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# The HNK-1 reactive sulfoglucuronyl glycolipids are ligands for L-selectin and P-selectin but not E-selectin

**(cell adhesion/glycosphingolipids/sialyl Lewis x/sulfatides)**

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ABSTRACT **E-selectin, L-selectin, and P-selectin are related cell adhesion molecules that bind via their lectin domains to sialyl Lewis x and related carbohydrate determinants. Reports have indicated that sulfated glycolipids and polysaccharides also bind selectins. To extend these findings, we compared binding of selectin–IgG chimeras to immobilized sulfated and sialylated glycosphingolipids. E-, L-, and P-selectin chimeras all bound to surfaces adsorbed with 2,3-sialyl Lewis x glycolipid or sulfatide (galactosylceramide $I^3$-sulfate) but not to surfaces adsorbed with control sulfated lipids (octadecyl sulfate, sphingosine sulfate). Notably, the L- and P-selectin chimeras but not E-selectin chimera bound to surfaces adsorbed with sulfoglucuronyl glycosphingolipids (SGNL lipids; e.g., $IV^3$glucuronylneolactotetraosylceramide $V^3$-sulfate). These unusual lipids have been reported as antigenic determinants for monoclonal IgM antibodies produced in patients with neuropathy associated with paraproteinemia and react with the mouse monoclonal antibody HNK-1. Binding of L- and P-selectin chimeras to SGNL lipids was specifically inhibited by appropriate anti-selectin antibodies. While binding of all three selectin chimeras to sialyl Lewis x was blocked by removal of calcium, binding to SGNL lipid was only modestly reduced by EDTA. Chemically desulfated SGNL lipid retained binding activity for L- and P-selectin chimeras, while methyl esterification of the glucuronic acid eliminated binding. We conclude that SGNL lipids, unlike sialyl Lewis x and sulfatides, selectively support L- and P-selectin but not E-selectin chimera binding. The presence of SGNL lipids on brain microvascular endothelium (and other endothelia) may implicate these molecules in leukocyte trafficking to the nervous system and elsewhere.**

Selectins are a family of structurally related cell adhesion molecules implicated in adhesive interactions of leukocytes and platelets with cells of the vascular endothelium (1–3). The three known selectins—E-selectin, L-selectin, and P-selectin—share a structural domain similar to that described in calcium-dependent vertebrate lectins (2, 4). This observation led to the investigation, by several groups, of the carbohydrate determinants responsible for selectin binding (5–13). These reports establish that the sialylated fucosylated neolactosylceramide, 2,3-sialyl Lewis x (2,3-S-$Le^x$), and related structures are binding determinants for all three known selectins. In addition to 2,3-S-$Le^x$, sulfated glycoconjugates, including glycosphingolipids, have been reported to interact with certain selectins (14–18).

An unusual class of sulfated glycosphingolipids, sulfoglucuronyl-containing neolactosylceramides (SGNL lipids), have been implicated in cell interactions in the nervous system (19–21). These glycolipids were first characterized as antigenic determinants for certain monoclonal IgMs from patients with peripheral neuropathy associated with plasma cell dyscrasia (22–24) and are also recognized by a mouse monoclonal antibody, HNK-1, raised against human natural killer cells. They are found (among other sites) in the embryonic cerebral cortex, in the mature cerebellum and peripheral nervous system, and on brain microvascular endothelium and vascular endothelial cells in culture (25–28). We report here a comparison of the recognition of S-$Le^x$, sulfatides, and SGNL lipids by the three known selectins.

## METHODS

**Selectin–IgG Chimeras and Anti-Selectin Antibodies.** The production and characterization of E-, L-, and P-selectin–IgG chimeras used in this study were as described (9). Functional equivalence with the native selectins was demonstrated by the ability of the E-selectin chimera to inhibit neutrophil adhesion to activated endothelial cells, the L-selectin chimera to inhibit lymphocyte binding to peripheral lymph node high endothelial venules *in vitro*, and the P-selectin chimera to inhibit platelet/HL-60 cell binding. The anti-L-selectin monoclonal antibody Mel-14 was purified from cell culture supernatants as described (9). Anti-E-selectin (BBA2) and anti-P-selectin (MON1137) monoclonal antibodies were purchased from British Biotechnologies (Abingdon, U.K.) and Sanbio (Unden, The Netherlands), respectively.

**Glycosphingolipids, SGNL Lipid Derivatives, and Control Lipids.** Bovine brain sulfatides, sphingosine sulfate, cholesterol sulfate, and ganglioside GM1 ($II^3$acetylneuraminylgangliotetraosylceramide) were from Sigma, and octadecyl sulfate was from Aldrich. Synthetic 2,3-S-$Le^x$ and 2,6-sialyl Lewis x (2,6-S-$Le^x$) glycosphingolipids (29) were the kind gifts of A. Hasegawa (Department of Applied Bioorganic Chemistry, Gifu University, Gifu, Japan).

SGNL lipid was purified from human cauda equina by modifications of published procedures (23, 24). Human cauda equina was obtained at autopsy and stored frozen at −70°C or was kindly provided by the National Neurological Research Specimen Bank (Los Angeles). Tissue was extracted with chloroform/methanol/water (4:8:3), precipitated protein was removed by centrifugation, and the clarified extract was evaporated to dryness. The resulting residue was extracted with chloroform/methanol (1:1), and the remaining solids were dissolved in chloroform/methanol/water (4:8:3) and partitioned into the upper phase (30) after addition of water to generate chloroform/methanol/water (4:8:5.6). SGNL lipids were purified by sequential DEAE-Sepharose, silicic acid, and C18 reverse-phase chromatography (31–33). Purified SGNL lipid (23, 24) consisted of two species re-

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Abbreviations: 2,3-S-$Le^x$, 2,3-sialyl Lewis x ($III^3$-α-fucosyl-$IV^3$-α-*N*-acetylneuraminylneolactotetraosylceramide); 2,6-S-$Le^x$, 2,6-sialyl Lewis x ($III^3$-α-fucosyl-$IV^6$-α-*N*-acetylneuraminylneolactotetraosylceramide); SGNL lipid, sulfoglucuronylneolactosylceramides (e.g., $IV^3$glucuronylneolactotetraosylceramide $V^3$-sulfate).

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solved by TLC, which correspond to the closely related structures IV$^3$glucuronylneolactotetraosylceramide V$^3$-sulfate (90%) and VI$^3$glucuronylneolactohexaosylceramide VII$^3$-sulfate (10%). Identification of SGNL lipids was via comigration with known standards (kindly provided by Robert Yu, Medical College of Virginia, Richmond) and by fast atom bombardment MS (see below). The concentration of purified SGNL lipids was determined by quantitative TLC on silica gel HPTLC plates (Merck) using chloroform/methanol/0.25% aqueous KCl (60:35:8) as developing solvent and 2% Azure A in 1 mM sulfuric acid for detection of sulfated compounds (34). Sulfatides were used as standards for Azure A quantitation of SGNL lipids. This method has been validated by carbohydrate compositional analysis (data not shown).

A portion of the SGNL lipid was treated with methanolic HCl, resulting in concomitant 3-O-desulfation and methyl esterification of the glucuronic acid moiety (24). The product, predominantly IV$^3$glucuronylneolactotetraosylceramide V$^6$-methyl ester, was purified by C18 reverse-phase chromatography. Some of the resulting desulfated methyl ester was treated with mild alkali to generate desulfated SGNL lipid, predominantly IV$^3$glucuronylneolactotetraosylceramide, which was purified by C18 reverse-phase chromatography. The concentrations of desulfated derivatives were determined by quantitative TLC on silica gel HPTLC plates using chloroform/methanol/0.25% aqueous KCl (5:4:1) as developing solvent, cupric sulfate/phosphoric acid char (35) for detection, and the parent SGNL lipids as quantitative standards.

SGNL lipids and their derivatives were subjected to fast atom bombardment MS in the negative mode with a Finnigan MAT 900 spectrometer with triethanolamine as matrix. We are grateful to Hans Schweingruber (Glycomed, Alameda, CA) for performing these analyses.

A polyvalent macromolecular radioligand derivatized with SGNL oligosaccharides was prepared as follows. SGNL oligosaccharide was released from the parent SGNL lipid by treatment with ceramide glycanase (V-Labs, Covington, LA) (36) and purified by chloroform extraction and C18 reverse-phase chromatography. The resulting oligosaccharide was added at a 50-fold molar excess to bovine serum albumin in 100 mM sodium phosphate buffer (pH 7) and coupled by reductive amination using pyridine borane as reducing agent (37). The resulting SGNL–bovine serum albumin conjugate was purified by gel-filtration chromatography and DEAE-HPLC as described (38). Preliminary estimates of the derivatization ratio were performed by acid hydrolysis followed by analysis of released sugars by Dionex HPLC, revealing ≈8 SGNL oligosaccharides attached per albumin molecule. The conjugate was radioiodinated with Na$^{125}$I (DuPont/NEN) using Iodo-Beads (Pierce) under the manufacturer's suggested conditions.

**Binding of Selectin Chimeras to Immobilized Lipids.** ELISAs were performed as described (9). Glycolipids (or control lipids) were dissolved in methanol, diluted with an equal volume of water, and added to 96-well polystyrene microwells; the solvents were allowed to evaporate. The plates were preblocked by incubation with calcium and magnesium-free phosphate-buffered saline (PBS) containing 5% bovine serum albumin. Soluble chimeras (1 μg/ml) were preincubated with 1:1000 dilutions of biotinylated goat F(ab') anti-human IgG Fc and alkaline phosphatase–streptavidin (Caltag, South San Francisco, CA) in PBS containing 1% bovine serum albumin and either 1 mM calcium chloride or 1–5 mM EDTA (as indicated). After 30 min at 37°C, the resulting complexes were added to the lipid-adsorbed preblocked wells and incubated at 37°C for 45 min. The plates were then washed with PBS and water and the bound chimera–anti-chimera–streptavidin complex was quantitated by addition of the alkaline phosphatase substrate, 2-(*p*-nitrophenyl) phosphate (1 mg/ml in 1 M diethanolamine/0.01% $MgCl_2$, pH 9.8). Color development was determined at 405 nm with a Molecular Devices microplate reader using either the kinetic mode or single point reading within the linear kinetic phase, which are directly comparable.

Generation of a multivalent complex before addition to the lipid-adsorbed wells enhanced the sensitivity of the assay. Although absolute levels of binding were increased by aggregation, relative binding of the different chimeras to different glycolipids can be accurately compared (9).

**Precipitation of $^{125}$I-Labeled SGNL–Bovine Serum Albumin by Selectin Chimeras.** Each selectin chimera (50 μg/ml) was mixed with goat anti-human IgG (Caltag; 60 μg/ml) in PBS containing 1% bovine serum albumin and either 1 mM calcium or 5 mM EDTA. After 15 min at 0°C to allow a multivalent complex to form, an aliquot (0.1 ml) of chimera–anti-chimera complex was mixed with 0.1 ml of $^{125}$I-labeled SGNL–bovine serum albumin (15 nM in the same buffer as the chimera). After 90 min at 37°C, the samples were chilled on ice and 0.5 ml of 20% polyethylene glycol in PBS was added, the samples were incubated for 15 min at 0°C, and precipitated conjugate was collected on glass fiber filters using a Brandel cell harvester and washed with 10% polyethylene glycol in PBS. Precipitated radiolabel was quantitated with a γ-radiation counter.

## RESULTS

**SGNL Lipids Are Recognized by L-Selectin and P-Selectin Chimeras but Not by E-Selectin Chimera.** When 2,3-S-Le$^x$, 2,6-S-Le$^x$, sulfatides, and SGNL lipids were adsorbed in comparable amounts to plastic microwells and probed with selectin chimeras, a distinctive pattern of binding emerged (Fig. 1). All three selectin chimeras bound to 2,3-S-Le$^x$ but not to 2,6-S-Le$^x$. Binding to 2,3-S-Le$^x$ was calcium dependent in each case. Sulfatide also supported binding of all three selectin chimeras, with E-selectin chimera binding showing calcium dependence, while L- and P-selectin chimera binding was only modestly inhibited by removal of calcium and addition of EDTA. Notably, SGNL lipids supported binding of the L- and P-selectin chimeras but not the E-selectin chimera. Again, binding was only modestly inhibited under calcium-free conditions. Control human IgG binding was negligible to surfaces adsorbed with any of the glycolipids, and none of the chimeras bound significantly to surfaces adsorbed with octadecyl sulfate, sphingosine sulfate, cholesterol sulfate, or ganglioside GM1 (data not shown).

Selective binding of the L- and P-selectin chimeras to SGNL lipids was confirmed by an independent method (Fig. 2). SGNL oligosaccharides, prepared from SGNL lipids by treatment with ceramide glycanase, were covalently attached to a carrier, bovine serum albumin, to create a multivalent ligand. When the resulting SGNL–bovine serum albumin was radioiodinated and incubated individually with the three selectin chimeras, only the L- and P-selectin chimeras resulted in precipitation of ligand, while E-selectin chimera was without effect. Removal of calcium and addition of EDTA again reduced P-selectin chimera binding moderately but did not affect L-selectin chimera binding.

Using the microplate binding assay, titration of glycolipid added per well versus selectin chimera binding emphasizes the selectivity of SGNL lipid recognition (Fig. 3). To compare recognition by different chimeras, the data are expressed relative to maximal binding of each to 2,3-S-Le$^x$ (compare with Fig. 1). While the E-selectin chimera is unable to bind to SGNL lipid even at the highest concentrations tested, binding of L- and P-selectin chimeras to SGNL-lipids exceeded that to 2,3-S-Le$^x$ under identical conditions. Half-maximal binding of either chimera required <20 pmol of SGNL lipid per well, while half-maximal binding required

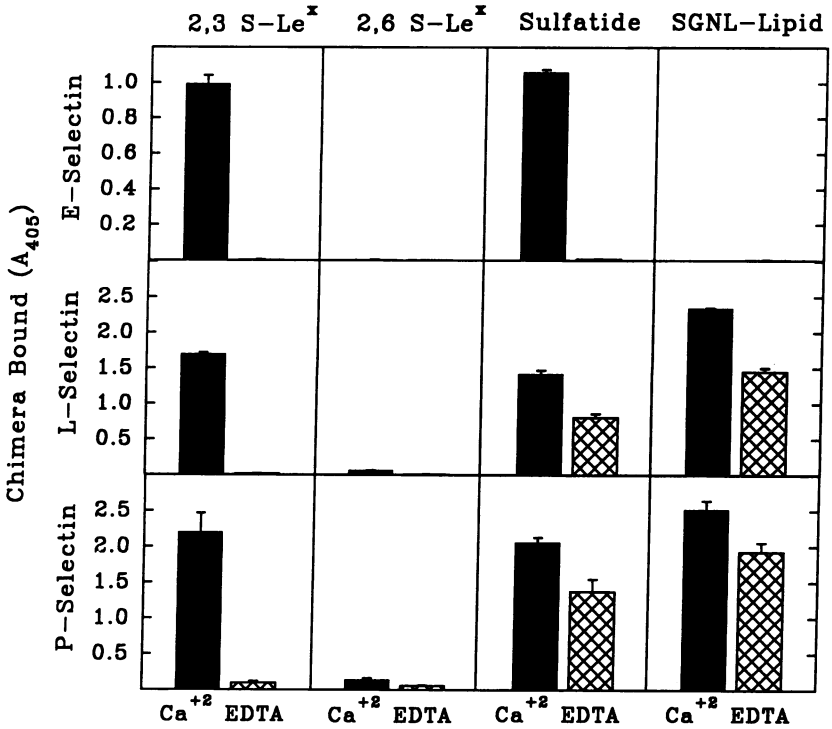


FIG. 1. Binding of E-, L-, and P-selectin–IgG chimeras to sialylated and sulfated glycosphingolipids. Lipids were adsorbed on microwell plates (see text) at the following concentrations: 2,3-S-Le$^x$ and 2,6-S-Le$^x$, 25 pmol per well; sulfatides, 31 pmol per well; SGNL lipids, 20 pmol per well. Each chimera was preincubated with anti-IgG and alkaline phosphatase–streptavidin in PBS containing either 1 mM calcium (solid bars) or 1 mM EDTA (cross-hatched bars). The chimera–anti-IgG–streptavidin complexes were added to lipid-adsorbed wells and incubated at 37°C for 45 min; the plates were then washed, colorimetric alkaline phosphatase substrate was added, the plates were incubated for 30 min at ambient temperature, and absorbance was read at 405 nm with a microplate spectrophotometer.

20–40 pmol of 2,3-S-Le$^x$ per well (compare with figure 3 of ref. 9).

Blocking antibodies were used to verify direct binding of L- and P-selectin chimeras to adsorbed SGNL lipid or sulfatide (Fig. 4). In each case, blocking monoclonal antibody specific for one of the selectins inhibited the ability of its chimera to bind to SGNL lipid (or sulfatide) but had no effect on binding by the other selectin chimera.

**SGNL Lipid Structural Determinants for Selectin Binding.** The molecular determinants on SGNL lipid responsible for recognition by L- and P-selectins were investigated by chemical modification of the parent lipid. Treatment of SGNL lipid with methanolic HCl resulted in concomitant removal of the 3-*O*-sulfate from the nonreducing terminal glucuronic acid and methyl esterification of the carboxylic acid at the 6-position of the glucuronic acid. A portion of the methyl esterified glycolipid was converted to the desulfated carboxylic acid by alkali treatment. The purity of the products was confirmed by silica gel TLC developed in chloroform/methanol/0.25% aqueous KCl (5:4:1). Each derivative appeared as a single major species that migrated distinctly from the parent SGNL lipid (SGNL lipid, $R_f$ = 0.35; desulfated

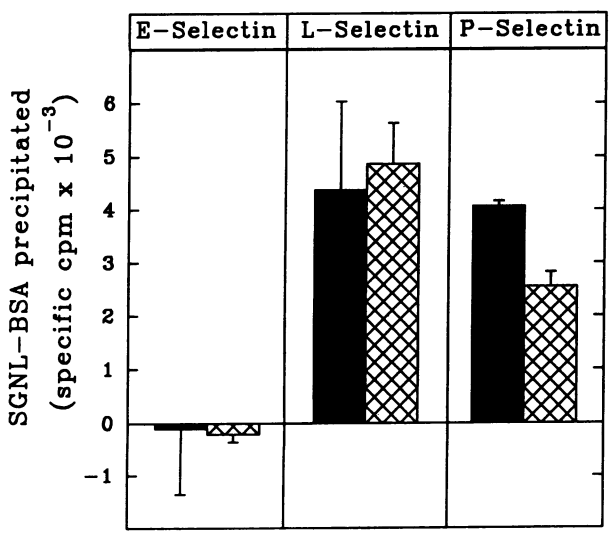


FIG. 2. Precipitation of $^{125}$I-labeled SGNL-derivatized bovine serum albumin by selectin chimeras. Selectin chimeras were preincubated with anti-IgG in PBS containing either 1 mM calcium (solid bars) or 5 mM EDTA (cross-hatched bars). The chimera–anti-IgG conjugates were added to $^{125}$I-labeled SGNL–bovine serum albumin (BSA) and incubated at 37°C for 90 min. Selectin-bound radioligand was precipitated with polyethylene glycol and collected on glass fiber filters; radiolabel was quantitated. Nonspecific binding was determined under identical conditions (in the presence of anti-IgG) but in the absence of any chimera.

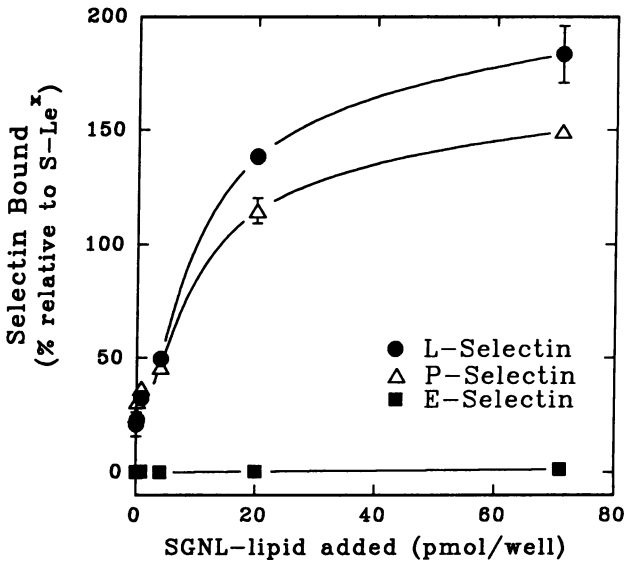


FIG. 3. Binding of E-, L-, and P-selectin–IgG chimeras as a function of SGNL lipid concentration. SGNL lipids (indicated amounts) or 2,3-S-Le$^x$ (25 pmol per well) were adsorbed to microwells as described in the text. Selectin chimera–anti-IgG–streptavidin conjugates were added and binding was measured as described in the legend to Fig. 1. Data are expressed relative to binding to 2,3-S-Le$^x$ for each chimera. Average $A_{405}$ values for binding to 2,3-S-Le$^x$ were as follows: E-selectin, 0.81; L-selectin, 1.16; P-selectin, 1.01.

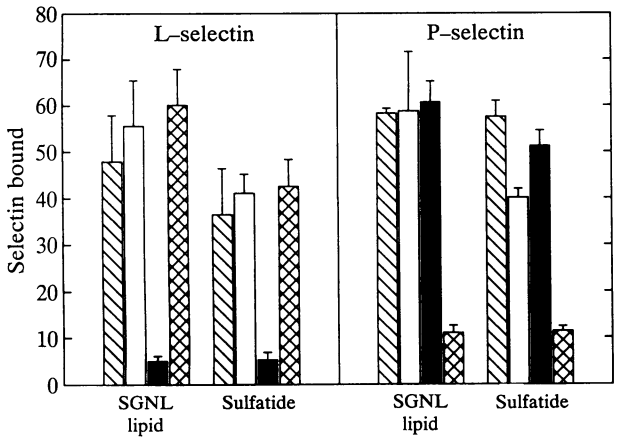


FIG. 4. Effects of anti-selectin antibodies on L-selectin chimera and P-selectin chimera binding to SGNL lipids and sulfatides. SGNL lipids or sulfatides were adsorbed to microwells at 25 pmol per well as described in the text. Selectin chimera–anti-IgG–streptavidin complexes (in PBS with 1 mM calcium) were added to wells either in the absence of anti-selectin antibodies (hatched bars) or in the presence of anti-E-selectin antibody BBA2 (open bars), anti-L-selectin antibody Mel-14 (solid bars), or anti-P-selectin antibody MON1137 (cross-hatched bars) (10 $\mu$g/ml each). Chimera binding was determined as described in the text, with bound alkaline phosphatase–streptavidin measured kinetically in a microplate reader. Binding is expressed as $\Delta A_{405}$/min $\times$ $10^3$.

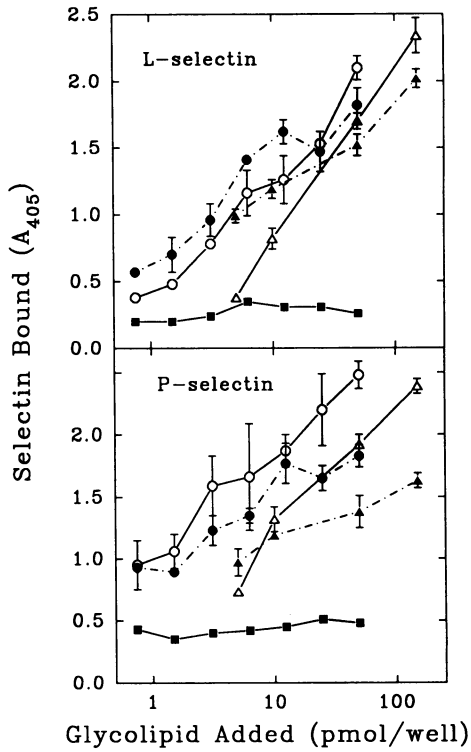


FIG. 5. L-selectin (*Upper*) and P-selectin (*Lower*) chimera binding to SGNL lipids and SGNL lipid derivatives. The following glycosphingolipids were adsorbed to microwells (see text) at the indicated concentrations: SGNL lipid (solid circles), desulfated SGNL lipid (open circles), desulfated SGNL lipid methyl ester (squares), sulfatides (open triangles), 2,3-S-Le$^x$ (solid triangles). L- or P-selectin chimera–anti-IgG–streptavidin conjugates in PBS containing 1 mM calcium were added and binding was measured as described in the legend to Fig. 1.

SGNL lipid, $R_f$ = 0.39; desulfated methyl esterified SGNL lipid, $R_f$ = 0.59). The identity of each derivative was confirmed by fast atom bombardment MS (negative ion mode; data not shown). As is characteristic of glycosphingolipids (39), the parent SGNL lipid produced a family of molecular ions representing heterogeneity in the ceramide (lipid) moiety, with the major molecular ion (M-1) at 1593, corresponding to a ceramide composed of sphingosine and a carbon 24:0 fatty acid amide as reported (24). The major molecular ion (M-1) generated by the desulfated species was 1513, as expected, and the methyl ester generated the same molecular species (M-$CH_3$) as well as the molecular ion (M-1) at 1527 (40).

Notably, desulfation of SGNL lipids did not reduce their ability to support L- and P-selectin chimera binding (Fig. 5). In contrast, methyl esterification of the glucuronic acid blocked the ability of desulfated SGNL lipid to support binding of L- or P-selectin chimeras.

To test the possibility that uronic acid groups and/or sulfate groups in close juxtaposition, rather than a specific orientation of binding determinants, are responsible for SGNL lipid binding, we tested the ability of various carbohydrate polymers with repeating carboxylic acid and/or sulfate groups to block binding of L- and P-selectin chimeras to SGNL lipids and sulfatide. At 20 $\mu$g/ml ($\approx$100 $\mu$M in saccharide groups), the following polymers did not significantly reduce binding of either chimera to either glycolipid: dextran sulfate, hyaluronic acid, chondroitin sulfate, or keratan sulfate (data not shown). While heparin did block binding of both chimeras to both sulfated glycolipids, it also blocked their binding to 2,3-S-Le$^x$, which has neither a uronic acid nor a sulfate group. Polyvinyl sulfate reduced binding of L-selectin chimera to all the glycolipids but did not reduce P-selectin chimera binding. We conclude that anionic charge density *per se* is not solely responsible for selectin chimera binding to any of the glycolipids tested.

## DISCUSSION

Selectins are a family of three structurally and functionally related cell surface carbohydrate binding proteins (E-, L-, and P-selectins) that have been implicated in leukocyte–endothelial cell adhesion, lymphocyte homing, and platelet adherence (1–3). The occurrence of a lectin domain in each selectin primary sequence led several groups to investigate carbohydrate ligands responsible for selectin-mediated cell adhesion. A consensus of data indicates that fucosylated sialylated glycoconjugates, specifically 2,3-S-Le$^x$ and related structures, are major carbohydrate determinants mediating cell adhesion via all three selectins (5–13). In addition to these data, some studies implicate sulfated glycolipids, proteoglycans, or glycoproteins as selectin ligands (14–18). A unique family of sulfated glycolipids, the HNK-1 reactive sulfoglucuronyl glycosphingolipids, have been characterized as determinants on peripheral nerve membranes. These glucuronyl glycosphingolipids may be evolutionarily more ancient than sialylated glycosphingolipids (gangliosides), since they have been demonstrated in invertebrates while gangliosides have not (41). Furthermore, vertebrate SGNL lipids are essentially absent from adult cerebral cortex, although they occur in the embryonic mammalian cortex and the adult cerebellum and peripheral nervous system (25–27). Recently, SGNL lipids have been reported to be present on endothelial cells from brain microvasculature (28). Therefore, we investigated the interaction between selectins and these sulfoglucuronyl lipids and compared them to interactions with S-Le$^x$ and sulfatide.

The most notable observation in our studies is that SGNL lipids support binding of L- and P-selectin chimeras but not the E-selectin chimera (Fig. 1). Even though all three selectin chimeras share considerable structural similarity (2), and all bound to 2,3-S-Le$^x$ and sulfatides, the E-selectin chimera failed to bind to SGNL lipids or a SGNL-derivatized protein

ligand (Fig. 2) under any condition tested. Even when added at concentrations far above those necessary to support significant L- or P-selectin chimera binding, the E-selectin chimera showed no hints of SGNL lipid recognition (Fig. 3). Further evidence of biochemical selectivity was the observation that anti-L- and anti-P-selectin antibodies blocked only the binding of their respective antigens to adsorbed SGNL lipid, while anti-E-selectin antibody was without effect on either selectin (Fig. 4).

Since selectins fall into the larger family of calcium-dependent vertebrate lectins (2, 4), calcium dependence has often been considered a criterion of valid *in vitro* binding assays for selectin recognition. However, lymphocyte adhesion to sulfated polysaccharides has been demonstrated to be calcium independent (42), although it is mediated by L-selectin (18). Sulfatide binding by L-selectin chimera *in vitro* has previously been shown to be calcium independent (9). In the current studies, L-selectin and P-selectin binding to both sulfatides and SGNL lipids was only modestly inhibited by removal of calcium ions. Of note, binding of all selectins to 2,3-S-Le$^x$ was calcium dependent, as was binding of E-selectin to sulfatides. The presence of calcium-dependent and calcium-independent modes of selectin-mediated binding may suggest that there are multiple binding sites on the selectins or that certain ligands can efficiently bind selectin active sites without the assistance of bound calcium ions. Only further investigations will resolve this important point.

Since the current studies were initiated to study sulfated glycoconjugates as selectin recognition molecules, structural modification studies with SGNL lipid were especially surprising. Remarkably, removal of the sulfate group from SGNL lipid did not significantly decrease its ability to support either L- or P-selectin binding (Fig. 5). These data demonstrate that a "neolacto" sugar core (Gal$\beta$1–4GlcNAc$\beta$1–3Gal$\beta$1–4Glc) substituted on the nonreducing galactose with a $\beta$1–3 glucuronic acid is sufficient to support L-selectin and P-selectin chimera binding.

Although functional *in vivo* roles for glucuronyl glycosphingolipids have not been proven, they are able to support cell adhesion of Schwann cells *in vitro* (20, 21) and have been implicated in neural cell adhesion (19). Whether L-selectin is involved in any SGNL lipid-mediated cell adhesions is not clear. However, the demonstration of SGNL lipids on brain microvascular endothelium and on human umbilical vein endothelial cells in culture (28) suggests that the SGNL determinant is available on appropriate tissues to interact with leukocytes carrying L- or P-selectins. In cases in which endothelial cells express SGNL lipids but not E-selectin, they may support selective adhesion of a desired subpopulation of leukocytes expressing L-selectin or P-selectin.

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