussed in more detail below.

In the spectrum of Fr 13-1, five different types of GlcNAcβ1→3 H-1 resonances are observed at 4.680, 4.710, 4.740, 4.750, and (minor) 4.760 ppm (Figure 1A). The spectrum of Fr 14 gave four types of GlcNAcβ1→3 H-1 resonances at 4.688, 4.705, 4.736, and 4.748 ppm, in an approximate ratio of 2:1:1:1, suggesting the presence of at least four LacNAc units attached to the usual lactosylCer core (Figure 1B). In addition to the extended Glcβ1→ H-1 resonance at 4.173 ppm, there appear to be three types of Galβ1→4 H-1 resonances at 4.214-4.215, 4.276-4.280, and 4.343-4.350 ppm, in the approximate ratio 1:3:2 [we are ignoring, for now, a number of weaker signals, including those at 4.309, 4.420, and 4.492, ascribing these to impurities (Figure 1B)]. Fr 13-1 gave very similar spectra in terms of Galβ1→4 H-1 resonances at 4.215, 4.280, and 4.347 ppm, which are tentatively assigned as shown in Figure 1A with reference to a similar spectrum given by Fr 11 (preceding paper, Figure 4D) and by Fr 14 (Figure 1B). The presence of a terminal NeuAcα2→3 residue is again clearly indicated by the observation of a doublet of doublets for H-3$_{eq}$ at the characteristic shift of 2.757 ppm, and by a NAc singlet at 1.888 ppm (data not shown) (Koerner et al., 1983; Levery et al., 1986, 1988) in spectra of both Fr 13-1 and 14 (data not shown). The resonance at 4.214 or 4.215 ppm in spectra of Fr 13-1 and 14 is characteristic for H-1 of the Galβ1→4 to which this NeuAc is linked, provided its aglyconic GlcNAcβ1→3 is not further substituted (*i.e.*, by Fucα1→3; Levery et al., 1988, 1989; Nudelman et al., 1988); *i.e.*, SLe^x structure is absent. The Galβ1→4 residue attached to Glcβ1→ can be expected to contribute one H-1 resonance to the overlapping signals at 4.276 ppm. This leaves four Galβ1→4 H-1 signals to be accounted for.

In the spectrum of Fr 14 (Figure 1B), the two remaining Galβ1→4 H-1 resonances in the group at 4.276−4.280 ppm, along with the two GlcNAcβ1→3 H-1 resonances at 4.680 ppm, clearly represent a pair of poly(*N*-acetyllactosamine) chain Galβ1→4GlcNAcβ1→3 units not influenced by Fucα1→3 substitutions (Levery et al., 1986; Nudelman et al., 1988). The other two H-1 resonances, at 4.343−4.350 ppm, are consistent with internal Galβ1→4 residues vicinal to Fucα1→3 on GlcNAcβ1→3 residues. The corresponding downfield shifted GlcNAcβ1→3 H-1 resonances are those found at 4.736 and 4.748 ppm. The fact that they are at

Table 2: Structures of Monosialogangliosides Present in Fr 12, 13, and 14 from HL60 Cells[a]

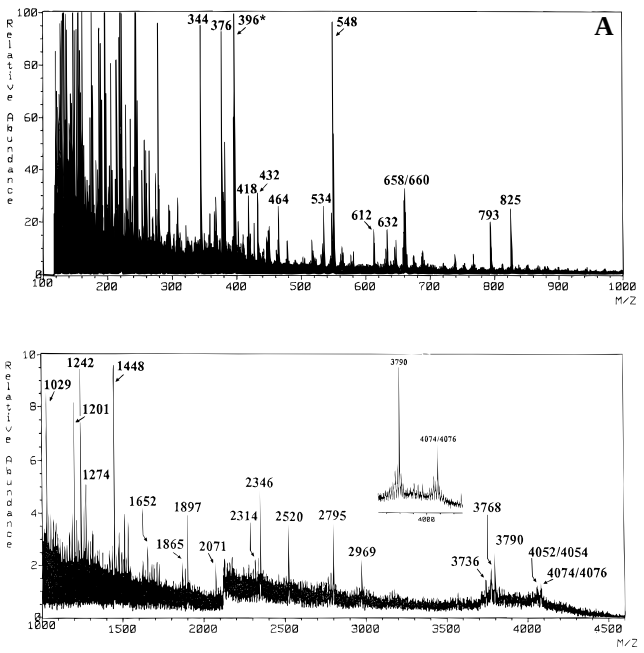
Fr.	Str.	Cer ion	EB*
12-2a	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα)	548	–
12-2b	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα)	548	–
12-3a	XVII Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα; 3 Fucα)	660	++
12-3b	XVIII Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα; 3 Fucα)	660	
13-1a	same as Str. XVII (Fr. 12-3a) but with different Cer	548	++
13-1b	same as Str. XVIII (Fr. 12-3b) but with different Cer	548	
13-2a	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα)	548	
13-2b	same as Str. XVII (Fr. 12-3a) but with different Cer	548	++
13-2c	XIX Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα; 3 Fucα)	660	
14-a	XX Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα)	548	++
14-b	XXI Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα)	548	
14-c	XXII Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα; 3 Fucα)	660	
14-d	XXIII Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (3 NeuAcα; 3 Fucα; 3 Fucα)	660	

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

different shifts indicates that they correspond to contiguous Galβ1→4(Fucα1→3)GlcNAcβ1→3 units; the difference arises from the long-range influence of Fucα1→3 on the next GlcNAcβ1→3 toward the nonreducing end. As previously noted (Levery et al., 1986), the additional shift for a GlcNAcβ1→3 already substituted by Fucα1→3 amounts to +0.12 ppm, consistent with the observed values. Furthermore, the chemical shifts are not consistent with the presence of SLe^x (preceding paper, Table 2, Str **XII**−**XVI**), which has the outermost Galβ1→4(Fucα1→3)GlcNAcβ1→3 group sialosylated due to the measurable shielding influence of NeuAcα2→3 on the nearest GlcNAcβ1→3 residue (Levery et al., 1988).

The values found previously for the anomeric protons of GlcNAcβ1→3 residues of a SLe^x−Le^x grouping were 4.739 and 4.726 ppm (Levery et al., 1986). These data are consistent only with the difucosyl ganglioside Str **XVII** (Table 2), with the last remaining β-GlcNAc H-1 resonance at 4.693 assignable to GlcNAcβ1→3 IX for the same reasons described previously for Fr 11 (preceding paper, Figure 4D) in relation to the ACFH-18 antigen: H-1 of β-GlcNAc XI would be expected to experience a downfield shift (on the order of +0.028 ppm) from a Fucα1→3 substituent on β-GlcNAc IX as well as a smaller upfield shift (on the order of −0.019 ppm) from the NeuAcα2→3 substituent on β-Gal XII (Levery et al., 1988). Mass spectrometric analysis of Fr 13-1 and 13-2 indicates that the majority of these fractions did not have SLe^x structure at the terminus (see following sections).

FABMS of Monosialogangliosides from HL60 Cells. Fr 14. The $^+$ion FABMS spectrum of permethylated Fr 14 is shown in Figure 2A. A predominant pseudomolecular ion (MH^+, nominal *m*/*z* 3768) is consistent with the sugar composition NeuAc·deoxyHex·Hex_7·$HexNAc_5$ plus Cer consisting of Sph/fatty acid combination d18:1/16:0. Cer ion observed at *m*/*z* 548. The spectrum clearly displays A_1-type fragments produced by cleavage of glycosyl linkages preferentially at *N*-acetylhexosaminyl linkages, with charge retention on the nonreducing portions. A_1 fragments observed at *m*/*z* 825, 1448, 1897, 2346, and 2795 established the sequence shown in Figure 2B, upper scheme, indicating a monofucosylated structure. The absence of *m*/*z* 999, representing NeuAc·Hex·deoxyHex·HexNAc, and the observed ion sequence place the deoxyHex residue on the second HexNAc from the nonreducing end. 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 presence of *m*/*z* 1274 in the sequence *m*/*z* 825, 1274, 1897, 2346, and 2795 suggests an isomeric structure as described in Figure



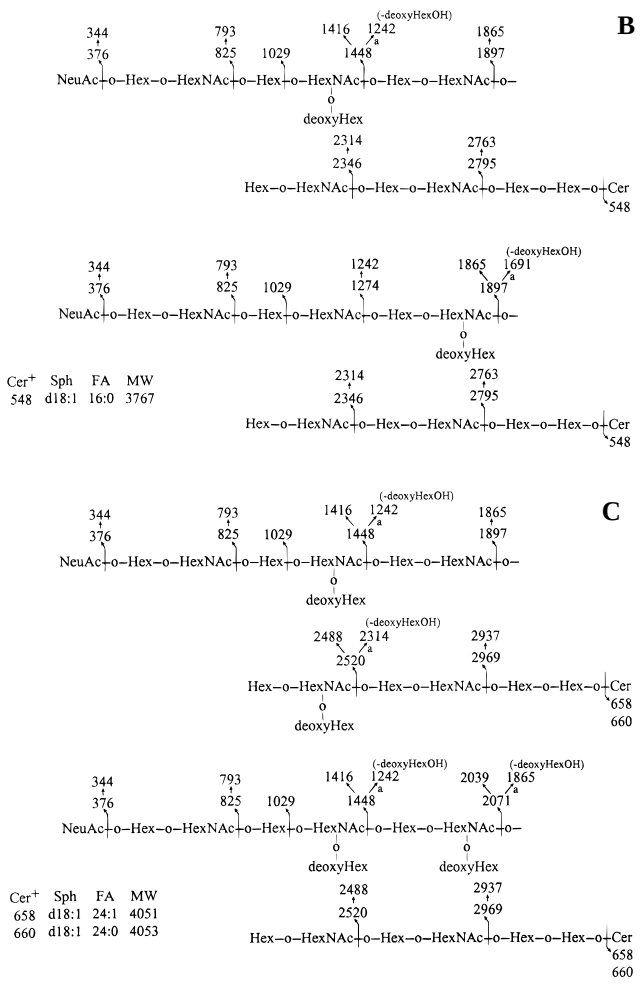


FIGURE 2: Fragmentation patterns and $^{+}$ion and $^{-}$ion FABMS spectra of various gangliosides isolated from HL60 cells. Panel A: $^{+}$Ion FABMS spectra of permethylated Fr 14 (mass range 100−4600 amu; scan slope 1′30″). Panel B: Upper and lower: two proposed isomeric monofucosylated structures and their fragmentation patterns. Neutral loss of MeOH yields secondary fragments; (a) neutral loss of 3-linked substituent. Panel C: Upper and lower: two proposed isomeric difucosylated structures and their fragmentation patterns. Neutral loss of MeOH yields secondary fragments; (a) neutral loss of 3-linked substituent.

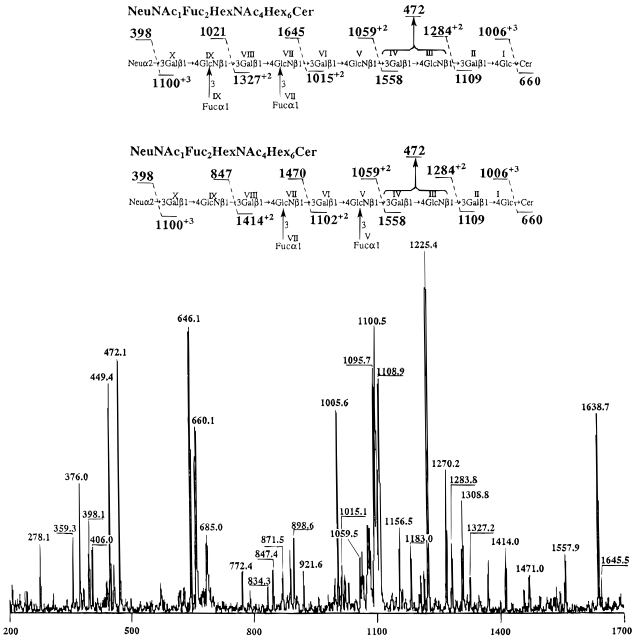


FIGURE 3: ES-MS and CID patterns of gangliosides present in Fr 12-3 and 13 isolated from HL60 •ells. CID spectrum of Fr 12-3 ion at *m/z* 1225.4 $[M + 3Na]^{3+}$. Upper and lower insets: two proposed isomeric structures and their fragmentation patterns.

2B, lower scheme. $[M + Na]^{+}$ and $[MH - CH_3OH]^{+}$ were observed at *m/z* 3790 and 3736, respectively.

A less abundant set of pseudomolecular ions (MH^{+}, nominal *m/z* 4052, 4054) are consistent with the composition NeuAc·deoxyHex$_2$·Hex$_7$·HexNAc$_5$ plus Cers of Sph/fatty acid combinations d18:1/24:1 and d18:1/24:0. Cer ions were observed at 658 and 660, respectively. A_1-type fragments established the sequence *m/z* 825, 1448, 1897, 2520, and 2969, indicating a difucosylated structure. See upper scheme in Figure 2C. The ion sequence supports placement of deoxyHex on the second and fourth HexNAc residues from the nonreducing end. Preferential neutral loss from the 3-position of HexNAc was observed at *m/z* 1242 (deoxyHexOH from *m/z* 1448) and *m/z* 2314 (deoxyHexOH from *m/z* 2520). The sequence *m/z* 825, 1448, 2071, 2520, and 2969 indicates the presence of an isomeric structure where deoxyHex units are placed on the second and third HexNAc residues from the nonreducing end. See lower scheme in Figure 2C. $[M + Na]^{+}$ and $[MH - CH_3OH]^{+}$ were observed at 4074, 4076 and 4020, 4022, respectively.

ES-MS Analysis of Monosialogangliosides from HL60 Cells. (A) Fr 12-3. Ganglioside(s) present in Fr 12-3 was analyzed by ES-MS following methylation and injection in the ES infusion solvent (data not shown). Distributions of sodium adducted ions were similar to those of Fr 12-2 (preceding paper, Figure 6A), but each peak had an additional satellite peak (ca. 50% abundance) 28 amu lower, which suggests alkane heterogeneity in the Cer moiety. Considering the molecular weight, the three major ions, *m/z* 925.6^{4+}, 1225.1^{3+}, and 1826.1^{2+}, were not related to gangliosides present in Fr 12-2 by differences in carbohydrate, and any compositions must include alterations in the Cer moiety as well. Collision of the triply charged ion, 1225.1^{3+}, provided the spectrum in Figure 3.

A LacN fragment ion series was observed from both termini, as well as an identical sphingenine fragment, *m/z* 287.1. Much larger was the Cer ion, *m/z* 660.1, attributed

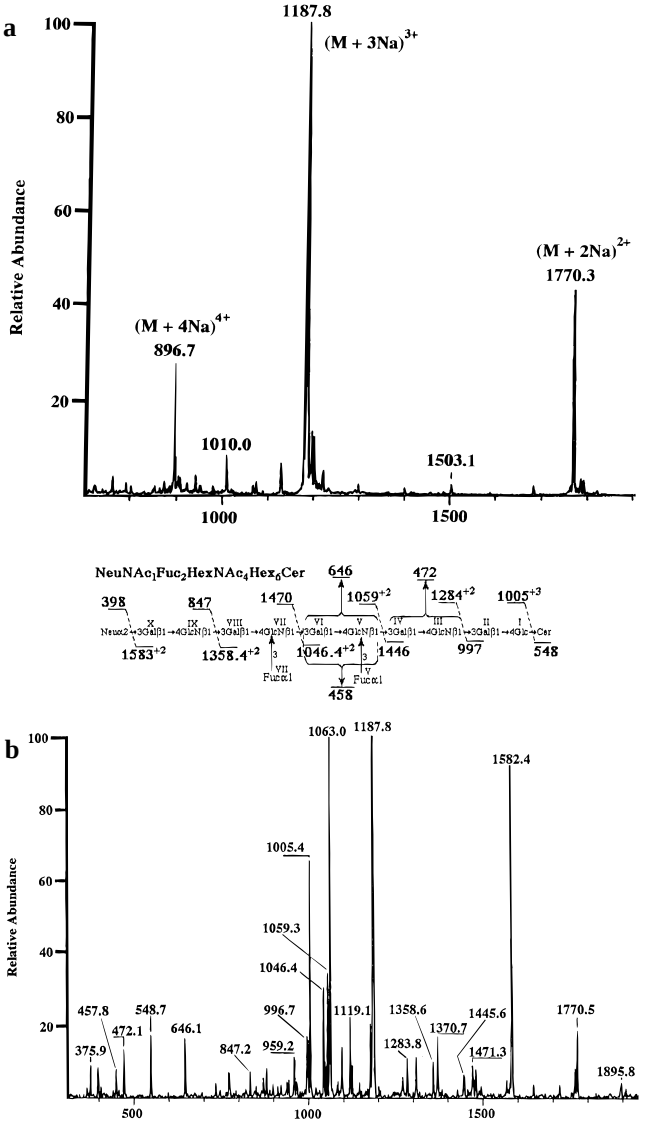


FIGURE 4: (a) ES-MS spectrum of Fr 13-1. (b) CID spectrum of *m*/*z* 1187.8 $[M + 3Na]^{3+}$. Inset scheme: proposed structure and fragmentation pattern.

by difference to the *N*-acyl tetracosanoyl moiety, $[CH_3(CH_2)_{22}C(O)-]$. Considering this difference, the molecular weight, 3606.3, will be satisfied by the addition of one more fucosyl residue. The sequence ions, *m*/*z* 1283.8, 1059.5, 1645.5, 1021.6, and 398, indicate fucosyl residues on the latter two nonreducing termini (upper inset scheme of Figure 3). Prominent LacN ions *m*/*z* 847.4 and 1471.0 indicate the selected ion, 1225.1^{3+}, to be composed of an isomeric mixture with a portion of the sample showing fucosylation shifted one residue toward the reducing end (lower inset scheme of Figure 3).

(B) Fr 13-1. The methylated ganglioside present in Fr 13-1 was dissolved in a methanol/water solution (6:4) containing 0.25 mM sodium hydroxide and profiled by ES-MS (Figure 4-a). Three major ions were observed in four, three, and two charge states, *m*/*z* 896.7^{4+}, 1187.8^{3+}, 1770.3^{2+}, indicating a single component of 3495.7 Da (Figure 4-a). The triply charged ion, *m*/*z* 1187.8, was analyzed by CID, which provided the spectrum shown in Figure 4-b. The product ions of this collision provided much of the same detailed information as in earlier figures (see inset scheme of Figure 4-b). The ion *m*/*z* 548.7 suggests an identical Cer as that detected in Fr 12-2 (preceding paper, Figure 6A) with an *N*-palmitoyl moiety. Thus, this single component in Fr 13-1 can be accounted for as a difucosylated *N*-palmitoyl analog. The LacN sequence ions from the nonreducing end indicate no fucosylation on the terminal LacN, *m*/*z* 847, whereas the next two LacN moieties are shifted up 174 amu, indicating that both carry a fucosyl residue. The carbohydrate residues on this myeloglycan appear to be identical with the difucosylated sample in Fr 12-3 (lower inset scheme of Figure 3).

(C) Fr 13-2. Direct ES-MS injection of permethylated gangliosides from Fr 13-2 provided the spectrum in Figure 5a. Triply charged ions were observed at *m*/*z* 1130.0^{3+}, 1188.1^{3+}, and 1282.9^{3+}, indicating three components of 3321.0, 3495.3, and 3779.7 Da, respectively. Each triply charged ion was analyzed by CID.

The CID spectrum of *m*/*z* 1130.0^{3+} is shown in Figure 5-b. Major fragments from the nonreducing end yielded the sequence Neu5Ac-LacN-LacN(deoxyHex)-LacN-LacN-Hex-Hex-Cer, where LacN = Gal′GlcNAc (see inset scheme of Figure 5-b). The observed ion at *m*/*z* 1470 (Neu5AcLacN-LacN(deoxyHex)) and the absence of *m*/*z* 1021 representing Neu5Ac-LacN(deoxyHex) positioned the fucosyl moiety on the penultimate lactosamine residue. The ganglioside described here is similar to that observed in Fr 12-2.

The CID spectrum of *m*/*z* 1188.1^{3+} is provided in Figure 5-c. Fragment ions from both nonreducing and reducing ends were observed satisfying the sequence Neu5Ac-LacN-LacN(deoxyHex)-LacN(deoxyHex)-LacN-Hex-Hex-Cer (see inset scheme of Figure 5-c). As observed in Fr 13-1, the sequence ions from the nonreducing end indicate no fucosylation on the distal LacN (*m*/*z* 1583^{2+}) but rather on the next two internal LacN moieties (1358^{2+} and 1046.4^{2+}). The carbohydrate composition and Cer at *m*/*z* 548 are consistent with the structure established in Fr 13-1.

The CID spectrum of *m*/*z* 1282.9^{3+} is shown in Figure 5-d. Cer-containing fragments positioned fucosyl residues at the penultimate LacN (*m*/*z* 1158^{3+}) and at the next two internal LacN moieties (*m*/*z* 1414^{2+} and 1102^{2+}). Reducing and nonreducing end fragments support the sequence Neu5Ac-LacN(deoxyHex)-LacN(deoxyHex)-LacN-(deoxyHex)-LacN-Hex-Hex-Cer (see inset scheme of Figure 5-d). The Cer ion at *m*/*z* 660 with an *N*-acyl tetracosanoyl moiety is consistent with the Cer ion observed in Fr 12-3.

Reactivities of Various Gangliosides with mAbs FH6, SNH3, SNH4, and CSLEX. These mAbs were originally identified as being directed to SLe^x or sialosyl dimeric Le^x structure. All the mAbs cross-reacted with Str **IV**, **VI**, **VII**, **VIII**, and **X** (preceding paper, Table 1) and with Str **XII**, **XIII**, and **XIV** (preceding paper, Table 2). They did not absolutely require SLe^x terminal structure for their reactivity. When CSLEX or FH6 was established, there was negligible reactivity reported with such structures as **IV** and **VI** (Fukushi et al., 1984b; Fukushima et al., 1984). However, obvious cross-reactivity with these structures is now demonstrated. This is seen with both solid-phase antibody binding assay on multiwell plates coated with GSL, and the TLC overlay technique. Figure 6 shows reactivities of FH6, SNH3, and SNH4 with sialosyl dimeric Le^x ($VI^3NeuAcV^3FucIII^3FucnLc_6Cer$), SLe^x hexasaccharide Cer ($IV^3NeuAcIII^3FucnLc_4Cer$), and sialosyl*nor*hexaosyl-Cer ($VI^3NeuAcnLc_6Cer$). mAb SNH3 showed high specificity with structures having SLe^x epitope, but still cross-reacted with sialosyl type 2 chain without fucose (see Discussion).

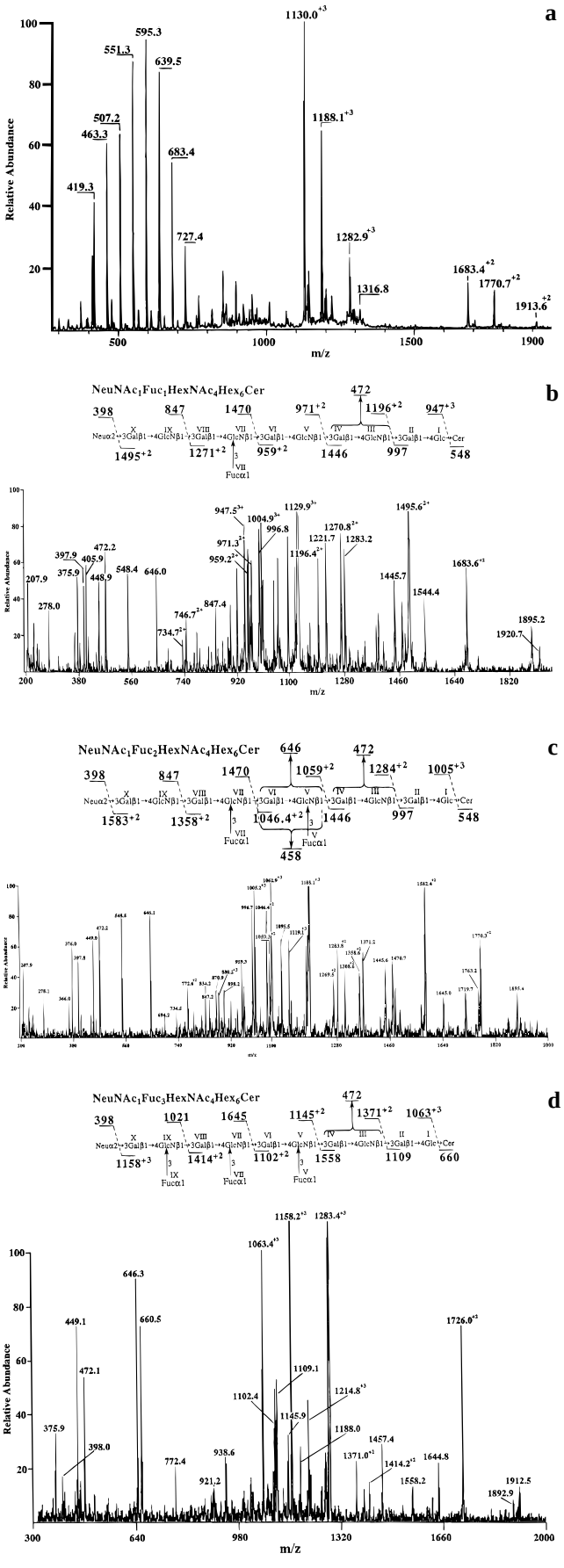


FIGURE 5: (a) ES-MS spectrum of Fr 13-2. (b) CID spectrum of *m/z* 1129.9 $[M + 3Na]^{3+}$. (c) CID spectrum of *m/z* 1188.1 $[M + 3Na]^{3+}$. (d) CID spectrum of *m/z* 1283.4 $[M + 3Na]^{3+}$. Inset schemes: proposed structures and fragmentation patterns.

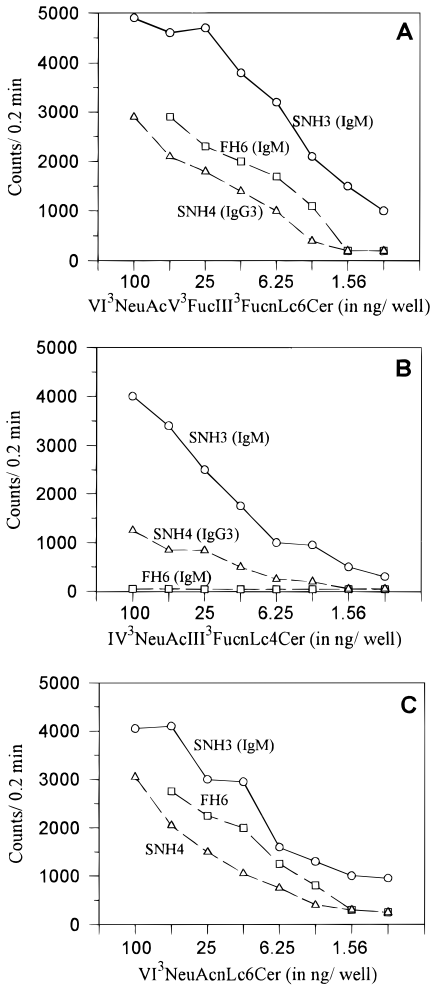


FIGURE 6: Reactivities of mAbs previously reported as being directed to SLe^x (FH6, SNH3, SNH4) with three GSL structures. Panel A: Sialosyl dimeric Le^x ($VI^3NeuAcV^3FucIII^3FucnLc_6Cer$). Panel B: SLe^x hexasaccharide Cer ($IV^3NeuAcIII^3FucnLc_4Cer$). Panel C: Sialosyl*nor*hexaosyl-Cer ($VI^3NeuAcnLc_6Cer$). 96-well plates were coated with the respective GSLs in the quantities shown on abscissa and subjected to antibody-binding assay as described previously (Fukushi et al., 1984b). Note that sialosyl*nor*hexaosyl-Cer reacted with all three mAbs.

DISCUSSION

Does SLe^x Represent a Physiological Ligand of E-Selectin in Neutrophils and HL60 Cells? The generally-accepted concept that SLe^x is the target epitope of E-selectin binding is based primarily on the following facts: (a) SLe^x is thought to be expressed on human neutrophils, leukemic leukocytes, myelogenous leukemia HL60 cells, monocytic leukemia U937 cells (Fukushi et al., 1984a; Symington et al., 1985; Ito et al., 1994), and mutants of CHO cells (Campbell & Stanley, 1984), because these cells are reactive with various mAbs claimed to be directed to SLe^x despite their strong cross-reactivity with other structures (see Specificity of Anti-SLe^x Antibodies..., below); (b) these "SLe^x-expressing cells" bind to TNFα- or IL-1-activated ECs which express E-selectin (Lowe et al., 1990; Phillips et al., 1990; Ito et al., 1994); (c) E-selectin-dependent adhesion of the above-listed cells to activated ECs is inhibited by liposomes containing GSLs or oligosaccharides with a terminal SLe^x structure (Phillips et al., 1990). Previous analytical data on E-selectin binding carbohydrates were N-linked structures of neutrophils and leukemic cells (Asada et al., 1991; Patel et al., 1994)

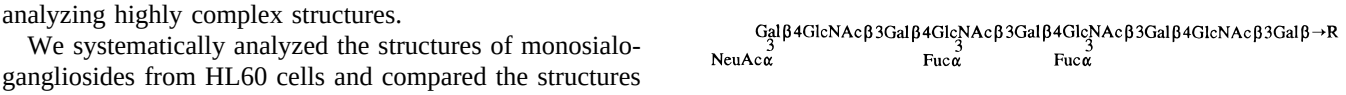
based on the enzymatic or chemical degradation patterns of oligosaccharides released from glycoproteins. However, there has been no unambiguous chemical analysis of carbohydrate structures expressed on normal human neutrophils or HL60 cells capable of binding to E-selectin, mainly because of the nonavailability of sufficient material and technical difficulties in separating pure compounds and analyzing highly complex structures.

We systematically analyzed the structures of monosialogangliosides from HL60 cells and compared the structures of gangliosides capable vs noncapable of E-selectin binding. E-selectin binding patterns were also compared with those of gangliosides from normal human neutrophils. For E-selectin binding capability, we obtained satisfactory results using a TLC staining method (Swank-Hill et al., 1987) with ^{32}P-labeled CHO cells which permanently express E-selectin. Based on this study, we were faced with the question of whether SLe^x acted as a physiological ligand of E-selectin, although our data were limited to GSLs expressed on normal leukocytes and HL60 cells. The major ganglioside (99%) of Fr 13-1 (which shows strongest E-selectin binding) is Str **XVII**. Fr 14, which contained Str **XXII** and **XXIII** but not even a trace quantity of SLe^x-containing gangliosides, nevertheless bound clearly to E-selectin. As further discussed in subsequent sections, human leukocytes (neutrophils) and HL60 cells express very little SLe^x but do have a relatively abundant structure (exemplified by Str **XVII** and **XXII** and collectively termed "myeloglycan"), which may represent a physicological ligand for E-selectin.

A novel ganglioside, X^{3}NeuAcVII^{3}FucV^{3}FucnLc$_{10}$Cer (Str **XVII**), was the major ganglioside component of Fr 13-1. The same ganglioside was also found as a major component in Fr 12-3 and 13-2. Another novel ganglioside X^3-NeuAcIX^{3}FucVII^{3}FucnLc$_{10}$Cer (Str **XVIII**) was found in Fr 12-3 and 13-1 as an extremely minor component, detected by ES-MS with CID. Novel ganglioside X^{3}NeuAcIX^3-FucVII^{3}FucV^{3}FucnLc$_{10}$Cer (Str **XIX**) was found as a very minor component in Fr 13-2. The major component of both Fr 13-1 and 13-2 was Str **XVII**.

Fr 14, containing a 12-sugar core, consisted of four components. The major components were Str **XX** and **XXII**. Str **XXI** and **XXIII** were very minor components. Because monofucosyl components had smaller Cer and difucosyl components had larger Cer, they migrated together as a single band on HPTLC. Not even trace quantities of SLe^x-containing gangliosides (*e.g.*, Str **XVIII** and **XIX**) were found in Fr 14.

Structural Requirements for E-Selectin Binding in HL60 Cells and Human Neutrophils. SLe^x gangliosides having a 4- to 8-monosaccharide core are absent from HL60 cells and human neutrophils. Comparison of E-selectin binding ability of various fractions containing gangliosides with a 10-sugar core, *i.e.*, Str **X** and **XI** (nonbinding) vs **XVII**, **XVIII**, and **XXII** (binding), indicates that terminal 2→3 sialylation and multiple fucosylation at GlcNAc-VII and -V, or GlcNAc-IX, are the structural requirements for E-selectin binding. The fractions showing the strongest E-selectin binding activity were Fr 13-1 and 13-2, followed by 12-3. The presence of SLe^x terminus with internal fucosylation (Table 1, Str **XVIII** and **XIX**) might be invoked to explain the E-selectin binding properties of these fractions. However, Fr 13-1 contains Str **XVII** and only a trace quantity (<1%) of Str **XVIII**. While Fr 12-3 contained much higher levels of Str **XVIII** than did 13-1, E-selectin binding activity of Fr 12-3 was less than that of Fr 13-1. Str **XVII**, therefore, plays a major role in E-selectin binding activity. This conclusion was supported by the fact that Fr 14 shows clear binding to E-selectin, completely lacks SLe^x structure, and contains Str **XXII**. Importantly, Str **XVII** and **XXII** share the common structure



Components having Str **XVII** or **XXII**, which lack SLe^x but have multiple internal fucosylation, collectively termed "myeloglycan", appear to be those responsible for physiological E-selectin binding under static conditions. TLC staining assay of gangliosides from neutrophils shows the same E-selectin binding pattern as for HL60 cells. These findings were based on the static adhesion conditions described for this study, *i.e.*, TLC overlay technique employing E-selectin-expressing CHO Cells. Involvement of sulfate in E-selectin-dependent adhesion has been suggested (Yuen et al., 1992). However, none of the E-selectin-binding GSLs in HL60 cells or neutrophils contain sulfate.

The present results apply only to Fr 1−14 separated on HPLC, particularly to Fr 12−14, which contain the E-selectin-binding structures termed "myeloglycan". A series of slower-migrating myeloglycans, as shown in Figure 2 of the preceding paper, may have analogous structures, *i.e.*, 16-, 18-, 20-, 22- or 24-monosaccharide cores with terminal α2→3 sialosylation and multiple α1→3 fucosylation at GlcNAc of LacNAc units. The myeloglycan core, consisting of unbranched LacNAc, is known to form a helical structure (Niemann et al., 1978), which provides a specific spatial relation between sialosyl and multiple fucosyl residues at specific sites of GlcNAc.

Specificity of Anti-SLe^x Antibodies, Enzymatic Synthesis of Myeloglycan, and Its Possible Presence in Glycoprotein Side Chains from HL60 Cells. Monoclonal antibodies FH6 (Fukushi et al., 1984b), CSLEX (Fukushima et al., 1984), SNH3, and SNH4 (Muroi et al., 1992), previously characterized as being directed to SLe^x, are known to react with neutrophils, monocytes, leukemic leukocytes, HL60 cells, and U937 cells (Ito et al., 1994; Fukushi et al., 1984a; Symington et al., 1985). However, we carefully reinvestigated the specificity of all these mAbs and found that they cross-react with not only myeloglycan-bearing and SLe^x-bearing GSLs, but also "VIM-2" epitope-bearing GSLs. Strong reactivity of cells with these mAbs obviously does not confirm the presence of SLe^x. Extensive reinvestigation of the specificity of these mAbs is needed.

This study is limited to GSLs because we could isolate them to near purity and obtain unambiguous structural analysis. Terminal structures present in glycoprotein glycans are not always the same as those in GSLs (Stroud et al., 1994). However, no previous study has provided unambiguous evidence that SLe^x is a *major* epitope on neutrophils or HL60 cells. The previous claim (Fukuda et al., 1984, 1985) for SLe^x terminus detected in $^+$ion FABMS of permethylated myelogenous leukemia cell glycan was based primarily on the extremely small intensity of *m*/*z* 999 signal (the specific signal for permethylated SLe^x in $^+$ion FABMS). Furthermore, the origin of the samples was leukemic leukocytes. PLA structure (NeuAcα2→3/6)$_{0-2}$(Galβ1→4GlcNAc)$_n$ with or without α1→3 fucosylation to GlcNAc of multiantennary

N-linked glycan was claimed to be present as a minor component of CD11/CD18 from normal human neutrophils (Asada et al., 1991). The presence of SLe^x was assumed, but no clear evidence was presented. Myeloglycan, rather than SLe^x, may have been present as the major epitope. More recently, N-linked radiolabeled carbohydrate profiles of various tumor cell lines including HL60 and U937 cells were compared with the known chromatographic behavior and digestibility of glycosylhydrolases. Oligosaccharides bound to an E-selectin column were found to be a very minor component having a tetraantennary structure with sialosyl dimeric Le^x present at the Manα1→3Man side chain. Detailed data in support of this claim were unclear (Patel et al., 1994).

The α1→3 fucosyltransferase (FT) from HL60 cells (myeloid type FT) (Potvin et al., 1990; Goelz et al., 1990) can create a Fucα1→3 linkage to the subterminal and internal GlcNAc of PLAs to form Le^x, dimeric Le^x (Le^x−Le^x), or trimeric Le^x (Le^x-Le^x-Le^x), but it cannot effectively synthesize SLe^x, *i.e.*, create a Fucα1→3 linkage to the penultimate GlcNAc when terminal α2→3 sialic acid is present. Under certain conditions, myeloid type FT IV is capable of preferentially transferring Fucα1→3 to the internal GlcNAc (Holmes & Macher, 1993). The α1→3FT capable of transferring Fuc to the penultimate GlcNAc when the terminal Gal is α2→3 sialylated has been clearly distinguished from myeloid type FT IV, and termed α1→3 FT VII (Sasaki et al., 1994; Natsuka et al., 1994). In these latter studies, only short-chain substrates were used, *i.e.*, LacNAc, sialosyl-LacNAc, and sialosyl-lacto-*N*-neotetraose. It is conceivable that FT IV and FT VII cooperatively synthesize myeloglycan, but not the SLe^x epitope alone, if a suitable PLA having a 10- to 12-sugar core is used as substrate.

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