Glycolipid Ligands For Selectins Support Leukocyte Tethering and Rolling Under Physiologic Flow Conditions[1]

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Selectin interactions with glycolipids have been examined previously under static conditions, whereas physiologic interactions mediated by selectins take place under flow. We find that under physiologic flow conditions, sialyl Lewis^x (sLe^x) glycolipid and sialyl Lewis^a (sLe^a) neoglycolipid support tethering and rolling adhesions of Chinese hamster ovary (CHO) cells expressing E-selectin and lymphoid and myeloid cells expressing L-selectin. These selectin-mediated adhesions persist at the highest shear stresses that occur in postcapillary venules in vivo and occur at lower site densities than found for sLe^x on neutrophils. The interactions are Ca^{2+}-dependent and can be specifically and completely blocked with anti-selectin mAbs. Asialo nonfucosylated glycolipids are inactive, and sulfatide supports weak tethering, but not rolling, of L-selectin-expressing cells. Rolling velocities and resistance to detachment are related to the glycolipid site density and fall within the range measured for neutrophil and myeloid cell rolling on substrates containing purified selectins. These observations are the first indication that glycolipids can interact with selectins in physiologic flow conditions, and can contribute to rolling adhesions. *The Journal of Immunology,* 1995, 154: 5356–5366.

Selectins are C-type lectins (1) capable of tethering leukocytes to vascular endothelium under shear flow (2, 3). E-selectin, P-selectin, and L-selectin are thought to function in flow by binding rapidly to specific carbohydrate determinants presented on various carrier molecules. Several glycoproteins have been shown to be carriers of the carbohydrate ligands of selectins (4–9). In the case of L-selectin, the isolated, appropriately glycosylated mucin-like carriers have been shown to mediate tethering and to support rolling adhesions of L-selectin-expressing leukocytes in shear flow (9a, 10). Studies on the ligands for L-selectin have focused on carbohydrates displayed by these mucin-like counter-receptors, which include GlyCAM-1, CD34, and MAdCAM-1 (4, 5, 10).

Distinct classes of neuraminidase-sensitive ligands for E-selectin on neutrophils have been found to mediate the initial tethering and subsequent rolling interactions (11). E-Selectin ligands important in tethering appear to be associated with protease-sensitive carriers, including L-selectin (6, 11, 12), whereas ligands that support rolling are resistant to a large variety of proteases (11, 13). Protease-resistant E-selectin ligands are also present on other leukocytes, including NK cells (14) and eosinophils (15). The nature of the protease-resistant class of ligands remains uncharacterized and may correspond to glycolipids.

Human neutrophils express glycolipids decorated with the Lewis^x and sialyl Lewis^x (sLe^x)[3] motifs (16–18). sLe^x-bearing glycolipids are quite abundant on neutrophils, with 2×10^7 copies/cell (17). Assuming that these are present in the outer leaflet of the plasma membrane, and dividing by the known surface area of the neutrophil of 450 μm^2 (19, 20), gives a density of 44,000 molecules of sLe^x glycolipid/μm^2. Naturally occurring (21) or synthetic glycolipids of this type (22–25) have been shown to support adhesion to E-selectin under static conditions. Sialyl Lewis^a (sLe^a), which is expressed on epithelial cells and carcinomas, rather than neutrophils, is closely related structurally to sLe^x and also has been shown to support E-selectin binding in static assays (22, 25, 26). P- and L-selectin have been reported to bind in static assays to sLe^x and sLe^a neoglycolipids or neoglycoproteins (27, 28).

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[3] Abbreviations used in this paper: sLe^a, sialyl Lewis^a; sLe^x, sialyl Lewis^x; LNT, lacto-*N*-tetraose; PC, phosphatidylcholine; H/H, HBSS and HEPES; HSA, human serum albumin; CHO, Chinese hamster ovary; CHO-E, CHO cells transfected with E-selectin; PPMe, polyphosphomonoester.

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However, selectin-expressing cells have not been shown previously to interact under shear flow and form rolling adhesions with any isolated glycolipid ligand. Because mucin-like molecules have a highly extended conformation and are likely to project from the leukocyte surface, and L-selectin is concentrated on microvilli, it has been emphasized that projection above the cell surface may facilitate interactions in flow (6, 29). By contrast, the ceramide moiety of glycolipids is present in the lipid bilayer and the carbohydrate moiety would be closer to the cell surface. In the present study, we directly assess whether glycolipid ligands for selectins can support tethering and rolling adhesions under flow. Cells transfected with full-length human E-selectin, as well as leukocytes expressing L-selectin, have been tested for their ability to interact under flow conditions with specific glycolipids. We report here that glycolipids bearing sLe^x or neoglycolipids bearing sLe^a (22) can specifically support selectin-mediated cell tethering and rolling under flow conditions. This is the first indication that short carbohydrates presented by a lipid carrier, rather than by a polypeptide backbone, can mediate initial tethering and support rolling of a selectin-expressing cell under shear conditions.

Materials and Methods

Preparation of lipid-linked oligosaccharide substrates

Neoglycolipids were prepared by conjugation of oligosaccharides to the lipid phosphatidylethanolamine dipalmitoate (DPPE; Sigma Chemical Co., St. Louis, MO) and monitored by thin layer chromatography-liquid secondary ion mass spectrometry, as described previously (22, 30). sLe^x glycolipid was a chemically synthesized glycosylceramide (a gift of Drs. S. A. Hasegawa and M. Kiso, Gifu University, Japan). The term glycolipid is used here for the neoglycolipids and glycosylceramides, collectively. Sulfatide (bovine brain) was purchased from Sigma Chemical Co. Purified glycolipids were dissolved at 1 to 0.1 μg/ml or, in one case, 2 μg/ml in 20:1 methanol:butanol solution containing 4 μg/ml (5.3 nmol/ml) of egg lecithin phosphatidylcholine (PC) (Sigma Chemical Co.) to give the indicated input densities.

A 3-μl drop was placed on a polystyrene Petri dish (Bio-Tek, Nunc) and formed a contact area 4 mm in diameter. The dish was air-dried and washed twice with PBS, and nonspecific binding sites were blocked by treatment overnight at 4°C with HBSS/10 mM HEPES, pH 7.4 (H/H), containing 20 mg/ml human serum albumin (HSA) (Calbiochem, San Diego, CA). Under these conditions, 15% of acidic glycolipids and 50% of neutral glycolipids are absorbed (22, 31). The input of sLe^x at 1 μg/ml corresponded to 85,000 molecules/μm^2 of the 4-mm coating circle; 15% adsorption of this gives 13,000 molecules/μm^2. No cell adhesions were observed to quenched substrates when the coated lipid was PC or when the lipid was omitted.

mAbs

The murine mAbs BB11 (anti-E-selectin (IgG2b) (32)) and DREG-56 (anti-L-selectin (IgG1) (33)) were the generous gift of Dr. R. Lobb (Biogen, Cambridge, MA) and of Dr. T. K. Kishimoto (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT), respectively. Both mAbs were used as purified Ig. CSLEX mAb (anti-sLe^x) (34) and FH6 (anti-difucosyl sLe^x) (35) IgM mAb were purified from the hybridoma line supernatants by ammonium sulfate precipitation and Sepharose CL-6B filtration. X63 control IgG1 was used as a 1/10 dilution of myeloma culture supernatant.

Cells

CHO cells stably transfected with full-length human E-selectin, termed CHO-E cells (32), were maintained in α-MEM with 10% fetal bovine serum, supplemented with glutamine and gentamicin. Cells were harvested by a 10-min incubation with H/H medium containing 5 mM EDTA at 37°C. Washed cells were resuspended in the same medium at 2×10^7 cells/ml and kept at room temperature until use. The human T cell line Jurkat was grown in RPMI 1640 supplemented with 10% FCS and gentamicin. Cells were washed with H/H twice and resuspended in H/H supplemented with 2 mM Ca^{2+} and 2 mg/ml HSA (binding medium).

PBLs and granulocytes were isolated from citrate-anticoagulated whole blood after dextran sedimentation and density separation over Ficoll-Hypaque (36). For lymphocyte isolation, the mononuclear cells were washed six times with RPMI 5% FCS medium containing 5 mM EDTA and depleted of monocytes, as previously described (37). Cells were maintained at room temperature in H/H medium in absence of divalent cations until use in the flow assay.

Cell treatments

Cells were incubated with 0.1 U/ml *Vibrio cholera* neuraminidase (Calbiochem) for 30 min at 25°C in HBSS, 10 mM HEPES, pH 7.4, and 2 mM Ca^{2+}. Control cells were incubated at 37°C in medium alone. Cleavage was terminated by washing the cells twice with H/H medium plus 5 mM EDTA. Removal of surface sialic acids by the enzyme was monitored by staining cells with the FH6 or CSLEX mAbs, which define neuraminidase-sensitive epitopes (35).

Immunofluorescence flow cytometry

Indirect immunofluorescence was performed on washed cells by using saturating amounts of selectin-specific Abs. CHO-E cells or leukocytes, suspended in L15 medium supplemented with 5% fetal bovine serum and 5 mM EDTA, were incubated at 4°C with 30 μg/ml of selectin-specific mAb (BB11 or DREG56, respectively), washed, and stained with fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Zymed, South San Francisco, CA).

Shear flow assays

Glycolipid substrates, prepared as described above, were assembled in a parallel flow chamber (260-μm gap thickness) (29) and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD, Nikon Inc., Garden City, NY). Cells (5×10^5/ml) in binding medium were perfused in the flow chamber with a syringe pump attached to the outlet side, and the number of cells that tethered per minute per field (0.43 mm^2) during flow, before the substrate became saturated with tethered cells, was quantitated by inspection of videotapes of two to five fields of view. The wall shear stress was calculated as previously described (29). For Ab inhibition studies, cells (10^7/ml) were preincubated for 10 min in Ca^{2+}-free (H/H) medium with 50 μg/ml mAb. The cell suspension was diluted 20-fold into binding medium before infusion in the flow chamber. The anionic polysaccharides fucoidin (Sigma Chemical Co.), heparin (Calbiochem), and PPME (polyphosphomonoester core from *Hansenula hostii* phosphomannan (38)) were included in the binding medium at indicated concentrations. Cells were preincubated for 5 min with the polysaccharide-containing medium and infused in the flow chamber without dilution.

For detachment assays, cells were perfused into the chamber at a wall shear stress of 0.73 dyne/cm^2 for 2 min to allow accumulation of tethered cells. The shear force was then increased every 20 s to a maximum of 36 dynes/cm^2, and the percentage of cells remaining tethered was determined at the end of each 20-s interval. Rolling velocities were measured on cells that were bound during flow at 0.73 dyne/cm^2 wall shear stress, unless otherwise indicated. Cell displacement was measured over 5- to 10-s intervals. Velocities were measured only for cells that remained adherent throughout the 20-s period during which a given shear was applied, as described above for detachment assays. For comparisons between cells treated with different reagents, rolling velocities were measured in identical fields of view on the substrate, to ensure that the results were not affected by inhomogeneity of the substrate. The same substrate was used repeatedly for one series of tethering, detachment, or rolling assays by flushing the chamber with 10 to 20 ml binding medium at 36 dynes/cm^2 between each assay.

		Mr
sLe^a	NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc-PE ↑ 1,4 Fuc α	1791
sLe^x	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer ↑ 1,3 Fuc α	1676
Le^x	Galβ1→4GlcNAcβ1→3Galβ1→4Glc-PE ↑ 1,3 Fuc α	1500
Sulfatide	SO_3-3Gal-Cer	814
LNT	Galβ1→3GlcNAcβ1→3Galβ1→4Glc-PE	1354

FIGURE 1. Lipid-linked oligosaccharides, referred to as glycolipids, used in this study. sLe^a, Le^x, and LNT were derived from human milk and conjugated to phosphatidylethanolamine lipid (PE). The sLe^x was synthesized chemically and was a glycosyl ceramide (Cer). Sulfatide was isolated from bovine brain.

Results

sLe^x and sLe^a glycolipids support E- and L-selectin-mediated cell tethering in shear flow

Different glycolipids (Fig. 1) were mixed with phosphatidylcholine and coated on a plastic substrate that was used to form one wall of a parallel wall flow chamber. To determine whether the glycolipids could support selectin-mediated tethering and rolling of cells under flow conditions, CHO-E cells were perfused at a representative wall shear stress (0.73 dyne/cm^2) through the chamber. CHO-E cells tethered efficiently to both sLe^x and sLe^a (Fig. 2*A*). Tethering in flow required Ca^{2+}, was abolished in the presence of EDTA (Fig. 2*C*; see below), was specific because it did not occur on substrates containing PC or lacking lipid, and was inhibited completely by mAb to E-selectin (Fig. 2*A*). CHO-E cells tethered to Le^x, and this was blocked by mAb to E-selectin. However, tethering to Le^x was less efficient than to sLe^x and sLe^a. There was no tethering to lacto-*N*-tetraose (LNT), an asialo, afucosyl analogue of sLe, nor to sulfatide. Glycolipids in this experiment were used at an input density of 80,000 to 175,000 molecules/μm^2 (Fig. 2 legend). The absorption efficiency for acidic glycolipids is 15% (22, 31); this gives 13,000 and 12,000 molecules/μm^2 for sLe^a and sLe^x, respectively. These values are comparable with the density of sLe^x glycolipid on neutrophils of 44,000 molecules/μm^2. The substrates we used were not saturated with lipid; a continuous monolayer of phospholipid has 1,330,000 molecules/μm^2.

Jurkat T cells that express L-selectin were measured similarly for efficiency of tethering at 0.73 dyne/cm^2 to the glycolipid substrates. Tethering was similar on sLe^x and sLe^a (Fig. 2*B*), required Ca^{2+} (Fig. 2*C*), and was blocked by mAb to L-selectin. Jurkat cells failed to tether in flow to LNT or PC. Jurkat cells could tether to sulfatide. Tethering to sulfatide was much less efficient than to sLe^a or sLe^x, yet it was specific, as shown by inhibition with mAb to L-selectin (Fig. 2*B*) and by EDTA (data not shown). By contrast to the results with sLe^x and sLe^a, Jurkat cells failed to interact under flow conditions with Le^x.

To further examine the specificity of lipid-linked oligosaccharide interaction with L-selectin and E-selectin, anionic polysaccharides were tested for their ability to inhibit tethering under flow to sLe^a (Fig. 2*C*). Fucoidin, heparin, and PPME all inhibited L-selectin-mediated adhesion of both the Jurkat T lymphoblast line and the JY B lymphoblast cell line, in agreement with blocking of L-selectin-mediated lymphocyte adhesion to HEV ligands, tested both in vitro and in vivo (39–41). However, fucoidin, heparin, and PPME did not inhibit E-selectin-mediated attachment to sLe^a (Fig. 2*B* and data not shown), nor to sLe^x (not shown). CHO-E cells also failed to tether under flow to fucoidin adsorbed to substrates in the form of an HSA conjugate, in contrast to various leukocyte types that efficiently tethered in an L-selectin-dependent manner to this substrate (data not shown).

Effect of shear flow on efficiency of cell tethering to glycolipid ligands of selectins

CHO-E cells tethered to sLe^x or sLe^a at wall shear stresses as high as 1.8 dynes/cm^2 (Fig. 3*A*). This was almost as efficient as neutrophil tethering to high densities of immobilized E-selectin, which occurred at wall shear stresses as high as 3.6 dynes/cm^2 (11).

Jurkat T lymphoblastoid cells were tethered to sLe^x and sLe^a at wall shear stresses as high as 0.73 dyne/cm^2 (Fig. 3*B*). Both E- and L-selectin-mediated tethering efficiency was related to the molar fraction of ligand on the substrate. In the case of Jurkat T cells, tethering efficiency decreased threefold as the glycolipid ligand (sLe^a) input density was diluted from 80,000 to 40,000 molecules/μm^2 (Fig. 3*B*).

Lipid-linked oligosaccharide ligands of E-selectin and L-selectin can support cell rolling at physiologic shear stresses

The ability of lipid-linked oligosaccharides to support rolling of tethered selectin-expressing cells provided further support for the physiologic nature of their interactions with selectins. The velocity of CHO-E cells rolling on all glycolipid ligands of E-selectin increased as shear stress was increased (Fig. 4*A*). Rolling persisted at the highest shear stresses at which neutrophils can roll on E-selectin substrates tested in an identical flow system (42). Rolling velocity on sLe^a glycolipid was slower than on sLe^x coated at identical input densities, in accordance with observations in static assays that E-selectin binds more

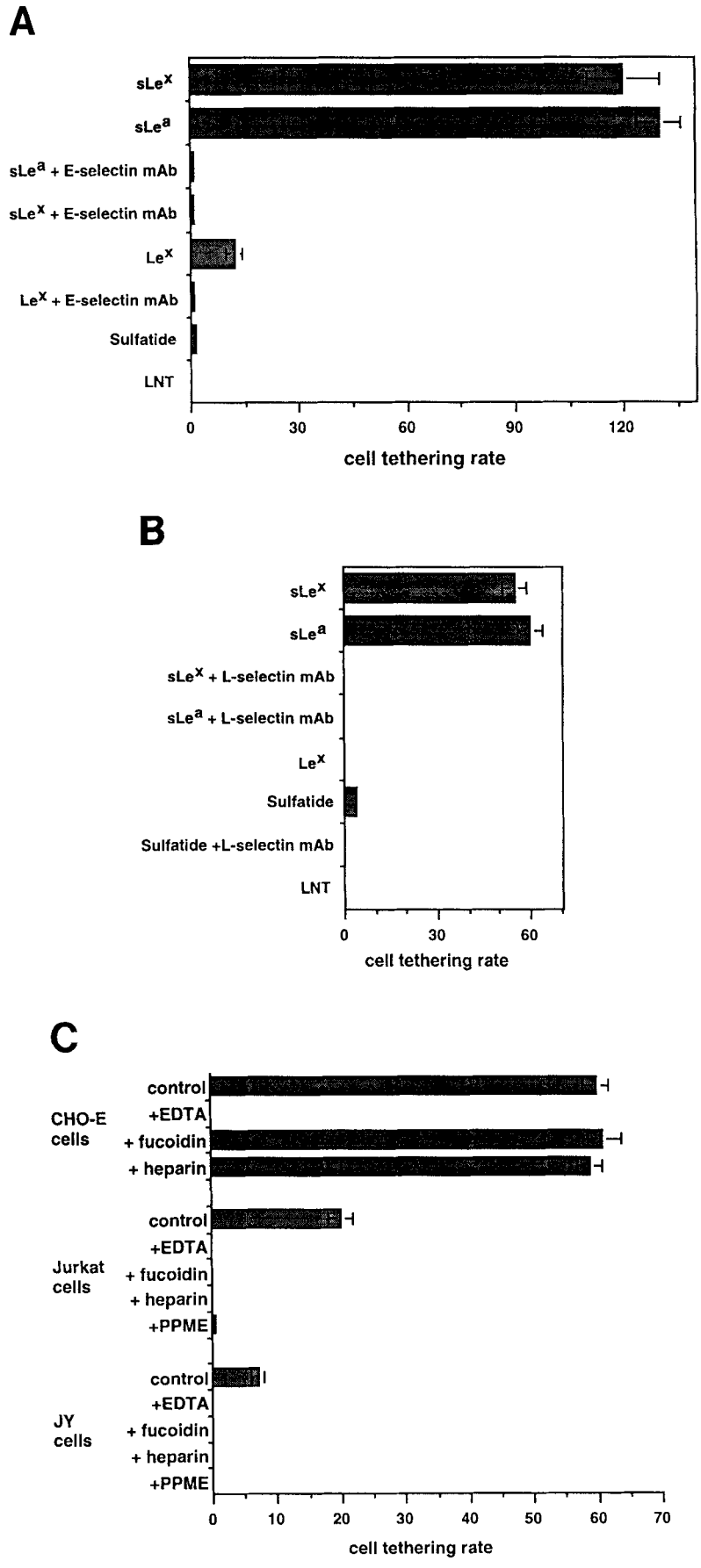

FIGURE 2. Lelectin-mediated cell tethering to different glycolipids. Lipid-linked oligosaccharides (1 μg) were dissolved in methanol containing 4 μg PC; the resulting input densities (molecules/μm^2) of glycolipid in the substrate were 80,000 for sLe^a, 85,000 for sLe^x, 95,000 for Le^x, 175,000 for sulfatide, and 105,000 for LNT. Cells were infused at a constant wall shear stress (0.73 dyne/cm^2) through a parallel plate flow chamber, and the rate of cell tethering to the substrate (cells/min/0.43 mm^2 field of view) was determined, as described in Materials and Methods. Tethering to all glycolipids was completely blocked in the presence of 5 mM EDTA. *A*: Tethering of CHO-E cells in presence or absence of BB11 mAb to E-selectin. *B*: Tethering of Jurkat T cells in presence or absence of DREG56 mAb to L-selectin. Results for *A* and *B* are averages of three experiments; bars show the SD. *C*: Inhibition by anionic polysaccharides of selectin-mediated tethering to sLe^a. The input densities of the sLe^a neoglycolipid in the coated lipid were 40,000 molecules/μm^2. Lymphocytes (Jurkat or JY cell lines) or CHO-E cells were preincubated in binding medium with anionic polysaccharides for 5 min and infused without dilution into the flow chamber. Concentrations of fucoidin, heparin, and PPME were 0.05, 0.1, and 0.3 mg/ml, respectively. Tethering was at a wall shear stress of 0.73 dyne/cm^2. Representative of two experiments. *Bars* show the mean ± range of two measurements.

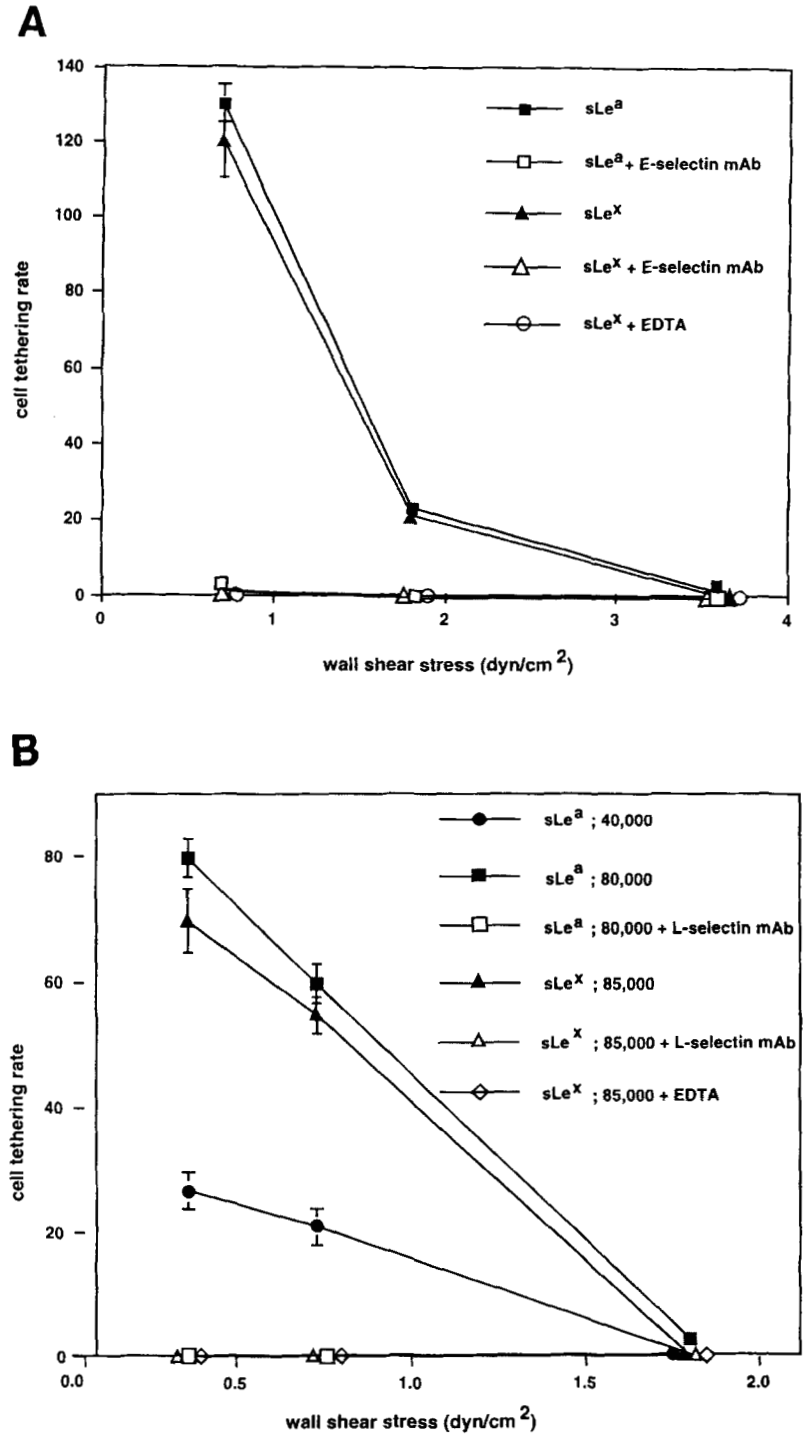

FIGURE 3. Effect of wall shear stress on tethering of CHO-E cells (*A*) or Jurkat lymphocytes (*B*) to sLe^a and sLe^x glycolipids. Cells were tethered to substrates containing sLe^a or sLe^x coated at input densities of 80,000 and 85,000 molecules/μm^2, respectively (*A*), or at the indicated input densities (*B*) at the indicated wall shear stresses, in the presence or absence of 5 mM EDTA or BB11 mAb to E-selectin or DREG56 mAb to L-selectin or control Ig, as indicated. Results are representative of five independent experiments. Each point represents the mean ± range of two measurements.

strongly to the sLe^a oligosaccharide (31). The rolling velocity of CHO-E cells at an input density of 85,000 molecules/μm^2 of sLe^x was in the range measured for neutrophil rolling on monolayers of CHO-E cells (not shown). The rolling velocity of CHO-E cells on Le^x was threefold higher than on sLe^x, corresponding to a weaker interaction of the selectin with Le^x. A twofold increase in the coating concentration of Le^x resulted in a reduced rolling velocity, suggesting that with more tethers being formed, cell rolling is slowed. By contrast to the above results, with an input density of 160,000 molecules $sLe^a/\mu m^2$, CHO-E cells arrested immediately upon attachment in flow and failed to roll even when the wall shear stress was increased to 10-fold the initial value (data not shown).

All Jurkat lymphocytes that tethered in flow to the sialylated Lewis glycolipids subsequently rolled, and rolling

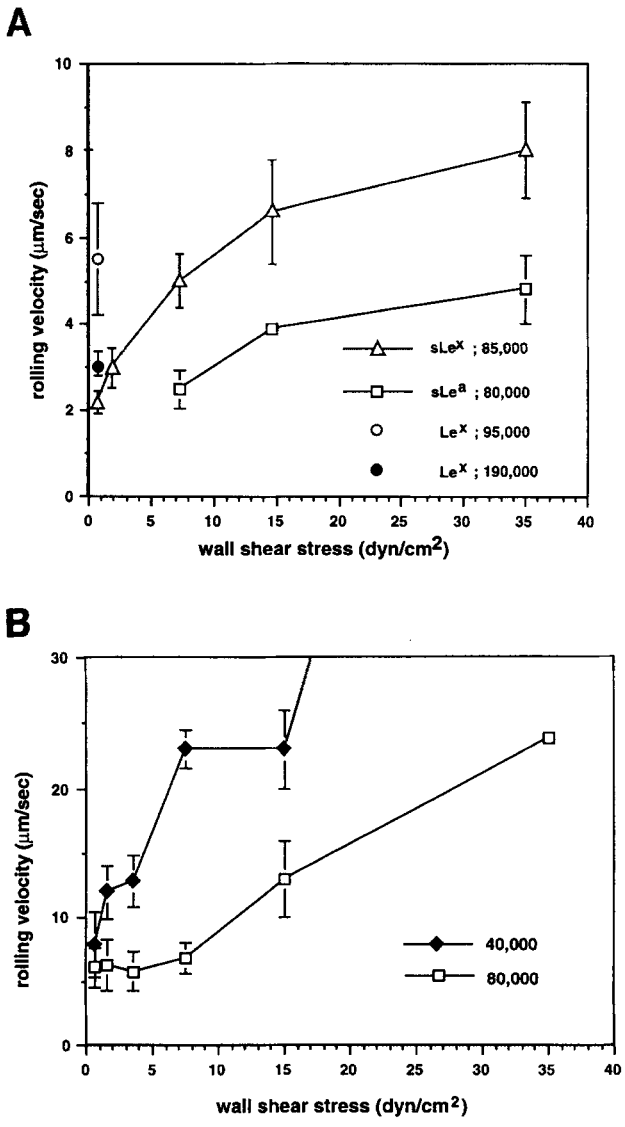

FIGURE 4. Rolling of CHO-E cells on the indicated glycolipids (*A*) and of Jurkat lymphocytes on sLe^a glycolipid (*B*) coated at the indicated input densities (molecules/μm^2). Cells were tethered at a wall shear stress of 0.73 dyne/cm^2 and subjected to increased wall shear stress every 20 s to a maximum of 36 dynes/cm^2 and rolling velocity was measured, as described in Materials and Methods. The results are representative of three experiments. Each value represents 30 cells and bars show the SE of the mean.

velocity increased with increasing wall shear stresses applied (Fig. 4*B*). When the input density of sLe^a was increased from 40,000 to 80,000 molecules/μm^2, rolling velocity decreased two- to fourfold. Similar results were observed with Jurkat cells rolling on sLe^x-coated substrates (not shown). The velocities measured for Jurkat cells on sLe^a (or sLe^x; data not shown) at any given flow condition were several fold higher than those of CHO-E cells on identical substrates (Fig. 4, *A* vs *B*). This correlated with higher surface expression of E-selectin on CHO-E cells than L-selectin on Jurkat cells, as shown by flow cytometry (data not shown).

Jurkat lymphocytes tethered minimally in flow to sulfatides through L-selectin (Fig. 2*B*); however, cells failed to roll, and either remained stationary or skipped a few cell diameters before detaching, suggesting weak interactions between L-selectin and sulfatide (data not shown).

Resistance of rolling adhesions on lipid-linked oligosaccharides to detachment

The strength of rolling adhesions can be measured by their resistance to detachment by increasing shear stress. Cells were allowed to tether at a wall shear stress of 0.73 dyne/cm^2, and after a sufficient number of cells had accumulated, the shear stress was incremented every 20 s through a range of wall shear stresses found in vivo (43), and the percentage of cells that remained adherent was determined. CHO-E cell rolling adhesions were quite resistant to shear, with most cells remaining rolling and adherent at wall shear stresses up to 15 to 35 dynes/cm^2 on sLe^a and sLe^x, at an input density of 85,000 and 80,000 molecules/μm^2 (Fig. 5*A*). The shear resistance of CHO-E cells on sLe^x was much greater than on its asialo analogue Le^x (Fig. 5A), consistent with its being a weak ligand of E-selectin (22, 26). CHO-E cells were markedly less resistant to detachment when the input density of sLe^a was decreased to 8,000 molecules/μm^2. Rolling adhesions of Jurkat T lymphoblasts on sLe^a were slightly less stable than CHO-E cells; however, most Jurkat T cells remained tethered at wall shear stresses up to 15 dynes/cm^2 (Fig. 5*B*). As the input density of sLe^a was decreased, a lower percentage of Jurkat T cells resisted detachment at higher shear stresses.

Variation among leukocytes in tethering through L-selectin

Leukocytes expressing L-selectin at similar levels (Fig. 6*A*) tethered with different efficiencies to the L-selectin glycolipid ligand, sLe^a (Fig. 6*B*). Tethering rates for JY B lymphoblast cells and neutrophils were 60 to 80% lower than for PBLs or Jurkat T lymphoblast cells, at any sLe^a glycolipid site density tested. Tethering for all cells was mediated by L-selectin, as shown by inhibition with mAb, and did not occur with LNT (Fig. 6*B*). Consistent with these data, L-selectin-mediated rolling adhesions of JY cells were considerably weaker than those of Jurkat cells; on identical sLe^a-containing substrates, the rolling velocities of JY cells were on average twofold higher than those measured for Jurkat cells (data not shown).

Treatment of leukocytes with neuraminidase to remove cell surface sialic acids, as verified by immunofluorescent flow cytometry with CSLEX and FH6 mAb to sLe^x (not shown) dramatically enhanced tethering efficiency through L-selectin (Fig. 6*C*). The strength of rolling adhesions was also enhanced, as indicated by slower rolling velocities

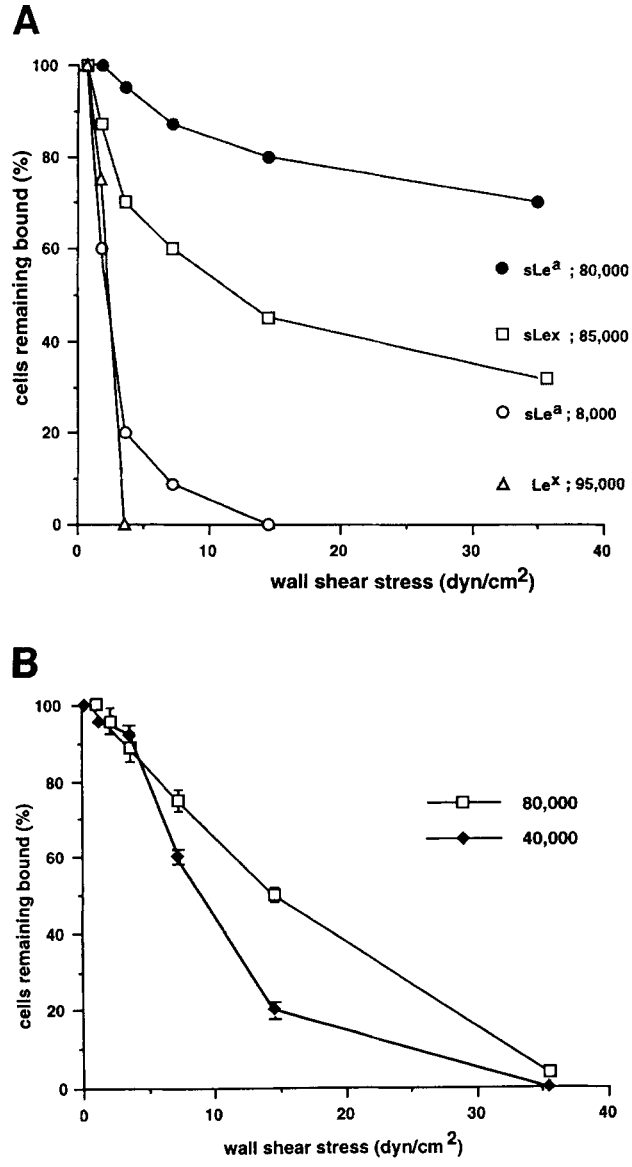

FIGURE 5. Resistance to detachment of CHO-E cells from various glycolipids (*A*) and detachment of Jurkat T cells from sLe^a substrate (*B*). Cells were infused into a chamber containing a PC substrate with the indicated input densities of lipid-linked oligosaccharide at 0.73 dyne/cm2, for 2 min. The shear stress was then increased every 20 s to a maximum of 35 dynes/cm2 and the percentage of cells remaining bound at each shear was determined.

after neuraminidase treatment (data not shown). Interestingly, the cells that were least efficient at tethering, neutrophils and JY cells, showed the greatest enhancement after neuraminidase treatment, of 10-fold or more.

Discussion

We have demonstrated that glycolipids bearing the sLe^x and sLe^a motifs can mediate cell tethering and rolling under physiologic flow conditions of E- and L-selectin-expressing cells. Tethering and rolling require Ca^{2+} and are blocked by mAbs to E- or L-selectin. This is the first indication that short carbohydrate ligands expressed on lipids can interact with cell surface selectins under flow and that it is not obligatory for the E- and L-selectin ligands to be presented on proteins (4, 8). Under continuous flow, glycolipid:selectin interactions result in reversible tethers that can resist detachment by hydrodynamic forces acting on the cell, over the entire range of physiologic shear stresses that occur in vivo in postcapillary venules.

We found that sLe^x and sLe^a were similarly efficient for tethering of E- and L-selectin-bearing cells. CHO-E cells rolled more slowly on sLe^a than sLe^x; this suggests a stronger interaction with sLe^a, as also reflected in the greater resistance to detachment. Le^x could mediate tethering and rolling for E-selectin-bearing cells, with substantially reduced efficiency compared with sLe^x or sLe^a, and was inactive as a substrate for L-selectin-bearing cells. Neither type of cell interacted with LNT substrates. These results are in agreement with previous studies on selectin-mediated adhesion under static conditions to lipid-linked (22, 44) and protein-linked (26, 28) oligosaccharide substrates.

In the present study, sulfatide was a poor ligand for L-selectin, supporting weak tethering interactions that failed to maintain cell rolling on the glycolipid. Thus, the binding of sulfatide micellar suspensions to L-selectin, indicated by ability to block L-selectin function (45), does not predict ability to support rolling adhesions when immobilized on surfaces. Heparin, which we found to efficiently block L-selectin-mediated tethering, mediated poor tethering that resulted in unstable attachments rather than rolling adhesions when immobilized as a porcine intestinal mucosal heparin-BSA neoglycoprotein (46) (data not shown). E-selectin did not interact under flow conditions with sulfatide. This is in accordance with the majority of observations on E-selectin binding under static conditions (47). The observation that sulfated polysaccharide blockers of L-selectin had no inhibitory effects on E-selectin tethers is consistent with lack of physiologic binding of nonfucosylated oligosaccharides to E-selectin (30).

Efficiencies of tethering, rolling velocity, and resistance to detachment at varying shear stresses were examined as a function of glycolipid density to test the relative strength of the tethers formed between the selectin and the glycolipid. The stronger the rolling adhesions of the selectin-expressing cell on a given lipid-linked oligosaccharide, as judged by resistance to shear stress, the slower was its rolling velocity, and the higher was the frequency of tethering. Thus, tethering frequency, rolling velocity and shear resistance serve as useful parameters to characterize under flow conditions the relative avidity of the interaction between the lipid-linked oligosaccharide and the selectin. This dynamic approach allows better assessment of a physiologic role for a putative selectin ligand. For example, sulfatide is active in static assays, but has little activity

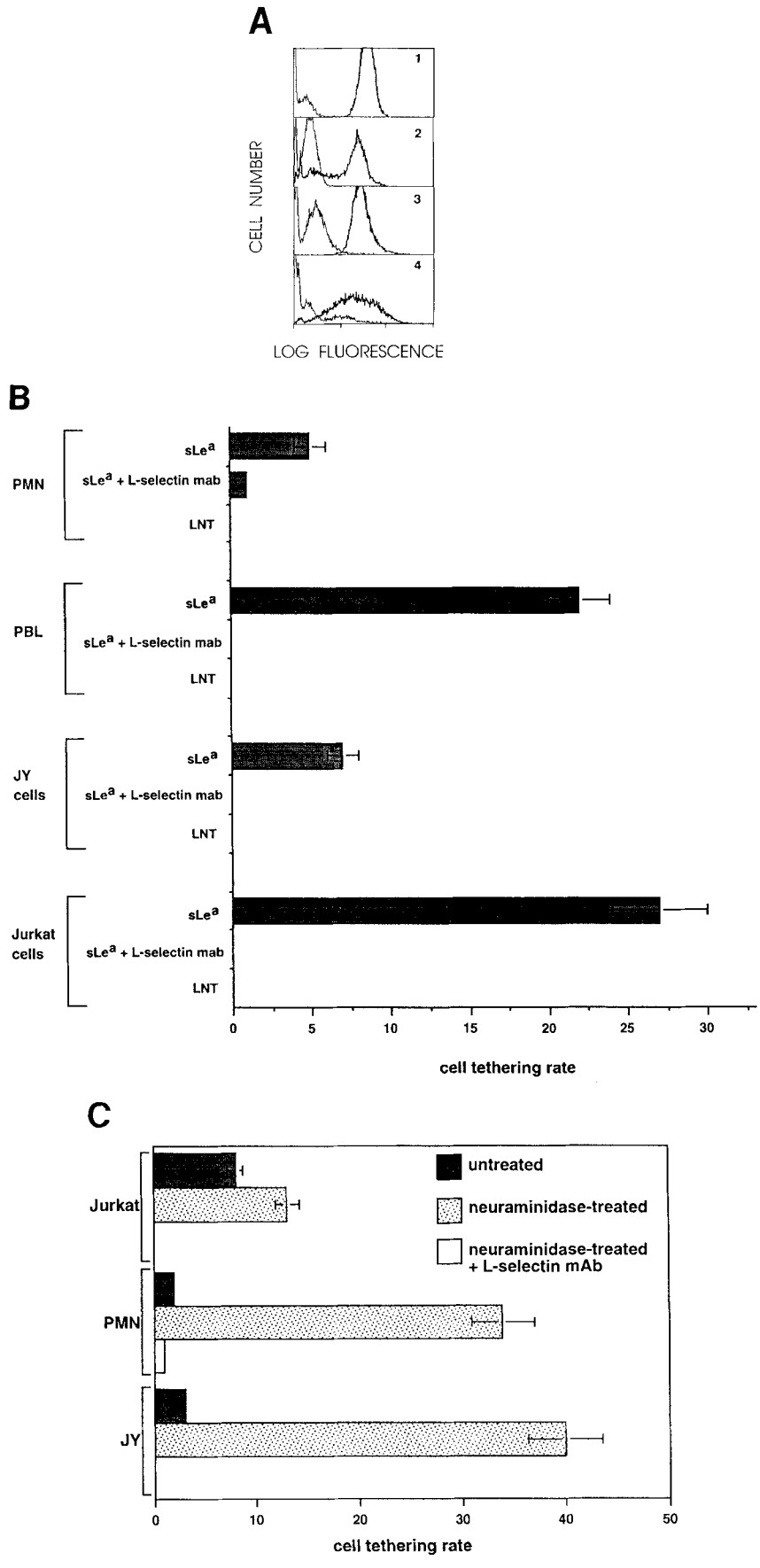

FIGURE 6. Variation among leukocytes in tethering through L-selectin and effect of neuraminidase treatment. *A*: L-selectin expression on various leukocytes. Immunofluorescent flow cytometry of neutrophils (*panel 1*), PBL (*panel 2*), JY cells (*panel 3*), and Jurkat cells (*panel 4*) stained with anti-L-selectin mAb DREG 56, followed by FITC-labeled secondary mAb (thick lines). Control staining with X63 (IgG1) is shown with thin lines. *B* : L-selectin-mediated cell tethering to glycolipids. Tethering was determined at a wall shear stress of 0.73 dyne/cm2. All cells were infused at a concentration of 1 × 10^6/ml, and started to roll upon tethering. The input densities of sLe^a and LNT glycolipids were 40,000 and 105,000 molecules/μm^2, respectively. *C*: Removal of cell surface sialic acid enhances L-selectin-mediated tethering to sLe^a glycolipid. Leukocytes were treated with or without neuraminidase, washed, and tested for tethering to sLe^a glycolipid (at input density of 24,000 molecules/μm^2) at a wall shear stress of 0.73 dyne/cm2. Tethering was performed in the absence or presence of DREG 56 mAb to L-selectin. Complete removal of sialylated epitopes was verified with CsLex mAb (neutrophils, JY cells) and FH6 mAb (Jurkat cells) by immunofluorescent flow cytometry (not shown).

in tethering or rolling in flow. Furthermore, whereas heparin is an inhibitor of L-selectin in solution and mediates binding in static assays, it is very weak in tethering assays and fails to mediate rolling. Flow assays on lipid-linked oligosaccharides may also prove useful in the functional characterization of individual oligosaccharides isolated from glycoprotein counter-receptors for selectins.

The densities of glycolipids that were absorbed on the wall of flow chambers in most assays were on the order of 13,000 sites/μm^2 and were comparable to the calculated density of sLe^x-bearing glycolipids on the neutrophil surface of 44,000 molecules/μm^2 (17, 19, 20). This calculation of surface density assumes all sLe^x glycolipid in the neutrophil is in the outer leaflet of the plasma membrane. In the case of lactosylceramide, less than 25% is plasma membrane-associated, and the majority is associated with primary and secondary granules (48). The subcellular distribution of sLe^x ceramide has not been reported; however, chemoattractants and phorbol esters that induce substantial fusion of primary and secondary granules with the plasma membrane have no effect ($\pm 10\%$) on the quantity of cell surface sLe^x Ag (11, 49). Most of the cell surface sLe^x, as well as E-selectin ligand activity in static and rolling assays, appears associated with glycolipids, because neither is affected by protease treatment (11, 13). Naturally occurring sLe^x and sLe^a glycolipids may have carbohydrate moieties longer in size and more complex than the hexasaccharide sLe^x glycolipid and sLe^a neoglycolipid molecules investigated in this study (35, 50), and may have higher affinities or enhanced tethering capabilities. The sLe^a hexasaccharide has higher affinity than sLe^a pentasaccharide (24), as do similar sulfated Le^a and sulfated Le^x pentasaccharides compared with tetrasaccharides (31).

Comparisons between leukocytes expressing comparable levels of L-selectin showed substantial differences in efficiency of tethering to sLe^a. Treatment of the cells with neuraminidase markedly enhanced tethering, and the improvement was most marked, by more than 10-fold, for the cells that were the least efficient in the absence of any treatment. Possible explanations include a decrease in charge-charge repulsion between the cells and the substrate, because of removal of sialic acid from the cell surface (51), removal of *cis* interactions on the surface of the cells between L-selectin and sialylated ligands (11), or alteration in distribution or conformation of L-selectin on the cell surface. It is noteworthy that both neutrophils and JY cells, which exhibited the least efficient L-selectin-mediated tethering in the absence of neuraminidase treatment, express significantly higher levels of sLe^x carbohydrate than Jurkat cells and PBLs. Thus, neuraminidase treatment could have resulted both in surface-charge alteration and elimination of the postulated *cis* interactions.

Previous studies have shown that protease-treated neutrophils and NK cells are fully active in attaching to E-selectin in static assays (13–15), and that chymotrypsin treatment of neutrophils greatly decreases tethering efficiency, but has no effect on the strength of rolling adhesions on E-selectin (11). Our results show that glycolipids can mediate rolling adhesions, and thus lend support for the concept that sLe^x glycolipids, and not exclusively protease-resistant glycoproteins, may be responsible for the rolling of protease-treated neutrophils. In contrast to E-selectin ligands, P-selectin ligands on various types of leukocytes can be eliminated by selective proteolysis and appear to be entirely associated with mucin-like sialoglycoproteins (7, 13, 52). Removal of the P-selectin glycoprotein ligand (PSGL-1) from polymorphonuclear leukocytes or HL-60 with *O*-glycoprotease resulted in complete abolishment of interactions under flow with P-selectin substrates (data not shown). Thus, sLe^x-bearing glycolipids expressed on leukocytes may have a significant physiologic role in E-selectin but not P-selectin-mediated rolling adhesions.

This study raises the possibility that glycolipids may contribute to physiologic L-selectin-dependent tethering or rolling of leukocytes. So far, mucin-like sialoglycoprotein carriers of L-selectin carbohydrate ligands, including CD34 (4), GlyCAM-1(5), and MAdCAM-1(10), have been isolated from HEV of peripheral lymph nodes. These and other glycoproteins that vary in size in SDS-PAGE and bear the MECA-79 carbohydrate epitope (53) have been proposed to function as the scaffolds for specific carbohydrate ligands of L-selectin; however, glycolipids have largely been excluded in previous analyses of L-selectin ligands. Our observations that glycolipids may contribute to L-selectin-mediated tethering and rolling emphasize the importance of examining the contribution in HEV of glycolipid ligands of L-selectin to tethering and rolling of lymphocytes.

Recently, neutrophils have been observed to roll on adherent neutrophils through an interaction of L-selectin on the rolling neutrophil with a neuraminidase-sensitive ligand on the adherent neutrophil (54). Because we have shown that sLe^x glycolipids can support rolling interactions at a density comparable with that found on the neutrophil surface, sLe^x glycolipids may be relevant ligands in this physiologic context.

In conclusion, this study points to the physiologic significance of the existence of different types of selectin counter-receptors cooperating in different steps of leukocyte tethering and rolling interactions. Slowing of a cell in shear flow in the bloodstream by tethering and rolling, and establishment of resistance to detachment by shear forces acting upon the rolling cell, seem essential to allow sufficient contact time to enable subsequent cell activation and development of adhesive interactions through integrins that result in firm adhesion (3, 55). We have shown that bonds formed between glycolipids and either E- or L-selectins can tolerate the entire range of physiologic shear stresses found in postcapillary venules. Although most attention to date has focused on carbohydrate ligands for selectins that are present on proteins, our data suggest that

glycolipids may be physiologically relevant ligands. It should be noted that although glycolipids may be less accessible, this may be compensated by their much greater abundance. The highest density proteins are present at approximately 100,000 copies per neutrophil, whereas sLe^x glycolipids are present at up to 2×10^7 copies, a difference of 200-fold. Most cell surface sLe^x may be associated with glycolipids, because protease treatment of neutrophils has little effect on staining with mAb to sLe^x. Glycoproteins may be more important than glycolipids in the initial tethering interaction because interactions of their cytoplasmic domains with the cytoskeleton can make them more resistant to extraction from the membrane under the high forces exerted on a single selectin and its ligand during tethering. By contrast, once a cell has tethered and further interactions occur, the force is distributed over multiple selectins and ligands. On the basis of previous studies in shear flow with E-selectin that show that protease-treated cells are reduced in tethering efficiency but are unaffected in strength of rolling adhesions (11), we propose that glycoprotein carriers of selectin ligands may be most important for the initial cell tethering, whereas glycolipids may be most important for subsequent rolling interactions and for strengthening tethers initiated by the glycoprotein ligands.

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