Sialosyl-Fucosyl Poly-LacNAc without the Sialosyl-Le^x Epitope as the Physiological Myeloid Cell Ligand in E-Selectin-Dependent Adhesion: Studies under Static and Dynamic Flow Conditions[†,‡]

Kazuko Handa, Mark R. Stroud,[§] and Sen-itiroh Hakomori*

Pacific Northwest Research Foundation, 720 Broadway, Seattle, Washington 98122, and Department of Pathobiology, University of Washington, Seattle, Washington 98195

Received May 20, 1997; Revised Manuscript Received July 15, 1997[⊗]

ABSTRACT: The majority of E- and P-selectin ligands in leukocytes and myelocytic or monocytic leukemia cells are carried by transmembrane glycoproteins having a tandem repeat mucin-like domain through which O-linked carbohydrate ligands are carried. However, determination of structure and adhesive function of carbohydrates in glycoproteins is extremely difficult because of the extensive structural heterogeneity and the scarcity of material for functional analysis. We have overcome this difficulty through use of poly-LacNAc gangliosides isolated from a large quantity of (∼1.2 L packed) HL60 cells [Stroud, M. R., Handa, K., Salyan, M. E. K., Ito, K., Levery, S. B., Hakomori, S., Reinhold, B. B., & Reinhold, V. N. (1996) *Biochemistry 35*, 758−769, 770−778]. We identified two major types of poly-LacNAc gangliosides without the sialosyl-Le^x epitope as being capable of binding to E-selectin: (i) those having a single α1→3 fucosylation at internal GlcNAcs but not at the penultimate GlcNAc and (ii) those having double α1→3 fucosylation at internal GlcNAcs, excluding the penultimate GlcNAc. Gangliosides from group i above did not show any adhesion under static conditions, but showed strong adhesion under dynamic flow conditions. Gangliosides from group ii above showed adhesion under both static and dynamic conditions, as did sialosyl-Le^x (SLe^x)-containing structures in previous studies. However, SLe^x-containing poly-LacNAc gangliosides are virtually absent or present in only trace quantities in leukocytes and HL60 cells. Poly-LacNAc gangliosides from groups i and ii above, lacking SLe^x structure, are the major membrane components of leukocytes and HL60 cells. These carbohydrates, bound to lipid or to protein, may therefore be the physiological epitope for E-selectin-dependent binding of these cells, particularly under dynamic flow conditions.

Sialosyl-Le^x (SLe^x)[1] binds to E- and P-selectin (Phillips et al., 1990; Polley et al., 1991) and is therefore generally believed to be a binding epitope for neutrophils and myeloid cells [for reviews, see Lasky (1995) and Varki (1994)]. However, there has been no unambiguous chemical demonstration of the presence of SLe^x in leukocytes, HL60 cells, or other hematopoietic cells, nor a demonstration of the fact that SLe^x functions as a binding epitope in neutrophils or myeloid cells under physiological conditions (see Discussion). The major carriers of E- and P-selectin-binding carbohydrate epitopes present in HL60 and U937 cells were believed to be transmembrane proteins having O-linked (but not N-linked) structure, since benzyl-GalNAc, but not castanospermine or swainsonine, reduces E- and P-selectin-dependent binding of these cells (Kojima et al., 1992a). The best-characterized carrier molecule, expressed in hematopoietic cells, is PSGL-1, a disulfide-dependent dimeric type 1 transmembrane protein having a tandem repeat O-glycosylation site (Moore et al., 1994; Sako et al., 1993). Many human tumor cell lines expressing SLe^x or SLe^a bind to E-selectin but do not bind to P-selectin unless PSGL-1 expression is induced through transfection of its gene (Handa et al., 1995). E-Selectin ligand carriers in human leukocytes and most human cell lines have not been clearly identified, although accumulated data suggest that they are O-linked glycoproteins, since E-selectin-dependent binding can be inhibited by incubation with benzyl-GalNAc.

Therefore, determination of O-linked carbohydrate structures as they relate to adhesive properties of carrier molecules is of crucial importance, although this is extremely difficult because of the extensive structural heterogeneity and the scarcity of material. An even greater problem is the fact that isolated O-linked oligosaccharide cannot be used for functional analysis (see Discussion).

To overcome these difficulties, we examined structures and E-selectin binding properties of lacto series gangliosides present in human leukocytes and HL60 cells. A series of unbranched poly-LacNAc gangliosides having more than eight core monosaccharide units were shown to bind to E-selectin with the thin-layer chromatography overlay technique (Stroud et al., 1996a,b). This paper describes comparative binding of poly-LacNAc gangliosides having (i) α1→3 fucosylation at a single internal GlcNAc or (ii) α1→3 fucosylation at two internal GlcNAc residues under static and dynamic flow conditions. Since poly-LacNAc chains without SLe^x structure comprise the major membrane component of human leukocytes and HL60 cells and are

[†] This work was supported by National Cancer Institute Outstanding Investigator Grant CA42505 (to S.H.), in part by Seikagaku Corp., and in part by a grant from the Mizutani Foundation.

[‡] Dedicated to Roger W. Jeanloz on the occasion of his 80th birthday.

* Author to whom correspondence should be addressed. Phone: 206-726-1222. Fax: 206-726-1212. E-mail: hakomori@u.washington.edu.

[§] Present address: Northwest Hospital, Seattle, WA 98125.

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1997.

[1] Abbreviations: BSA, bovine serum albumin; FCS, fetal calf serum; Fr., fraction; GSL, glycosphingolipid; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PBS(+), PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$; SLe^a, sialosyl-Le^a; SLe^x, sialosyl-Le^x; Str., structure.

S0006-2960(97)01181-1 CCC: $14.00

Table 1: Structures of Poly-LacNAc Gangliosides Used in This Study

Fr.	Str.	Cer ion*
7	1 NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer	546
8	2 Galβ4GlcNAcβ (6) Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3)	658/660
**	3 Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3), Fucα (3)	
10-1	4† Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3)	546/548
10-2	5† Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3)	546/548
12-2	6 Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3) (same as ACFH-18 antigen)	658/660
12-3a	7‡ Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3), Fucα (3)	660
13-1	7‡ Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3), Fucα (3)	548
14-a	9§ Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3)	548
14-b	10§ Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3)	548
14-c	11§ Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3), Fucα (3)	660
14-d	12§ Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer; NeuAcα (3), Fucα (3), Fucα (3)	660

*Cer ions indicate the following combinations of sphingosine (Sph) and fatty acids (FA): 546, Sph d18:1, FA 16:1; 548, Sph d18:1, FA 16:0; 658, Sph d18:1, FA 24:1; and 660, Sph d18:1, FA 24:0. **The compound containing this structure was originally isolated from colonic adenocarcinoma (Fukushi et al., 1984). The compound used in the present study was enzymatically synthesized by fucosylation of Str. 1 (Holmes et al., 1986). † The molar ratio of Str. 4 and 5 in Fr. 10 was 1:1. The subfractions are designated 10-1 and 10-2. ‡ The molar ratio of Str. 7 and X^{3}NeuAcIX^{3}FucVII^{3}FucnLc$_{10}$Cer in Fr. 12-3 was roughly 85:15. The subfractions are designated 12-3a and 12-3b. The molar ratio of Str. 7 and X^{3}NeuAcIX^{3}FucVII^{3}FucnLc$_{10}$Cer in Fr. 13-1 was roughly 99:1. § The molar ratio of Str. 9−12 in Fr. 14 was roughly 5:1:3:1. The subfractions are designated 14-a−d, respectively (Stroud et al., 1996b).

usually shared between glycoproteins and GSLs, their E-selectin adhesive properties are obviously of physiological importance.

MATERIALS AND METHODS

GSLs and Monosialogangliosides. Monosialoganglioside fractions used for the adhesion assay are shown in Table 1. All these compounds (except SLe^x-Le^x) were prepared from a large quantity of HL60 cells as described previously (Stroud et al., 1996a,b). Structures were verified by proton nuclear magnetic resonance spectroscopy, positive-ion fast atom bombardment mass spectrometry, and electrospray mass spectrometry with collision-induced dissociation of permethylated compounds as described previously (Stroud et al., 1995, 1996a,b). Structures of Fr. 10-1 and 10-2 were further confirmed by endo-β-galactosidase digestion (Fukuda et al., 1978). Lc$_3$Cer (GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; shown in boldface in structure **1** below) was the major degradation product released from Fr. 10-1, while a compound with TLC mobility similar to that of Cer hexasaccharide was the major product from Fr. 10-2. This compound was identified as GlcNAc-Le^x (shown in boldface in structure **2** below) on the basis of its conversion to the Le^x Cer pentasaccharide with the jackbean β-*N*-acetylhexosaminidase treatment. Thus, the major endo-β-galactosidase cleavage sites for Fr. 10-1 and 10-2 are as follows:

1 NeuAcα2→3Galβ4GNβ3Galβ4↓**GNβ3Galβ4GlcCer**; Fucα (3); **Lc$_3$**

2 NeuAcα2→3Galβ4GNβ3Galβ4↓**GNβ3Galβ4GNβ3Galβ4GlcCer**; **Fucα (3)**; **GlcNAc-Le^x**

Details of structural determination based on enzymatic degradation will be described elsewhere.

SLe^x-Le^x ($VI^3NeuAcV^3FucIII^3FucnLc_6Cer$; Str. 3) was produced from $VI^3NeuAcnLc_6Cer$ by enzymatic fucosylation using Colo205 fucosyltransferase.

To determine quantity of gangliosides adhering to polystyrene beads affixed to glass plates, and the resistance of this adhesion to washing with water, gangliosides (*e.g.* Fr. 9) were labeled by the periodate/$NaB(^3H)_4$ method as follows. Gangliosides (50−100 μg) were dissolved in 100 μL of 1 mM acetate buffer at pH 5.0 and kept in ice/water. One hundred microliters of 1 mM $NaIO_4$ was added, the mixture kept on ice for 10 min, and glycerol added. The solution was dialyzed overnight in a "Spectra/Por 3" dialysis tube (Spectrum Medical Industries, Houston, TX), reduced for 1 h in 1 mM NaOH containing $NaB(^3H)_4$ (0.5 mCi), further reduced for 1 h in cold $NaBH_4$, and dialyzed against distilled water. The ^3H-labeled gangliosides (~5000 cpm per 10 μg), purified on C-18 silica gel column, were used for quantification of gangliosides remaining on plastic surfaces in dynamic flow experiments.

E- or P-Selectin-Expressing Cells and mAbs Which Block E- or P-Selectin-Dependent Adhesion. CHO cells transfected with E- or P-selectin cDNA were established as described previously (Handa et al., 1995). E- or P-selectin-expressing transfectants were isolated by cytofluorometry using anti-E-selectin mAb E1C or anti-P-selectin mAb P5A. These mAbs were established through immunization of BALB/c mice with E- or P-selectin-expressing NS1 cells followed by fusion with NS1 cells (Handa et al., 1997). Inhibition of E- or P-selectin-dependent cell adhesion was performed using these mAbs at 10 μg/mL.

E-Selectin-Dependent Cell Binding to Various Poly-LacNAc Gangliosides under Static Conditions. (1) Static Adhesion Assay Using 96-Well Plates. Gangliosides were dissolved in 50% ethanol and serially diluted in 96-well plates (the first well contained 200 ng), and plates were dried at 37 °C for 5 h. Gangliosides used were SLe^x-Le^x (Str. 3 in Table 1); poly-LacNAc ganglioside Fr. 10, containing 10-1 (Str. 4) and 10-2 (Str. 5); Fr. 12, containing 12-3a (Str. 7) and 12-2 (Str. 6) (molar ratio of 12-3a:12-2 is ~4:1); pure Fr. 12-2 (Str. 6); and Fr. 13-1 (Str. 7 with a Cer different from 12-3a; see Table 1). ^3H-labeled poly-LacNAc ganglioside, coated on a 96-well plate, cannot be washed off in any aqueous medium and is recovered at 100%. Plates with the same serial dilutions of SLe^x-Le^x and poly-LacNAc gangliosides were prepared for control cell adhesion in the presence of EDTA or anti-E-selectin mAb E1C. Plates, both experimental and control, were washed with PBS(+) and blocked with 3% BSA in PBS(+). E-selectin-expressing CHO cells (Handa et al., 1997) were metabolically labeled with [^3H]thymidine overnight, detached with 0.02% EDTA, and resuspended in the binding buffer (1% FCS, RPMI, and 10 mM HEPES at pH 7.4) at 2×10^6 per milliliter. In inhibition experiments, EDTA-detached cells were preincubated with mAb E1C (10 μg/mL) at room temperature for 30 min. A 50 μL aliquot of this cell suspension (containing 1×10^5 cells; ~5000 cpm) was added to ganglioside-coated wells and incubated at room temperature for 30 min. After incubation, each well was filled with PBS(+). The 96-well plates were immersed in PBS(+) in a large container and suspended upside-down above the bottom of the container for 10 min to allow nonadherent cells to sediment (fall) out of the plates. The plates were then turned right-side up and removed from the container, and PBS in each well was removed by gentle aspiration. Bound cells were detached with trypsin−EDTA and counted with a scintillation counter.

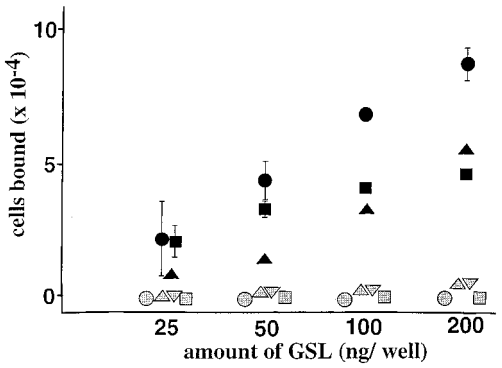

FIGURE 1: Comparative adhesion of E-selectin-expressing CHO cells to poly-LacNAc gangliosides with SLe^x structure and to those without SLe^x structure and having internal multiple fucosylation (myeloglycan), coated on flat-bottom 96-well plates (Falcon, Becton-Dickinson, Lincoln Park, NJ). Various quantities of gangliosides as shown in Table 1 were coated on plates, and ^3H-labeled E-selectin-expressing CHO cells (1×10^5 per 50 μL per well; ~5000 cpm) in the presence or absence of mAb E1C (0.5 μg of Ig per 50 μL per well) were overlaid for 1 h without agitation, followed by a specific washing procedure as described in Materials and Methods. Cells adhered to the bottom of each well were estimated by ^3H activity and converted to cell number (ordinate). Points represent mean experimental minus control value in the presence of mAb E1C, ± SE of triplicate experiments. No binding was observed with P-selectin-expressing cells: (●) SLe^x-Le^x (Table 1, Str. 3); (■) Fr. 13-1 (Str. 7); and (▲) Fr. 12, a mixture of Fr. 12-2 (Str. 6) and Fr. 12-3a (Str. 7). The approximate molar ratio of Fr. 12-2:12-3a is 1:4: (shaded ○) Fr. 7 (Str. 1), (shaded △) Fr. 10-1 (Str. 4), (shaded ▽) Fr. 10-2 (Str. 5), and (shaded □) Fr. 12-2 (Str. 6).

(2) Static Adhesion Assay Using Polystyrene Latex Beads. To observe static adhesion with the same matrix used for the dynamic adhesion assay, the following procedure was used. Polystyrene latex beads with 1 μm diameters (IDC Sphere; IDC, Portland, OR), affixed to objective microscope slides, were used as carriers of poly-LacNAc GSLs. Sixty microliters of bead suspension (containing 1×10^{11} beads/mL) was placed in Eppendorf tubes and washed with absolute ethanol three times. Sedimented 1 μm beads were suspended in 2 mL of ethanol. One microliter aliquots of the suspension were placed on freshly opened microscope slides (Labcraft Superfrost Plus, Curtin Matheson Scientific, Houston, TX). Beads were distributed homogeneously on the glass surface within a circular spot with a diameter of ~1 cm. Slides were heated at 150 °C for 50 s, which caused the beads to adhere strongly to the surface so that they could not be washed off by a water stream at various velocities. Gangliosides dissolved in 2-propanol/hexane/water (55:25:20 by volume) were applied to latex beads affixed to the slides; namely, 1 μL aliquots containing 50−100 ng of ganglioside were placed on the center of the circular spot. The ganglioside thus became affixed to the bead surface. Plates were immersed in 3% BSA in PBS(+) for 1 h at room temperature and washed three times with PBS(+).

Plates were overlaid with E-selectin-expressing CHO cells freshly harvested and suspended in the binding buffer (5×10^5 cells/mL), and left for 15 min without moving. Washing three times with binding buffer was usually sufficient to eliminate nonadherent cells from beads. However, careful

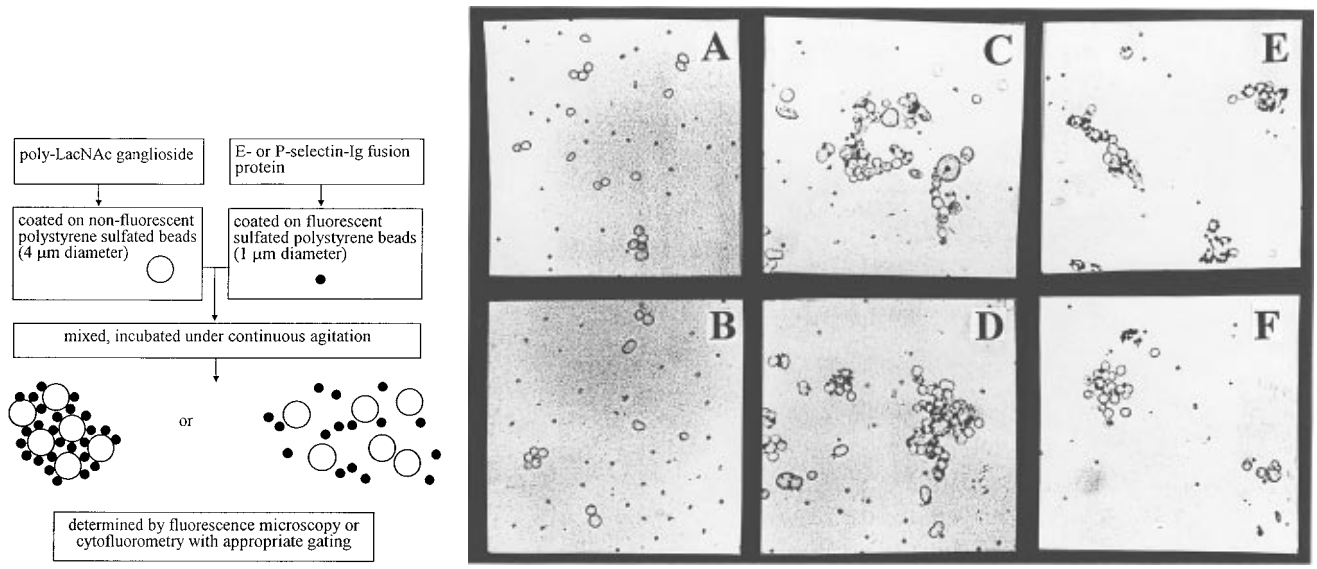

FIGURE 2: (Left) Procedure for measuring binding of poly-LacNAc gangliosides to E- and P-selectin under continuous motion. (Right) Fluorescence micrographs showing aggregation of nonfluorescent polystyrene beads coated with poly-LacNAc gangliosides and fluorescent beads coated with the E−selectin-Ig fusion protein, under motion. Coated poly-LacNAc gangliosides were as follows, for each micrograph: (A) VI^{3}NeuAcnLc$_6$Cer (Str. 1 in Table 1), (B) IV^{3}NeuAcnLc$_4$Cer, (C) Fr. 13-1 (X^{3}NeuAcVII^{3}FucV^{3}FucnLc$_{10}$Cer; Str. 7), (D) Fr. 10 (mixture of VIII^{3}NeuAcIII^{3}FucnLc$_8$Cer and VIII^{3}NeuAcV^{3}FucnLc$_8$Cer; Str. 4 and 5; approximate molar ratio of 1:1), (E) SLe^x-Le^x (VI^{3}NeuAcV^{3}FucIII^3-FucnLc$_6$Cer; Str. 3), and (F) SLe^x (IV^{3}NeuAcIII^{3}FucnLc$_4$Cer).

microscopic examination had to be repeated until the cells placed on control polystyrene beads were washed out. Plates were then fixed with 1% glutaraldehyde in PBS(+), and the number of cells adhered to the layer of poly-LacNAc ganglioside-coated beads was counted. Essentially all the ^{3}H-labeled poly-LacNAc ganglioside coated on beads was recovered after plates were washed three times with binding buffer.

Determination of Selectin−Poly-LacNAc Ganglioside Interaction Based on Aggregation of Coated Beads under Continuous Agitation. To observe the binding of selectin to poly-LacNAc gangliosides under motion, a sensitive method was used (illustrated in Figure 2, left panel) in which two polystyrene bead systems (i and ii as follows) were mixed. (i) Poly-LacNAc gangliosides having a single or double internal Fuc or SLe^x were coated on nonfluorescent beads (diameter of 4 μm). (ii) Fluorescent beads (diameter of 1 μm) were coated with the E- or P-selectin−Ig fusion protein. A mixture from methods i and ii was put on a gyratory shaker. Interaction was revealed under fluorescence microscopy by the presence of nonfluorescent beads surrounded with fluorescent beads, and aggregates consisting of products from methods i and ii. The number and intensity of fluorescent bead aggregates were also measured by flow cytometry with appropriate gating. An example of the overall procedure, for measuring binding of E- or P-selectin to gangliosides, is described below.

(1) Preparation of Ganglioside-Coated Beads. Polystyrene beads (5×10^6, diameter of 4.2 μm) (IDC Spheres, IDC) in suspension were washed with ethanol by centrifugation. One microgram of GSL in 50 μL of ethanol was added to the washed beads, and the mixture was evaporated under a nitrogen stream. Beads were resuspended in 1% BSA/PBS, washed by centrifugation, blocked with 3% BSA/PBS(+) at room temperature for 2 h, centrifuged, resuspended in 1% BSA/PBS(+)/0.1% azide, and stored at 4 °C.

(2) Preparation of Selectin-Coated Fluorescent Beads. Yellow-green fluorescent sulfated latex beads (diameter of 1 μm) (Molecular Probes, Portland, OR) were coated with goat anti-human IgG (Fc fragment specific) antibody (Jackson Immunoresearch Lab, West Grove, PA) according to the manufacturer's protocol. Beads were washed three times with PBS and blocked with 3% BSA/PBS at 4 °C for 2 h. Blocked beads ($\sim 5 \times 10^8$) were mixed with 5 mL of the E- or P-selectin−Ig fusion protein containing culture supernatant ($\sim$1 μg/mL fusion protein) from CHO transfectants, at 4 °C for 12−18 h. This mixing procedure was repeated twice more using new culture supernatant containing the fusion protein. Beads were washed with PBS and incubated in 1 mL of PBS containing 50 μg of human IgG (Jackson Immunoresearch Lab). For control beads, human IgG was used at 1 μg/mL, instead of the fusion protein.

(3) Reactivity of the Two Bead Systems under Motion. Ganglioside-coated beads were mixed with fluorescent beads, in PBS(+) or in the presence of 5 mM EDTA at room temperature for various durations on a gyratory shaker. For inhibition experiments, E-selectin-coated fluorescent beads were preincubated with mAb E1C. The resulting suspension was subjected to fluorescence microscopy and flow cytometer (Coulter) analysis.

E-Selectin-Dependent Cell Rolling and Tethering to Various Poly-LacNAc Gangliosides under Defined Dynamic Flow Conditions. The experimental system employed is similar to that previously described (Alon et al., 1995); *i.e.* ganglioside is placed on a plastic plate and exposed to a flow of E-selectin-expressing cells. Specifically, polystyrene latex beads (diameter of 4 or 1 μm) were spread on glass slides and affixed by heating, and 1 μL aliquots of the ganglioside solution were added as described under Static Adhesion Assay Using Polystyrene Latex Beads. The quantity of ganglioside used was 100 ng in most experiments (*e.g.* those shown in Figures 4A−E and 5A) or 1 ng in some experiments (*e.g.* that shown in Figure 5B). Plates coated with poly-LacNAc gangliosides as described above were incubated with 3% BSA, incubated at room temperature for at least 1 h, and washed with PBS(+). Slides were placed in

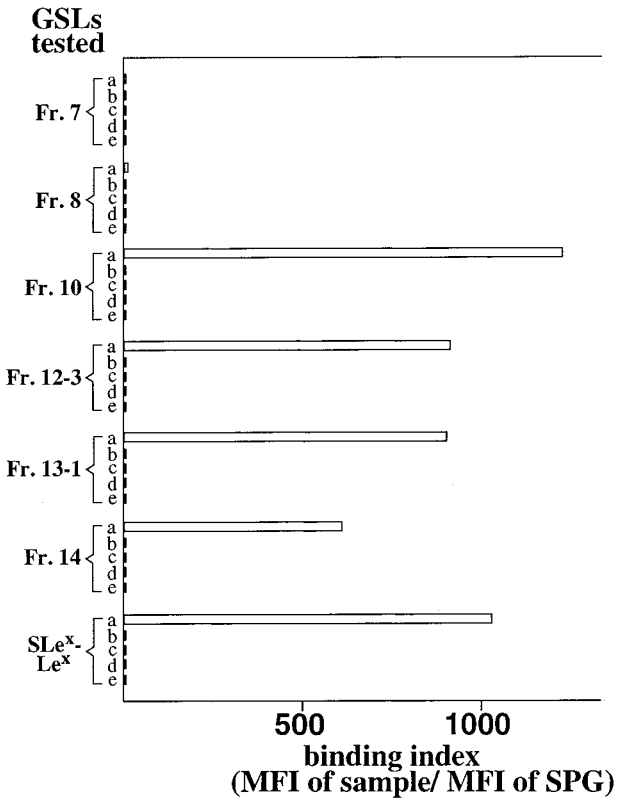

FIGURE 3: Binding of poly-LacNAc gangliosides to E- and P-selectin-coated fluorescent beads under motion. One microgram of various poly-LacNAc gangliosides (as indicated on the ordinate) was coated on polystyrene beads (diameter of 4 μm), which were then mixed with fluorescent beads (diameter of 1 μm) coated with the E-selectin−IgG or P-selectin−IgG fusion protein as described in Materials and Methods. Mixtures were shaken on a rotary shaker for 30 min at room temperature and then analyzed by flow cytometry: (a) E-selectin-coated beads, (b) E-selectin-coated beads in the presence of 5 mM EDTA, (c) P-selectin-coated beads, (d) human IgG-coated beads (control), and (e) E-selectin-coated beads in the presence of anti-E-selectin mAb E1C. The length of the column represents the binding index, *i.e.* the mean fluorescence intensity (MFI) of ganglioside-coated beads divided by the MFI of SPG ($IV^3NeuAcnLc_4Cer$)-coated beads. All poly-LacNAc gangliosides with single internal fucosylation (Fr. 10; Str. 4 and 5 in Table 1) or double internal fucosylation, *i.e.* Fr. 12-3 (Str. 7 with a larger Cer), Fr. 13-1 (Str. 7 with a smaller Cer), and Str. 14 (a mixture of 14-a−d), showed clear binding. In contrast, Fr. 7 (Str. 1) and Fr. 8 (Str. 2), which have no internal fucosylation, failed to bind.

a parallel plate laminar flow chamber connected to an infusion pump (model 935, Harvard Apparatus, Cambridge, MA). The assembly, originally described by Lawrence et al. (1990) and Lawrence and Springer (1991), simulates the flow shear stress present in physiological microvascular environments. The inlet of the flow chamber was connected to a cell suspension pool. A laminar flow with a defined rate and wall shear stress was achieved by backward manipulation of the infusion pump with a 50 or 30 mL syringe connected to the outlet of the chamber. A suspension of E- or P-selectin-expressing CHO cells (1×10^5/mL), harvested with EDTA, washed, and resuspended in binding buffer, was infused into the assembly at various laminar flow rates. Cell movements were observed under an inverted phase-contrast microscope (Diaphot-TMD, Nikon) and recorded with a time-lapse videocassette recorder. Numbers of rolling and tethering cells during a 2 min period at shear stresses from 0.4 to 12.0 dyn/cm^2 were observed and counted from at least 10 fields on videotape. Wall shear stress (T) was calculated as described previously (Kojima et al., 1992b), using the equation of Lawrence et al. (1987, 1990). Fifty to sixty percent of ^{3}H-labeled Fr. 7 ganglioside adsorbed on beads was recovered even after infusion under high shear stress (12.0 dyn/cm^2).

RESULTS

Adhesion of E-Selectin-Expressing CHO Cells to Poly-LacNAc Gangliosides under Static Conditions. Adhesion of E-selectin-expressing CHO cells to various poly-LacNAc gangliosides, including those containing the SLe^x determinant, was examined under two different static conditions as described in Materials and Methods. Under one condition, various quantities (25−200 ng/well) of poly-LacNAc gangliosides were coated on 96-well plates. Equal quantities (1×10^5 cells per 100 μL) of ^{3}H-labeled E-selectin-expressing CHO cells were added in the presence or absence of anti-E-selectin mAb and kept without movement for 30 min. Strong binding was observed with SLe^x-Le^x. Moderate binding was observed with Fr. 12 (a mixture of 12-2 and 12-3, *i.e.* Str. 6 and 7 in Table 1) and Fr. 13-1 (Str. 7), which have two Fuc residues at internal (but not the penultimate) GlcNAcs (Figure 1A). No binding was observed with Fr. 10 (mixture of 10-1 and 10-2, *i.e.* Str. 4 and 5) or Fr. 12-2 (Str. 6), which have one Fuc residue at internal GlcNAcs, or with Fr. 7, which has no Fuc residue (Figure 1).

A similar trend of adhesion was observed under an alternative static condition using polystyrene beads as described in Materials and Methods. Adhesion to SLe^x-Le^x was strongest, followed by adhesion to Fr. 13-1 and 14. There was no adhesion to Fr. 10-1 (Str. 4), 10-2 (Str. 5), or Fr. 7 (Str. 1) (data not shown).

Binding of Poly-LacNAc Gangliosides to E-Selectin-Coated Fluorescent Beads under Continuous Agitation. A sensitive assay method using fluorescence microscopy or flow cytometry to detect interaction of poly-LacNAc gangliosides with selectin was developed (see Materials and Methods). In contrast to the results under static conditions (see above), poly-LacNAc gangliosides having a single Fuc residue $\alpha1\rightarrow3$ linked to internal GlcNAc (*e.g.* Fr. 9, 10, and 12-2 in Table 1) bound clearly to E-selectin. Fr. 10, which contains approximately equal quantities of Str. 4 (10-1) and Str. 5 (10-2) (Table 1) showed a degree of E-selectin binding, as measured by fluorescence microscopy and by flow cytometry, roughly equal to that of poly-LacNAc gangliosides having two Fuc residues $\alpha1\rightarrow3$ linked to internal GlcNAc (*e.g.* Fr. 13-1 and 14) or those having the SLe^x determinant (Figure 2, right panel). Fr. 7 (Str. 1 in Table 1) and Fr. 8 (Str. 2) showed no binding to E-selectin, even under this dynamic condition (column a of each fraction in Figure 3). None of these fractions bound to P-selectin (column c of each fraction) under this condition. The E-selectin binding was completely inhibited by the presence of 5 mM EDTA (column b) or anti-E-selectin mAb E1C (column e). No binding was observed to control beads coated with human IgG (column d). These quantitative findings suggest that E-selectin binding properties of poly-LacNAc gangliosides are very different under static and dynamic flow conditions. This concept was confirmed by subsequent experiments as described below.

E-Selectin-Dependent Cell Tethering and Rolling on Poly-LacNAc Gangliosides under Dynamic Flow Conditions.

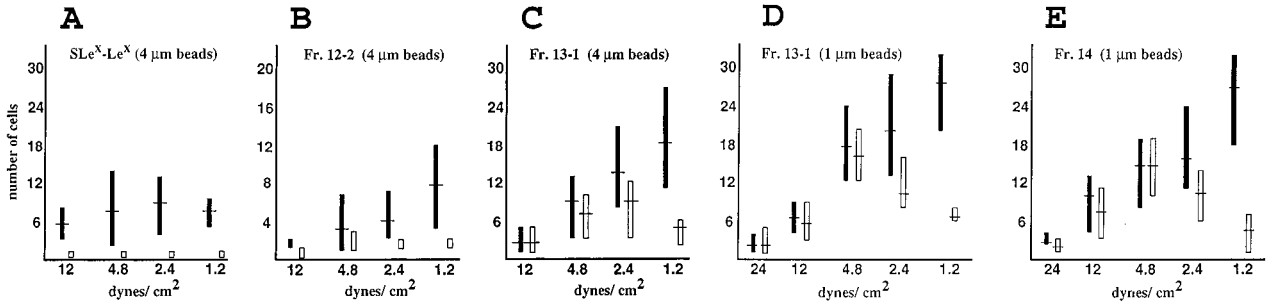

FIGURE 4: Rolling and tethering of E-selectin-expressing CHO cells under dynamic flow conditions. Various poly-LacNAc gangliosides were quantitatively adsorbed on beads affixed to glass microscope slides as described in Materials and Methods. One microliter of a solution (IHW, 50:25:20 by volume) containing 100 ng of ganglioside was added at the center of a polystyrene bead layer affixed to a glass slide throughout the experiments. One microliter of the solution spread to a circular area with a diameter of ~1 cm. Gangliosides adsorbed on beads were resistant to washing in water (see Materials and Methods). Slides were blocked by placing in 3% BSA in PBS at room temperature for 1 h and assembled in a parallel-plate laminar flow chamber. E-selectin-expressing CHO cells were freshly harvested and suspended in the binding buffer (1×10^5 cells/mL). The cell suspensions were placed in an infusion pump connected to the flow chamber and infused into the assembly at various laminar flow rates. Cell movement and tethering were observed under a phase-contrast microscope and recorded with a videocassette recorder. The range (minimum to maximum) of the number of cells in 10−20 microscope fields is shown by the bar: (open bar) rolling cells and (solid bar) tethering cells. Because of the extensive variation in cell numbers (see the text), the mean value of cell numbers (rather than the midpoint of the range) is indicated by a horizontal line for each bar. (A) Rolling and tethering of cells on SLe^x-Le^x (Str. 3 in Table 1) adsorbed on 4 μm beads affixed to a glass plate. There were adherent, but no rolling (*i.e.* rolling number = 0), cells in every field at all shear stresses. **(**B) Fr. 12-2 (Str. 6) on 4 μm beads. Rolling was highest at 4.8 dyn/cm^2. Both rolling and tethering were lower than those for Fr. 13-1 and 14 (panels C and E) but comparable to those of SLe^x-Le^x. **(**C) Fr. 13-1 (Str. 7) on 4 μm beads. Rolling was more frequent at 4.8 and 2.4 dyn/cm^2 than at 1.2 dyn/cm^2. **(**D) Fr. 13-1 on 1 μm beads. Rolling was highest at 4.8 dyn/cm^2. Tethering was higher and rolling was lower at 1.2 dyn/cm^2. **(**E) Fr. 14 (Str. 9-12) on 1 μm beads. Rolling and tethering were highest at 2.4 and 4.8 dyn/cm^2. Rolling was lower at 1.2 dyn/cm^2. Note that the numbers of rolling and tethering cells were higher with 1 μm than with 4 μm beads, in general. The number of tethering cells increased as shear stress decreased. The number of rolling cells was maximal at 4.8 or 2.4 dyn/cm^2.

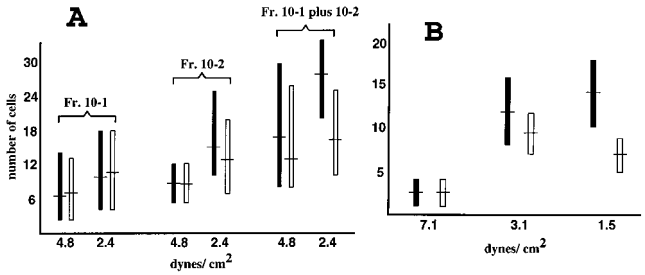

FIGURE 5: Rolling and tethering of E-selectin-expressing cells on Fr. 10-1 or Fr. 10-2 poly-LacNAc gangliosides or a mixture of the two. **(**A) One hundred nanograms of Fr. 10-1 (Str. 4 in Table 1) (left plot) or Fr. 10-2 (Str. 5) (middle plot) or a mixture of 50 ng each of Fr. 10-1 and 10-2 (right plot). **(**B) A mixture of 0.5 ng each of Fr. 10-1 and 10-2 (total quantity of 1 ng). In both experiments, gangliosides were added to 1 μm polystyrene beads affixed to glass slides as described in Materials and Methods and the Figure 4 legend. Data are expressed as described for Figure 4.

Various poly-LacNAc gangliosides having one or two Fuc residues α1→3 linked to internal (not the penultimate) GlcNAcs, or having SLe^x at the terminus, were coated on polystyrene beads affixed to slides assembled in a parallel-plate laminar flow chamber as described in Materials and Methods. The number of cells rolling and tethering were counted in 10 or more microscopic fields recorded on videotape. Stronger rolling and tethering were observed when poly-LacNAc gangliosides were adsorbed on 1 μm beads (Figure 4D,E) than when they were observed on 4 μm beads (Figure 4B,C). Strong rolling and tethering were observed with Fr. 13-1 and 14, which have two α1→3 residues in internal GlcNAc (Figure 4C−E), followed by Fr. 12-2 (Figure 4B) and SLe^x-Le^x (Figure 4A). For SLe^x-Le^x-coated plates, there were essentially no rolling cells, and the number of tethering cells was essentially the same at different shear stresses (Figure 4A). For plates coated with Fr. 12-2 (Str. 6), the number of tethering cells increased as shear stress decreased (Figure 4B). The difference between panels A and B of Figure 4 is small but was reproduced in independent experiments. The same trend seen for Fr. 12-2 was more clearly observed for Fr. 13-1 (Str. 7) and 14 (a mixture of Str. 9−12) (Figure 4C−E). Fr. 10-1 (Str. 4), 10-2 (Str. 5), and 12-2, having single internal fucosylation, showed clear rolling and tethering under dynamic conditions, although they showed no adhesion under static conditions. With Fr. 12-2, 13-1, and 14, rolling was maximal at 2.4−4.8 dyn/cm^2 (in the range of physiological shear stress), whereas tethering was higher at lower shear stress. Tethering was nearly constant with SLe^x-Le^x-coated beads, and rolling was negligible (Figure 4A). Rolling and tethering were slightly enhanced for a mixture (50 ng each) of Fr. 10-1 and 10-2 as compared to that with 100 ng of 10-1 or 10-2 alone (Figure 5). Rolling and tethering were observed clearly when various smaller quantities of ganglioside were coated on plates. Data from the experiment in which 0.5 ng each of Fr. 10-1 and 10-2 were applied on plates, under the same experimental conditions as for the 100 (50 + 50) ng experiment, are shown in Figure 5B.

DISCUSSION

A large variety of carbohydrate structures, *e.g.* SLe^x, sialosyl dimeric Le^x (SLe^x-Le^x), sulfated Le^x, SLe^a, and their respective synthetic derivatives, were found to bind to E- and P-selectin in previous studies [for review, see Lasky (1995) and Varki (1994)]. The binding ability of these structures is of pharmacological interest, but it is unlikely that many of them are physiological ligands. For example, SLe^a is completely absent in neutrophils and myelocytes. The claim for sulfated Le^x was based on binding of sulfated Le^x-neoglycolipid (the oligosaccharide incidentally isolated from ovarian pseudomucin) to E-selectin (Yuen et al., 1992). The claim for SLe^x and sialosyl dimeric Le^x was based on their inhibitory effect on E- and P-selectin-dependent adhesion (Phillips et al., 1990; Polley et al., 1991) or their ability

to bind directly to E- and P-selectin (Handa et al., 1991). In these experiments, epitopes were presented as gangliosides, isolated from tumors or obtained by chemical or enzymatic synthesis, and incorporated in liposomes or adsorbed on latex beads.

The presence of SLe^x or sialosyl dimeric Le^x in neutrophils and HL60 cells has been simply imagined, because of the strong reactivity of these cells with mAbs known to be reactive with SLe^x or sialosyl dimeric Le^x (Symington et al., 1985), despite the fact that these mAbs were later found to be strongly cross-reactive with fucosyl-poly-LacNAc gangliosides, termed "myeloglycan", which are the major component of neutrophils and HL60 cells (Stroud et al., 1996b). Only a few chemical studies examining the presence of SLe^x in these cells have been carried out. It is clear that the quantity of SLe^x chemically detectable in these cells is extremely small. Positive-ion FAB-MS of methylated N-linked structures in leukemic leukocytes gave a barely detectable *m*/*z* 999, representing the SLe^x structure (Fukuda et al., 1984), even though a large, extended scale was used. SLe^x in N-linked side chains of leukocytes and HL60 cells was assumed to be present on the basis of enzymatic hydrolysis (Asada et al., 1991) and MALDI mass spectrometry (Patel et al., 1994), although <1% of the total carbohydrate chains bound to the E-selectin column.

The major physiological P-selectin ligand, PSGL-1, expressed in neutrophils and HL60 cells, has been identified as a carrier of O-linked but not N-linked oligosaccharides (Moore et al., 1994; Sako et al., 1993). While PSGL-1 also functions as an E-selectin ligand, it may not be the major ligand. In mouse myeloid cells, a glycoprotein "E-selectin ligand 1" (ESL-1) was identified (Levinovitz et al., 1993). The human equivalent to ESL-1 is unknown. In these ligand glycoproteins, a sufficient number of oligosaccharides are assembled to display optimal density and orientation. It is of crucial importance to elucidate (a) the primary structures, (b) the functional (binding) activity of each oligosaccharide, and (c) their assembly in these glycoproteins. Unfortunately, this information is unavailable because of the extreme technical difficulty in obtaining a sufficient quantity of these ligand glycoproteins.

For example, recent studies of O-linked side chains present in PSGL-1 indicate the presence of SLe^x carried by difucosylated polylactosamine and by the mucin type 2 core structure. The proposed structures, however, are based solely on the change of the radiolabeled oligosaccharide peak by enzymatic degradation (Wilkins et al., 1996). Direct chemical analysis of the structures involved in adhesion was not performed, presumably because of the scarcity of material. Myeloglycan-type structures without the SLe^x terminus cannot be excluded by the methods used in the above study. The extreme scarcity of purified O-linked oligosaccharide in PSGL-1 does not allow determination of its binding ability to E- or P-selectin. Oligosaccharides are capable of inhibiting selectin-dependent adhesion only at millimolar levels. Therefore, it is impossible to perform a functional assay of liberated oligosaccharide.

To overcome the difficulties involved in structural and functional analysis of O-linked side chains of a defined glycoprotein, we utilized GSLs present in leukocytes and HL60 cells. GSLs and gangliosides have the novel property of forming clusters when they are applied in solution on plastic surfaces or incorporated in liposomes. Clustered GSLs, regardless of the chain length of carbohydrates, display a strong ability to bind to both E- and P-selectin (Handa et al., 1991). Utilizing this property in the TLC overlay technique, radiolabeled E-selectin-expressing CHO cells bound only to poly-LacNAc gangliosides having more than eight monosaccharide core units. Through systematic isolation and characterization of poly-LacNAc gangliosides prepared from >1.2 L of HL60 cells and from ~100 mL of human leukocytes, we were able to identify two types of major structures (I and II): (I) those having a single α1→3 fucosylation at internal GlcNAcs (as below):

a. Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
3 3
↑ ↑
NeuAcα2 Fucα1

b. Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
3 3
↑ ↑
NeuAcα2 Fucα1

c. Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
3 3
↑ ↑
NeuAcα2 Fucα1

and (II) those having double α1→3 fucosylation at internal GlcNAcs, excluding the penultimate GlcNAc (as below):

Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
3 3 3
↑ ↑ ↑
NeuAcα2 Fucα1 Fucα1

Structures of type Ia−c above, which comprise about 70−75% of total poly-LacNAc gangliosides from HL60 cells, did not show E-selectin binding under static conditions. In contrast, these structures bound to E-selectin strongly or clearly under motile conditions using a two-bead aggregation system as described in this paper or under dynamic flow conditions using a Lawrence−McIntire assembly (Lawrence et al., 1990). Structures of type Ia and -c contain a single α1→3 fucosylation at the second internal GlcNAc from the terminus. A ganglioside with the same structure was originally isolated from chronic myelogenous leukemia cells and characterized (Fukuda et al., 1986). The terminal structure, including 2→3 NeuAc and 1→3 Fuc linked to two LacNAc units, was identified as an epitope defined by mAb VIM-2 (Macher et al., 1988) and was thought to be the E-selectin binding epitope (Tiemeyer et al., 1991). This structure does not bind to E-selectin under static conditions, as shown in our previous (Stroud et al., 1996a) and present study. Surprisingly, the same structure binds clearly to E-selectin under dynamic flow conditions in two entirely different experimental systems (Figures 2−5).

Structures of type II above, which comprise about 20−24% of total poly-LacNAc gangliosides from HL60 cells, showed clear E-selectin binding under both static and dynamic flow conditions, as did poly-LacNAc gangliosides having SLe^x structure in previous studies.

In addition to types I and II, we found a type III having multiple α1→3 fucosylation at internal GlcNAcs and at the penultimate GlcNAc:

III Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
3 3 3 3
↑ ↑ ↑ ↑
NeuAcα2 Fucα1 Fucα1 Fucα1

Structures of type III, which comprise 1% or less of total poly-LacNAc gangliosides from HL60 cells, showed E-

selectin binding under both static and dynamic flow conditions, similar to that of type II. Because the quantity of type III was so small, we could not compare its binding activity quantitatively with that of type I or II. Binding activity of SLe^x-Le^x (Str. 3, Table 1) under dynamic conditions is less than that of type I and II above.

We did not find poly-LacNAc ganglioside with α1→3 fucosylation at the penultimate GlcNAc only (*i.e.* SLe^x without internal fucosylation), having a carbohydrate chain shorter than eight monosaccharides. Recently, Müthing et al. (1996) detected SLe^x ganglioside having a ceramide hexasaccharide backbone without internal fucosylation in neutrophils. The reason for the discrepancy between their findings and our findings is unclear, but the quantity of such a ganglioside in neutrophils and myelocytes, if present, must be extremely small relative to the quantity of poly-LacNAc ganglioside.

Lacto-series type 2 chain, particularly poly-LacNAc, is present at O-linked, N-linked, and lipid-linked structures. It is reasonable to assume that structures of types I and II above are present in glycoprotein as well. Ganglio- and globo-series gangliosides, in contrast, are found exclusively in GSLs. It should be noted that the type III structure, as typified by SLe^x-Le^x, caused tethering but not rolling under dynamic conditions, whereas types I and II caused rolling and tethering. Since type I and II structures comprise over 95% of poly-LacNAc gangliosides in leukocytes and HL60 cells, they obviously have greater physiological relevance than SLe^x or SLe^x-Le^x.

The quantity of poly-LacNAc ganglioside coated on plates in most experiments (*e.g.* those shown in Figures 4A−E and 5A) was 100 ng. However, even 1 ng of ganglioside was sufficient to cause obvious rolling and tethering (Figure 5B). Rolling and tethering could not be observed when 0.05 ng was coated (data not shown). Thus, the optimal quantity of poly-LacNAc ganglioside presented on the solid phase is in the 1−100 ng range.[2]

Our results suggest an explanation of why a series of poly-LacNAc structures with differing location of α1→3 fucosylation, and terminal sialylation, are present and form arrays on the neutrophil surface. Combinations of specific structures may form high-, middle-, or low-affinity binding sites in order to optimally bind E-selectin under high-, middle-, or low-shear stress dynamic flow conditions. Poly-LacNAc is known to form helical structures (Atkins et al., 1974; Rees, 1975). Poly-LacNAc gangliosides, including myeloglycan (Stroud et al., 1995, 1996a), may have helical backbone structures onto which multiple or single fucosyl residues are linked and oriented in different directions. Such helical structures, based on the positioning of the fucosyl residues, could interact with each other and may form high-affinity binding sites under dynamic flow conditions *in vivo*. Under dynamic flow conditions, the spatial relationship between terminal sialic acid and internal Fuc arranged on a helical poly-LacNAc chain may create flexibility in distance and orientation of these two functional groups, which are recognized by the lectin domain of selectin. The rationale for increased rolling and tethering displayed by poly-LacNAc fucogangliosides relative to that by SLe^x-containing structures may reside in the above relationship.

[2] The minimum quantity of poly-LacNAc ganglioside coated on a 1 cm diameter circle (area of $\sim 7.8 \times 10^7$ μm^2) which causes obvious rolling and tethering is 0.05−0.1 ng; the optimal quantity (used in most of these experiments) is within the 1−100 ng range. The 7.8×10^7 μm^2 area is roughly equal to the combined surface area of 4×10^4 HL60 cells, assuming that a single HL60 cell is spherical, with a diameter of 25 μm and a surface area of 1963 μm^2 ($4\pi r^2$). The amount of poly-LacNAc ganglioside present in 4×10^4 HL60 cells is estimated to be ∼1−2 ng, which is within the range (1−100 ng) causing rolling and tethering.

ACKNOWLEDGMENT

We thank Dr. Stephen Anderson for scientific editing and preparation of the manuscript.

REFERENCES

Alon, R., Feizi, T., Yuen, C.-T., Fulbrigge, R. C., & Springer, T. A. (1995) *J. Immunol. 154*, 5356−5366.

Asada, M., Furukawa, K., Kantor, C., Gahmberg, C. G., & Kobata, A. (1991) *Biochemistry 30*, 1561−1571.

Atkins, E. D. T., Isaac, D. H., Nieduszynski, I. A., Phelps, C. E., & Sheehan, J. K. (1974) *Polymer 15*, 263−271.

Fukuda, M. N., Watanabe, K., & Hakomori, S. (1978) *J. Biol. Chem. 253*, 6814−6819.

Fukuda, M. N., Spooncer, E., Oates, J. E., Dell, A., & Klock, J. C. (1984) *J. Biol. Chem. 259*, 10925−10935.

Fukuda, M. N., Dell, A., Tiller, P. R., Varki, A., Klock, J. C., & Fukuda, M. (1986) *J. Biol. Chem. 261*, 2376−2383.

Fukushi, Y., Nudelman, E. D., Levery, S. B., Rauvala, H., & Hakomori, S. (1984) *J. Biol. Chem. 259*, 10511−10517.

Handa, K., Nudelman, E. D., Stroud, M. R., Shiozawa, T., & Hakomori, S. (1991) *Biochem. Biophys. Res. Commun. 181*, 1223−1230.

Handa, K., White, T., Ito, K., Fang, H., Wang, S., & Hakomori, S. (1995) *Int. J. Oncol. 6*, 773−781.

Handa, K., White, T., Hakomori, S., & Hirohashi, S. (1997) *Glycoconjugate J. 14*, 39−43.

Holmes, E. H., Ostrander, G. K., & Hakomori, S. (1986) *J. Biol. Chem. 261*, 3737−3743.

Kojima, N., Handa, K., Newman, W., & Hakomori, S. (1992a) *Biochem. Biophys. Res. Commun. 182*, 1288−1295.

Kojima, N., Shiota, M., Sadahira, Y., Handa, K., & Hakomori, S. (1992b) *J. Biol. Chem. 267*, 17264−17270.

Lasky, L. A. (1995) *Annu. Rev. Biochem. 64*, 113−139.

Lawrence, M. B., & Springer, T. A. (1991) *Cell 65*, 859−873.

Lawrence, M. B., McIntire, L. V., & Eskin, S. G. (1987) *Blood 70*, 1284−1290.

Lawrence, M. B., Smith, C. W., Eskin, S. G., & McIntire, L. V. (1990) *Blood 75*, 227−237.

Levinovitz, A., Muhlhoff, J., Isenmann, S., & Vestweber, D. (1993) *J. Cell Biol. 121*, 449−459.

Macher, B. A., Buehler, J., Scudder, P., Knapp, W., & Feizi, T. (1988) *J. Biol. Chem. 263*, 10186−10191.

Moore, K. L., Eaton, S. F., Lyons, D. E., Lichenstein, H. S., Cummings, R. D., & McEver, R. P. (1994) *J. Biol. Chem. 269*, 23318−23327.

Müthing, J., Spanbroek, R., Peter-Katalinic, J., Hanisch, F.-G., Hanski, C., Hasegawa, A., Unland, F., Lehmann, J., Tschesche, H., & Egge, H. (1996) *Glycobiology 6*, 147−156.

Patel, T. P., Goelz, S. E., Lobb, R. R., & Parekh, R. B. (1994) *Biochemistry 33*, 14815−14824.

Phillips, M. L., Nudelman, E. D., Gaeta, F. C. A., Perez, M., Singhal, A. K., Hakomori, S., & Paulson, J. C. (1990) *Science 250*, 1130−1132.

Polley, M. J., Phillips, M. L., Wayner, E. A., Nudelman, E. D., Singhal, A. K., Hakomori, S., & Paulson, J. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6224−6228.

Rees, D. A. (1975) in *MTP International Review of Science* (Whelan, W. J., Ed.) Vol. 5, pp 1−42, University Park Press, Baltimore.

Sako, D., Chang, X.-J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cumming, D. A., & Larsen, G. R. (1993) *Cell 75*, 1179−1186.

Stroud, M. R., Handa, K., Ito, K., Salyan, M. E. K., Fang, H., Levery, S. B., Hakomori, S., Reinhold, B. B., & Reinhold, V. N. (1995) *Biochem. Biophys. Res. Commun. 209*, 777−787.

Stroud, M. R., Handa, K., Salyan, M. E. K., Ito, K., Levery, S. B., & Hakomori, S. (1996a) *Biochemistry 35*, 758−769.

Stroud, M. R., Handa, K., Salyan, M. E. K., Ito, K., Levery, S. B., & Hakomori, S. (1996b) *Biochemistry 35*, 770−778.

Symington, F. W., Hedges, D. L., & Hakomori, S. (1985) *J. Immunol. 134*, 2498−2506.

Tiemeyer, M., Swiedler, S. J., Ishihara, M., Moreland, M., Schweingruber, H., Hirtzer, P., & Brandley, B. K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1138−1142.

Varki, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7390−7397.

Wilkins, P. P., McEver, R. P., & Cummings, R. D. (1996) *J. Biol. Chem. 271*, 18732−18742.

Yuen, C.-T., Lawson, A. M., Chai, W., Larkin, M., Stoll, M. S., Stuart, A. C., Sullivan, F. X., Ahern, T. J., & Feizi, T. (1992) *Biochemistry 31*, 9126−9131.

BI971181T