Higher-Affinity Oligosaccharide Ligands for E-Selectin

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Abstract

A series of synthetic oligosaccharides based on sialyl Lewis x (sLe^x; Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc) and sialyl Lewis a (sLe^a; Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAc) was used to study the binding interactions of selectins. E-selectin-immunoglobulin fusion protein (E-selectin-Ig) bound to immobilized bovine serum albumin (BSA)-neoglycoproteins containing sLe^x or sLe^a in a Ca^{2+}-dependent manner. Solution-phase sLe^x tetrasaccharide blocked this interaction by 50% at a concentration of 750±20 μM (IC_{50}). sLe^a was more effective (IC_{50} = 220±20 μM), while nonsialylated, nonfucosylated derivatives showed little or no activity at concentrations up to 1 mM. Attachment of an 8-methoxycarbonyloctyl aglycone in a β linkage to the anomeric carbon of the GlcNAc of sLe^x or sLe^a increased their blocking activity nearly twofold. Finally, replacement of the 2-*N*-acetyl substituent of the GlcNAc by an azido or amino group resulted in substantial increases in activity, with the most potent inhibitor being amino substituted sLe^a, which was 36-fold more active (IC_{50} = 21±3 μM) than the reducing tetrasaccharide sLe^x. In contrast to results obtained with E-selectin-Ig, P-selectin-Ig binding to immobilized BSA-sLe^a was blocked modestly by most oligosaccharides at 1 mM, with no substantial differences among them. IC_{50} values of soluble oligosaccharides determined in competitive binding studies accurately predicted blocking of leukocyte adhesion to recombinant E-selectin-Ig and to cytokine-activated endothelium. (*J. Clin. Invest.* 1993. 91:1157–1166.) Key words: adhesion • carbohydrate • P-selectin (CD62) • sialyl Lewis a • sialyl Lewis x

Introduction

The selectin family of molecules supports adhesion of leukocytes to the blood vessel wall—a key step in the inflammatory response to injury and infection. The three known members, designated E-, P-, and L-selectin (for nomenclature summary, see reference 1), are structurally similar transmembrane glycoproteins, each containing an amino-terminal lectin-like domain, an EGF repeat, and a variable number of complement regulatory-like repeats (2–7). E-selectin transcription and surface expression by vascular endothelial cells are transiently upregulated (maximal at 2–6 h) after stimulation by bacterial endotoxin and by cytokines associated with inflammation, including IL-1 and TNF (2, 8). Experiments in vitro have demonstrated that E-selectin can support the adhesion of PMN, monocytes, and a subpopulation of T lymphocytes (2, 8–18). P-selectin (CD62) is stored in α-granules and dense granules of platelets as well as in Weibel-Palade bodies of endothelial cells, and is rapidly (minutes) redistributed to the cell surface after activation by thrombin and other mediators (19–23), where it supports leukocyte binding (24, 25). L-selectin is present on the surface of most lymphocytes and mediates their adhesion to high endothelial venules found in peripheral lymph nodes (reviewed in references 26 and 27). L-selectin is also found on the surface of circulating PMN and monocytes, and can support their adhesion to cytokine-activated endothelium (28–32). Recent reports have suggested that the selectins are especially important in leukocyte rolling on the vessel wall, a process that can precede firm attachment and extravasation during inflammation (33–35).

Carbohydrate recognition by selectins has been demonstrated in several studies (reviewed in reference 36). E-selectin binds sialylated, fucosylated lactosamine structures, notably sialyl-Lewis x (sLe^x, Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc)[1] (37–41). E-selectin may also bind to oligosaccharides related to sLe^x and sLe^a in which the sialic acid is replaced by a sulfate group (42), and to fucosylated structures that lack sialic acid (43). The pentasaccharide lacto-*N*-fucopentaose III (LNF-III) contains the Lewis x (Le^x) determinant and was found to block the rosetting of activated platelets to leukocytes, a process involving P-selectin (44). Other investigations have demonstrated that sialic acid is a component of some P-selectin ligands (45, 46), and that oligosaccharides containing sLe^x are recognized by P-selectin (47, 48). More recently, the recognition of the sLe^x structure was extended to murine L-selectin (49, 50). Human E- and P-selectin and murine L-selectin also bind to molecules containing sLe^a (Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAc), an isomer of sLe^x (50–53). L- and P-selectin have also been shown to interact with sulfated polysaccharides such as heparin, fucoidan, and dextran sulfate, and with sulfatides, glycosphingolipids containing monosulfated

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1. *Abbreviations used in this paper:* Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetyl glucosamine; IC_{50}, concentration of inhibitor resulting in the reduction of binding to 50% of maximum; LNF-III, lacto-*N*-fucopentaose III (Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc); mOD_{450}, milli-optical density units at 450 nm; Neu5Ac, *N*-acetyl neuraminic acid (for abbreviations of oligosaccharides, see Table I); OPD, *o*-phenylenediamine dihydrochloride; selectin-Ig, selectin immunoglobulin fusion protein (see Fig. 1, *left panel*); sLe^a, sialyl Lewis a; sLe^x, sialyl Lewis x; thimerosal, mercury-[(*o*-carboxyphenyl)thio]-ethyl sodium salt; Tween-20, polyoxyethylenesorbitan monolaurate.

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galactose (53–57). The molecular details of selectin recognition of carbohydrates, both oligosaccharides and polysaccharides, remain to be determined.

We have examined carbohydrate binding interactions of E- and P-selectin using rationally designed synthetic oligosaccharides in three assays of increasing biological complexity. First, a competitive ELISA was developed to quantitate the ability of solution-phase carbohydrates to inhibit the binding of selectin-Ig fusion proteins to immobilized BSA-sLe^x or BSA-sLe^a neoglycoproteins in a purified component system. Second, a cell–protein adhesion assay was used to determine the ability of soluble carbohydrates to inhibit the adhesion of cells bearing carbohydrate ligands to purified, immobilized recombinant selectin-Ig fusion proteins. Third, a cell–cell adhesion assay was employed to determine the ability of carbohydrates to inhibit the adhesion of PMN to cytokine-activated cultured human umbilical vein endothelial cells, known to express E-selectin on their surface. Our results indicate that E- and P-selectin have distinct carbohydrate recognition characteristics, and that selected modifications of the oligosaccharides sLe^x and sLe^a result in higher-affinity ligands for E-selectin. These findings suggest avenues for the development of novel anti-inflammatory therapeutic agents.

Methods

Proteins

Recombinant fusion proteins composed of extracellular portions of the human selectins joined to human immunoglobulin heavy chain CH3, CH2, and hinge regions (Fig. 1, *left panel*) were constructed, expressed, and purified as previously described (39, 57, 58). These soluble selectin–immunoglobulin fusion proteins (selectin-Ig) contain the signal sequence, lectin-like domain, and EGF repeat along with six, two, and two of the complement regulatory-like modules for E-, P-, and L-selectin-Ig, respectively. Selectin-Ig purity was assessed by SDS-PAGE followed by silver staining; protein concentration was determined using the Bradford reagent. Molecular weights used for calculation of molar concentrations are: E-selectin-Ig, 170,000; P-selectin-Ig and L-selectin-Ig, 120,000. BSA was protease-free grade from Boehringer-Mannheim Biochemicals, Indianapolis, IN. BSA-neoglycoproteins containing sLe^x, sLe^a, Le^x, or Le^a (kindly provided by Chembiomed Ltd., Edmonton, Alberta) were made as previously described (59). Oligosaccharides were synthesized with an aliphatic aglycone (8-methoxycarbonyloctyl; $-(CH_2)_8CO_2CH_3$) attached in a β linkage to carbon 1 of the GlcNAc (Fig. 1, *right panel*) (60). This modification facilitated covalent attachment of oligosaccharides to BSA through the formation of acyl azides. Incorporation of the aglycone glycoside forms of sLe^x, sLe^a, Le^x, and Le^a into the respective BSA-neoglycoproteins (mole ratio) was 9, 13, 13, and 17. Antibody G559 against P-selectin was provided by R. McEver, University of Oklahoma Health Science Center, Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK.

Carbohydrates

A majority of the oligosaccharides used in these studies were provided by Chembiomed, Ltd., and were unambiguously synthesized using a chemoenzymatic route, purified by chromatographic methods with the final step on Biogel P-2, and freeze-dried in vacuo (P. V. Nikrad, M. A. Kasham, K. Wlasichuk, and A. P. Venot, Chembiomed Ltd., manuscript in preparation). In most cases, these compounds were synthesized with an aliphatic aglycone (8-methoxycarbonyloctyl; $-(CH_2)_8CO_2CH_3$) attached in a β linkage to the reducing sugar (59, 60) (Fig. 1, *right panel;* Table I). All of these compounds were pure, as assessed by ^{1}H-nuclear magnetic resonance spectroscopy. Reducing tetrasaccharides sLe^x and sLe^a (sLe^x—OH and sLe^a—OH; see Table I) were purchased from Oxford Glycosystems Ltd. (Rosedale, NY). Oligosaccharides, heparin, and fucoidan (Sigma Chemical Co., St. Louis, MO) were reconstituted in Dulbecco's phosphate-buffered saline (PBS) and stored at −80°C. Sulfatides (heterogeneous 3-O sulfated galactosyl ceramide) and cerebrosides (galactosyl ceramide) from bovine brain (Sigma Chemical Co.) were reconstituted in chloroform:methanol (1:1) at 10 mg/ml and stored at −20°C.

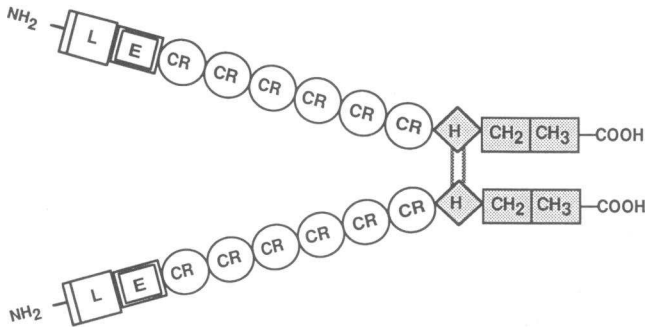

Figure 1.

ELISA measuring selectin–carbohydrate interactions

Polystyrene 96-well microtiter ELISA plates (no. 25801, Corning Glass, Newark, CA) were coated with unconjugated BSA or BSA-neoglycoproteins by incubation overnight at 4°C with 1.5 μg/ml in 75 μl per well of 50 mM sodium carbonate/bicarbonate buffer, pH 9.5. For coating sulfatides or cerebrosides, the required volume of stock solution was dried in a glass test tube and redissolved in methanol (HPLC grade) at a final concentration of 20 μg/ml; 50 μl was added to each microtiter plate well and evaporated to dryness in a 37°C incubator. All coated plates were blocked using 200 μl per well of 20 mg BSA/ml in assay buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.25 mM thimerosal) for a minimum of 2 h. The remaining steps of the assay were performed at room temperature. Wells were washed twice with assay buffer followed by addition of 50 μl of test sample (containing either purified selectin-Ig or selectin-Ig and carbohydrate) in assay buffer supplemented with 10 mg BSA/ml, 0.05% (vol/vol) Tween-20, and either 2 mM $CaCl_2$ or 5 mM EGTA. After incubation for 3 h on a rotating platform shaker at 75 rpm, the plates were washed twice, and 100 μl per well added of peroxidase-conjugated rabbit F(ab')$_2$ anti-human IgG (H+L) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 1:3,000 in assay buffer supplemented with 2 mM $CaCl_2$ and 10 mg BSA/ml. After 30 min the plates were washed 3 times with assay buffer containing 2 mM $CaCl_2$, followed by addition of 200 μl/well of 0.8 mg *o*-phenylenediamine dihydrochloride (OPD)/ml in 50 mM sodium citrate, 50 mM sodium phosphate buffer, pH 5.0, containing 0.015% (vol/vol) hydrogen peroxide. The rate of the enzymatic reaction was determined in duplicate or triplicate wells by measuring the absorbance at 450 nm at 15–30-s time intervals using a V_{max} kinetic microplate reader and Softmax software (Molecular Devices, Inc., Menlo Park, CA). An endpoint determination at 490 nm was made after stopping the reaction in the linear range with 50 μl per well of 4 N H_2SO_4. Specific binding was calculated by subtraction of the signal in wells coated with underivatized BSA (nonspecific binding) from the signal in wells coated with BSA-neoglycoproteins. For determination of IC_{50} values, the data were iteratively fit to the equation: Fraction of maximal binding = $IC_{50} \div (IC_{50} + [\text{oligosaccharide}])$, using non-linear least squares analysis software (Enzfitter, Elsevier-Biosoft, Cambridge, England).

Cell isolation and culture

PMN (neutrophils). Blood from healthy human donors was collected in heparinized tubes and layered over a double-density gradient of Histopaque 1077 and 1119 (Sigma, St. Louis, MO), essentially as described (61). After centrifugation for 20 min at 700 *g*, PMN were

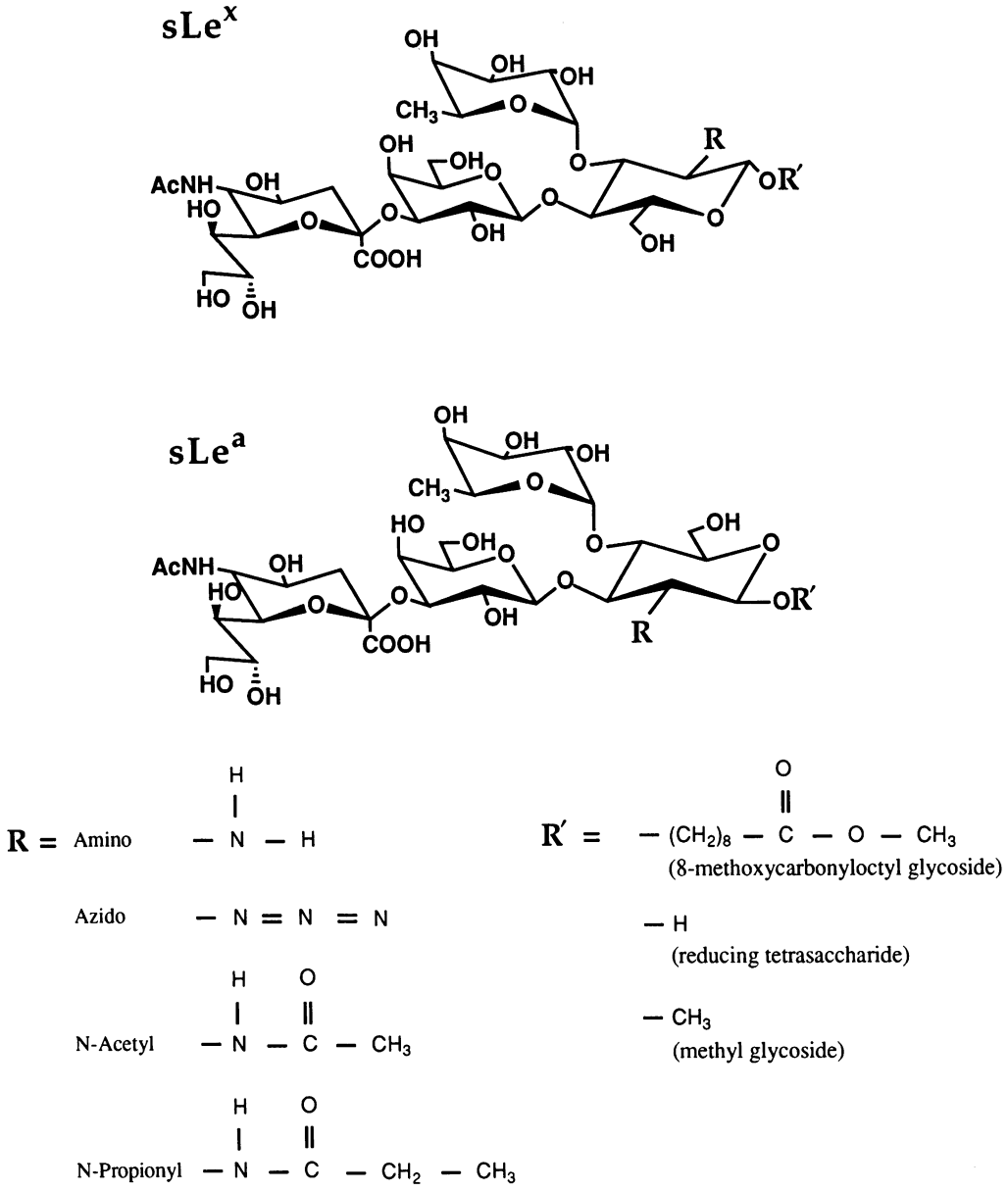

Figure 1. (*Opposite page*) Structure of E-selectin-Ig fusion protein. Shaded regions indicate the human immunoglobulin sequences; unshaded portions represent human E-selectin domains. This fusion protein is secreted from transfected COS cells as a dimeric molecule, disulfide linked in the immunoglobulin hinge region. *Abbreviations:* L, lectin-like domain; E, epidermal growth factor repeat; CR, complement regulatory-like domain; H, immunoglobulin hinge region; CH_2 and CH_3, immunoglobulin heavy chain constant regions. Analogous immunoglobulin fusion proteins containing human P- and L-selectin sequences were also used in some studies, and these molecules contained two CRs in each selectin polypeptide. (*Above*) Structures of sLe^x, sLe^a, and derivatives. sLe^x, sLe^a, and substituents used in modifications at carbons 1 and 2 of GlcNAc are depicted. In naturally occurring sLe^x and sLe^a, R = *N*-acetyl. For reducing oligosaccharides, R′ = H. The majority of oligosaccharides used in the present studies were 8-methoxycarbonyloctyl glycosides (R′ = $(CH_2)_8CO_2CH_3$). See Table I for a complete list of abbreviations and structures of compounds.

collected and washed once with cold PBS. Contaminating red blood cells were removed via hypotonic lysis. Cell viability was assessed using trypan blue exclusion and the cells resuspended at a concentration of 2×10^6 cells/ml in PBS containing 0.5 mg of human serum albumin (HSA)/ml, 3 mM glucose, and 0.3 U aprotinin/ml (adhesion assay buffer).

Endothelial cells. Human umbilical vein endothelial cells (Clonetics Corp., San Diego, CA) were seeded in tissue culture wells that had been coated with 0.1% gelatin in water and allowed to grow to confluence in media M199 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 5 U heparin/ml and endothelial cell growth supplement (Sigma Chemical Co.) at 15 μg/ml. Before adhesion assays, endothelial cell monolayers were washed once to remove cellular debris and activated with 200 U TNF-α/ml (gift from Biogen, Cambridge, MA) in M199 −10% FCS for 4 h at 37°C (8).

Cell–protein and cell–cell adhesion assays

Leukocyte adhesion assays were performed in Nunclon MicroWell Terasaki plates (No. 136528, Nunc, Naperville, IL) containing an endothelial cell monolayer (see above) or coated with recombinant selectin-Ig fusion proteins. To capture selectin-Ig molecules, wells were incubated with 10 μl of a 10 μg/ml solution of polyclonal goat anti-human IgG (γ-chain specific) antibody (Organon Teknika, Malverne, PA) or protein A (Chemicon, Temecula, CA) in 50 mM carbonate/bicarbonate buffer, pH 9.5, for 1 h at room temperature or overnight at 4°C. Plates were washed twice with PBS by flooding, and aspirated; 5 μl of the appropriate selectin-Ig at 10 μg/ml in PBS were added to each well and allowed to incubate for 1 h at room temperature. Plates were washed, incubated for 1 h with PBS containing 1% HSA, and washed again. Wells coated with selectin-Ig fusion proteins or containing confluent endothelial cell monolayers were incubated with 5 μl of PBS or PBS containing carbohydrate for 30 min at 4°C prior to the addition of PMN. PMN (2×10^6 cells/ml) were added in 5 μl of adhesion assay buffer and allowed to adhere for 15–30 min at 4°C. Nonadherent cells were removed by washing with PBS. Adherent cells were fixed by adding 2.5% glutaraldehyde in PBS, and counted over a 0.24-mm^2 area in each of four replicate wells. Cell counts were averaged and expressed as adherent cells per square millimeter. Adhesion of PMN to E- and P-selectin-Ig fusion proteins was inhibitable by specific monoclonal antibodies H18/7 and G559, respectively. Adhesion to cytokine-activated endothelial cells was partially blocked by anti-E-selectin antibody H18/7 as previously described (8).

Table I. Oligosaccharide Abbreviations and Structures

Abbreviation*	Structure‡
sLacNAc	Neu5Ac α2-3 Galβ1-4GlcNAcβ—OR′
sLe^x	Neu5Ac α2-3 Galβ1-4(Fuc α1-3)GlcNAcβ—OR′
sLe^x—OH	.....GlcNAc
sLe^x—OCH_3	.....GlcNAcβ—OCH_3
$sLe^x(GlcN_3)$	.....$GlcN_3$β—OR′
$sLe^x(GlcNH_2)$	.....$GlcNH_2$β—OR′
sLe^x(GlcNHPr)	.....GlcNHPrβ—OR′
$9NH_2$-sLe^x	$9NH_2$Neu5Ac.....GlcNAcβ—OR′
$KDNLe^x$	Neu5OH .....β—OR′
Fuc1-6sLacNAc	Neu5Ac α2-3 Galβ1-4(Fuc α1-6)GlcNAcβ—OR′
sLe^c	Neu5Ac α2-3 Galβ1-3GlcNAcβ—OR′
sLe^a	Neu5Ac α2-3 Galβ1-3(Fuc α1-4)GlcNAcβ—OR′
sLe^a—OH	.....GlcNAc
$sLe^a(GlcN_3)$	.....$GlcN_3$β—OR′
$sLe^a(GlcNH_2)$	.....$GlcNH_2$β—OR′
sLe^a(GlcNHPr)	.....GlcNHPrβ—OR′
2-6sLe^a	Neu5Ac α2-6.....GlcNAcβ—OR′

* s, Neu5Ac; Pr, $C(O)CH_2CH_3$; KDN, keto-deoxy-nonulosonic acid (Neu5OH). ‡ Dots indicate identity with the structure above; R′ = $(CH_2)_8CO_2CH_3$.

Results

E- and P-selectin-Ig bind to immobilized BSA-sLe^a and BSA-sLe^x. Soluble E- and P-selectin-Ig bound to BSA-sLe^a immobilized on microtiter plates in a concentration-dependent manner (Fig. 2). E-selectin-Ig binding appeared to depend entirely on the covalently attached oligosaccharide moiety of the neoglycoprotein, in that it failed to bind to BSA alone. Furthermore, this binding was abolished in the presence of 5 mM EGTA, consistent with the function of E-selectin as a C-type lectin (62). In similar studies, E-selectin-Ig bound to immobilized BSA-sLe^x in a concentration- and Ca^{2+}-dependent fashion, but displayed very little binding to BSA-neoglycoproteins containing Le^a or Le^x (at 100 nM E-selectin-Ig, ~ 6 and < 2 mOD_{450}/min, respectively).

P-selectin also bound to immobilized BSA-sLe^a (Fig. 2) and BSA-sLe^x (not shown) in a concentration-dependent manner. Unlike E-selectin-Ig, P-selectin-Ig showed a small amount of binding to immobilized BSA that had not been derivatized with oligosaccharide. In a typical ELISA, 100 nM P-selectin-Ig binding to BSA alone gave a signal of 9 mOD_{450}/min., whereas the same concentration of E-selectin-Ig produced a BSA signal of 3 mOD_{450}/min (background signal in the absence of selectin-Ig was 2–4 mOD_{450}/min). This P-selectin-Ig binding to unconjugated BSA was apparently unrelated to a Ca^{2+}-dependent lectin activity since it was enhanced severalfold in the presence of EGTA. With this high background binding to BSA subtracted, the binding of P-selectin-Ig to BSA-sLe^a (Fig. 2) and BSA-sLe^x was largely blocked by 5 mM EGTA.

Human L-selectin-Ig displayed little or no detectable binding to BSA-neoglycoconjugates containing sLe^a, sLe^x, Le^a, or Le^x in the presence of 0.05% Tween-20. Some preparations of L-selectin-Ig displayed modest binding to BSA-sLe^x and BSA-sLe^a in the absence of detergent (at 100 nM, 20–50 mOD_{450}/min), possibly reflecting the enhanced avidity of aggregates. Consistent with previous reports (53, 57), both L-selectin-Ig and P-selectin-Ig bound to immobilized sulfatides (not shown). In the presence of EGTA, L-selectin-Ig binding to sulfatides was reduced by ~ 50%, while P-selectin-Ig binding was largely unaffected.

Sialic acid and fucose are key components of sLe^a and sLe^x binding to E-selectin-Ig. We have examined the ability of solution-phase synthetic oligosaccharides to block the binding of E-selectin-Ig to solid-phase BSA-sLe^a. At 500 μM, the tetrasaccharides sLe^x and sLe^a with attached aliphatic aglycone (see Table I) blocked specific binding by more than 50%, with sLe^a

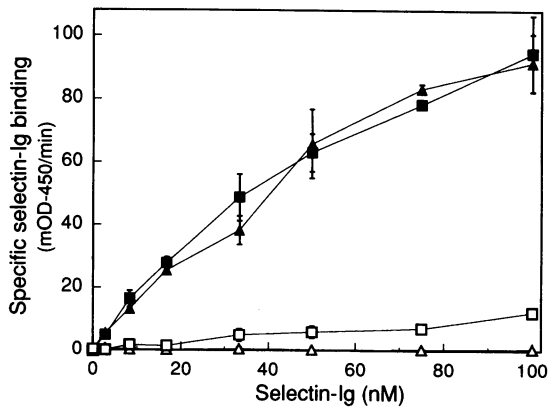

Figure 2. Specific binding of E- and P-selectin-Ig to immobilized BSA-sLe^a. An ELISA was used to measure concentration-dependent binding to BSA-sLe^a of E-selectin-Ig (*triangles*) and P-selectin-Ig (*squares*) in the presence of 2 mM $CaCl_2$ (*filled symbols*) or 5 mM EGTA (*open symbols*). Specific binding to BSA-sLe^a was determined by subtraction of binding to unconjugated BSA at each concentration and buffer condition. As noted in the text, P-selectin-Ig demonstrated an unexpectedly high binding to unconjugated BSA in the presence of EGTA. Symbols and error bars are the mean and standard deviation of measurements of rate of color generation from triplicate wells.

exhibiting higher activity than sLe^x (Fig. 3). The non-sialylated trisaccharide Le^x showed no blocking activity at this concentration, while Le^a demonstrated slight activity (13–20% inhibition, four experiments). The ability of solution-phase tetrasaccharides sLe^a and sLe^x to block binding of E-selectin-Ig to immobilized BSA-neoglycoproteins was interpreted as competitive inhibition.

To determine structural requirements for carbohydrate recognition by E-selectin, we tested a variety of synthetic oligosaccharides with modifications of the sialic acid and/or fucose groups. The importance of the linkage of sialic acid to the saccharide backbone was demonstrated using an isomer of sLe^a, designated 2-6sLe^a, in which the sialic acid is attached to galactose in an α2-6 rather than an α2-3 linkage. This carbohydrate displayed no significant blocking activity at a concentration of 500 μM. Two additional modifications to the sialic acid of sLe^x, namely attachment of an amino group on carbon 9 ($9NH_2sLe^x$) or replacement of the N-acetyl group at carbon 5 by a hydroxyl group ($KDNLe^x$), resulted in compounds that had less activity than unmodified sLe^x.

The importance of fucose position and linkage in sLe^a and sLe^x was examined using selected oligosaccharides (Fig. 3). sLe^x- and sLe^a-related trisaccharides that lack fucose, namely sLacNAc (Neu5Acα2-3Galβ1-4GlcNAcβ—OR′) and sLe^c (Neu5Acα2-3Galβ1-3GlcNAcβ—OR′) respectively, had no blocking activity. In addition, Fuc1-6sLacNAc, a compound structurally similar to sLe^x with fucose linked α1-6 to the GlcNAc, showed only a small amount of blocking at 500 μM.

Higher-affinity carbohydrate ligands for E-selectin. The addition of hydrophobic sequences to certain mono- and oligosaccharides has been shown to increase their binding affinity for lectins (63, 64). The majority of oligosaccharides employed in our experiments were synthesized with an aliphatic aglycone

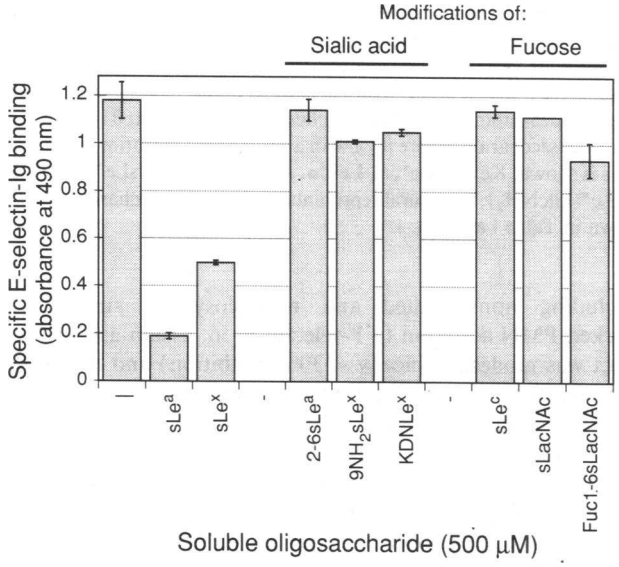

Figure 3. Inhibition of E-selectin-Ig binding to immobilized BSA-sLe^a by sLe^a, sLe^x, and oligosaccharides modified in sialic acid and fucose. Soluble oligosaccharides (500 μM) were incubated together with 25 nM E-selectin-Ig in microtiter wells coated with BSA-sLe^a. After washing, bound E-selectin-Ig was detected using an enzyme-conjugated antibody and colorimetric substrate, as described in Methods. Mean endpoint absorbance measurements (±SD) from triplicate wells are shown.

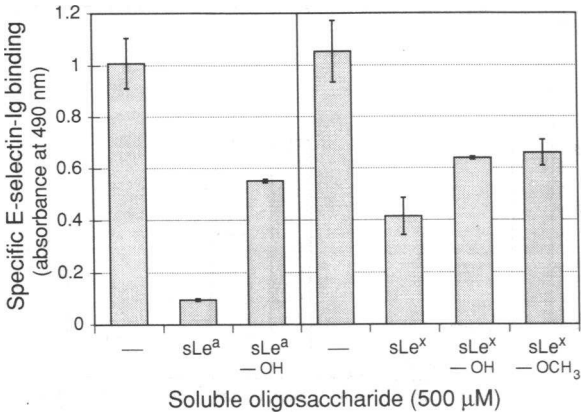

Figure 4. Effect of the aliphatic aglycone on blocking activity of sLe^a and sLe^x. Solution-phase oligosaccharide (500 μM) was used to block the direct binding of 25 nM E-selectin-Ig to immobilized BSA-sLe^a. sLe^a and sLe^x (*second bar of each panel*) were synthesized with the aliphatic aglycone, $(CH_2)_8CO_2CH_3$ (8-methoxycarbonyloctyl), attached via β linkage to carbon 1 of GlcNAc. In the experiment depicted in the left panel, sLe^a with the aglycone was compared to the reducing tetrasaccharide (sLe^a—OH). The right panel compares the activity of sLe^x with the aglycone to the reducing tetrasaccharide and the methyl glycoside.

(—$(CH2)_8CO_2CH_3$) attached in a β-glycosidic linkage to the reducing sugar (Table I). The presence of this aglycone was found to measurably enhance the blocking activity of sLe^a and of sLe^x (Fig. 4). The methyl β-glycoside of sLe^x (sLe^x—OCH_3) and the reducing tetrasaccharide (sLe^x—OH)appeared to have identical inhibitory activities. Titration experiments indicated that sLe^a and sLe^x with the aglycone were nearly twofold more active than the corresponding reducing tetrasaccharides (sLe^a—OH and sLe^x—OH), as determined by the concentration required to inhibit 50% of E-selectin-Ig binding (IC_{50}).

By examining tetrasaccharides based on the sLe^a and sLe^x structures with substitutions at the 2 position of GlcNAc, we identified several compounds having substantially greater blocking activity against E-selectin. Replacement of the *N*-acetyl group with either amino or azido moieties produced the most potent blockers. As shown in Fig. 5, $sLe^a(GlcNH_2)$ and $sLe^x(GlcN_3)$ at concentrations of 500 μM almost completely blocked direct binding of E-selectin-Ig to BSA-sLe^a. Analogues of sLe^a and sLe^x synthesized with a propionyl substitution at the same position showed decreased [sLe^a(GlcNHPr)] or unchanged [sLe^x(GlcNHPr)] blocking relative to the parent tetrasaccharides. Titration of these tetrasaccharides (Fig. 6, *top;* Table II) indicated that amino substitution for the *N*-acetyl group of GlcNAc reduced the IC_{50} to 21±3 μM, an apparent sixfold increase in activity. Further, this represents a 36-fold increase in activity over the reducing tetrasaccharide, sLe^x—OH, which has an IC_{50} of 750±20 μM. The analogous azido substitution displayed activity between sLe^a and $sLe^a(GlcNH_2)$, with an IC_{50} of 55±14 μM. As noted above, sLe^a was a better blocker of E-selectin-Ig binding than was sLe^x; the IC_{50} of sLe^a was about one-third that of sLe^x, comparing either the reducing sugars themselves or their aglycone glycosides. In contrast to their effects on E-selectin-Ig, none of the synthetic oligosaccharides tested were especially effective in blocking P-selectin-Ig binding to BSA-sLe^a, including the amino and azido deriva-

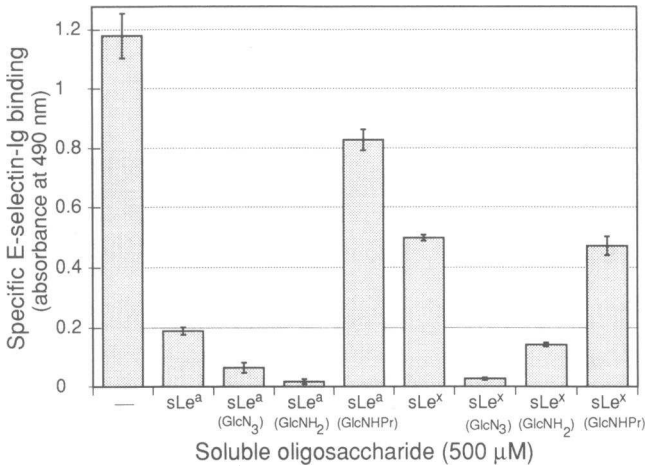

Figure 5. Analogues of sLe^a and sLe^x with amino and azido substitutions at carbon 2 of GlcNAc are potent blockers of E-selectin-Ig. Solution-phase sLe^a, sLe^x, and derivatives (500 μM) were used to block the binding of 25 nM E-selectin-Ig to immobilized BSA-sLe^a. Structures of azido, amino, and *N*-propionyl constituents used to replace the *N*-acetyl group of GlcNAc in sLe^a and sLe^x are shown in Fig. 1, *right panel*. Symbols and error bars represent the mean and standard deviation of measurements made in triplicate wells.

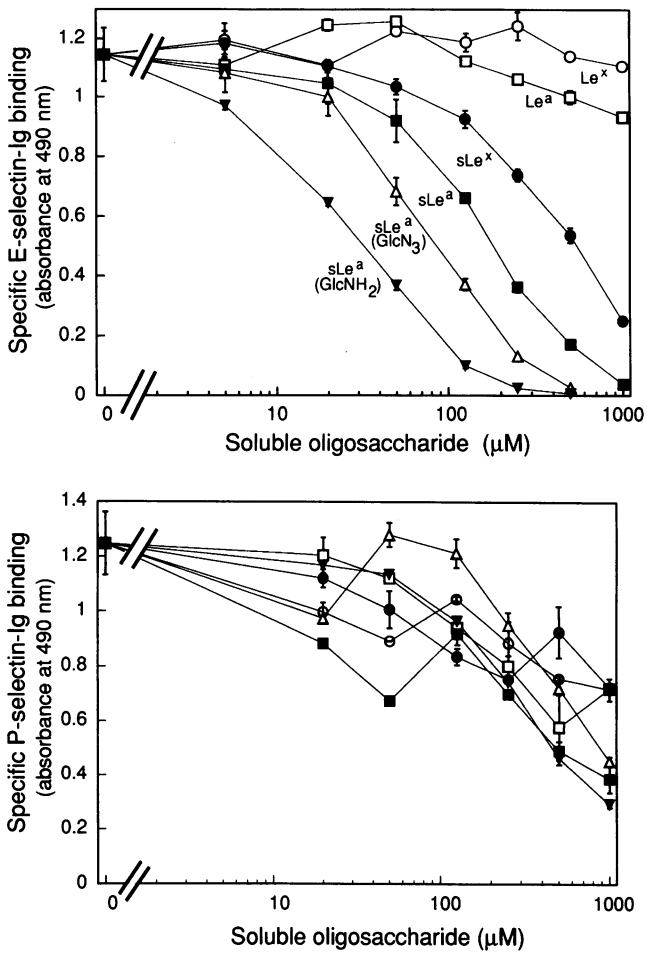

Figure 6. Concentration-dependent inhibition of E- and P-selectin-Ig binding to immobilized BSA-sLe^a by synthetic oligosaccharides. (*Top*) Data from a representative competitive binding assay measuring the inhibition of specific E-selectin-Ig binding to BSA-sLe^a by six related oligosaccharides. (*Bottom*) Inhibition of specific P-selectin-Ig binding by the same oligosaccharides. Assays were performed in the presence of 2 mM $CaCl_2$. Symbols and bars represent the mean and range of measurements made in duplicate wells, except at 0 mM soluble oligosaccharide, where the mean and standard deviation of six wells is shown. Key: ○, Le^x; □, Le^a; ●, sLe^x; ■, sLe^a; △, $sLe^a(GlcN_3)$; ▼, $sLe^a(GlcNH_2)$. Structural representations of oligosaccharides are shown in Table I and Fig. 1.

tives of sLe^a and sLe^x (Fig. 6, *bottom*). It is noteworthy that among solution-phase oligosaccharide inhibitors of P-selectin-Ig, both nonsialylated and sialylated structures had some blocking activity, with the latter being moderately more effective. As noted above, however, P-selectin-Ig exhibited a substantially higher level of binding to immobilized BSA-sLe^x and BSA-sLe^a than to their nonsialylated counterparts BSA-Le^x and BSA-Le^a. In separate experiments, both heparin (a glycosaminoglycan composed of alternating, sulfated residues of glucosamine and uronic acid) and fucoidan (a homopolymer of sulfated L-fucose) blocked the binding of P-selectin-Ig to BSA-sLe^a but had no effect on E-selectin-Ig binding (Fig. 7).

Blocking of cell adhesion by oligosaccharides in vitro. Results of competitive binding studies described above accurately predicted relative effects of solution-phase carbohydrates on leukocyte adhesion to purified selectin-Ig fusion proteins and to cytokine-activated endothelial monolayers. At a concentration of 1 mM, sLe^a blocked adhesion of PMN to immobilized recombinant E-selectin-Ig by ~ 50% (Fig. 8). sLe^x showed little or no blocking of PMN adhesion at concentrations up to 1 mM, a result consistent with its IC_{50} being threefold higher than that of sLe^a in the competitive binding ELISA (Table II). In further agreement with the competitive binding results, the ability of oligosaccharides to block cell adhesion to E-selectin-Ig required sialic acid linked α2-3 to galactose, and fucose linked to the GlcNAc. Compounds with sialic acid linked α2-6 to galactose as well as compounds lacking sialic acid or fucose failed to block PMN adhesion at concentrations up to 1 mM (data not shown). Further, $sLe^a(GlcN_3)$ and $sLe^a(GlcNH_2)$ were much more active than sLe^a in blocking the adhesion of PMN to E-selectin-Ig. sLe^a(GlcNHPr), which has an *N*-propionyl group substituted for the *N*-acetyl group of GlcNAc, failed to block adhesion at a concentration of 1 mM (Fig. 8). In separate experiments, PMN adhesion to immobilized P-selectin-Ig was poorly blocked by soluble oligosaccharides related to sLe^x and sLe^a (not shown). Although several oligosaccharides (including nonsialylated and nonfucosylated structures) blocked PMN adhesion to P-selectin-Ig in certain assays, the effect was modest (typically < 30% inhibition) and inconsistent. Neither azido nor amino substitutions at carbon 2 of GlcNAc enhanced the ability of the compounds to block leukocyte adhesion to P-selectin-Ig.

In a more complex adhesion assay, we examined the effects of solution-phase oligosaccharides on the adhesion of PMN to human umbilical vein endothelial cell monolayers. Cytokine activated endothelial cells express E-selectin in abundance, and thereby support leukocyte adhesion (8). As shown in Fig. 9, sLe^a at a concentration of 1 mM partially blocked PMN adhesion. $sLe^a(GlcNH_2)$ was the most active blocker in this system, followed by $sLe^a(GlcN_3)$. Moreover, sLe^x was consistently less active than sLe^a in blocking PMN adhesion to activated endothelial cell monolayers, typically blocking 0–30% of adhesion. Oligosaccharide inhibition data from the three assays were in

Table II. Comparison of Inhibitory Potency of Soluble Oligosaccharides Assessed in E-Selectin-Ig Competitive Binding Studies

Oligosaccharide	IC_{50}*	Relative blocking activity
	μM	
sLe^x—OH	750±20 (2)	1.0
sLe^x	380±70 (4)	2.0
sLe^a—OH	220±20 (1)	3.4
sLe^a	130±20 (4)	5.8
sLex($GlcNH_2$)	77±7 (1)	9.7
sLe^a($GlcN_3$)	55±14 (2)	14
sLe^a($GlcNH_2$)	21±3 (4)	36

* Concentration of oligosaccharide that resulted in 50% inhibition of the binding of solution-phase E-selectin-Ig to immobilized BSA-sLe^a, as measured in an ELISA (see text for assay details and Fig. 6 for representative binding data). IC_{50} values were determined using nonlinear least squares analysis of the data, as described in Methods. The number of titration experiments performed (n) is shown in parentheses for each oligosaccharide. Where $n > 1$, IC_{50} data represent the mean of n extracted values±SD; where $n = 1$, the extracted IC_{50}±SE is shown.

general agreement: inhibition of PMN adhesion to natural (nonrecombinant) E-selectin expressed by endothelial cells corresponded to inhibition of PMN adhesion to recombinant E-selectin-Ig, and to inhibition of solution-phase E-selectin-Ig binding to immobilized BSA-neoglycoproteins. Of these assays, the competitive binding ELISA was the most sensitive. An example of the difference in sensitivity of the assays can be seen by comparing the degree of inhibition in each assay system (Figs. 5, 8, and 9).

Discussion

Knowledge that the selectins contain regions homologous to carbohydrate recognition domains of C-type lectins has prompted an intensive effort to identify carbohydrate ligands (36). To date, most data on selectin–carbohydrate binding interactions have been generated using cellular systems to facilitate the characterization of natural ligands. A major success of this work has been the recognition that sLe^x, a structure found in abundance on PMN and monocytes (65–67) is a ligand of E-selectin (37–41, 51). Other studies have demonstrated that

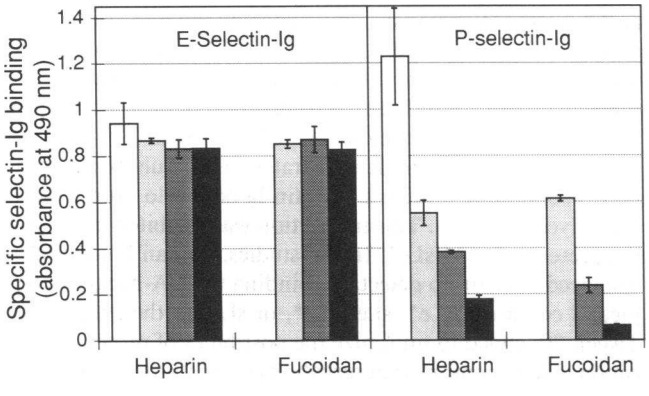

Figure 7. Inhibition of E- and P-selectin-Ig binding to immobilized BSA-sLe^a by sulfated polysaccharides. E- and P-selectin-Ig (25 nM) were incubated with 0 (open bars), 0.1, 1.0, and 10 (*increasingly darker bars*) μg/ml of heparin or fucoidan in a competitive binding ELISA. Symbols and error bars represent the mean and standard deviation of measurements made in triplicate wells.

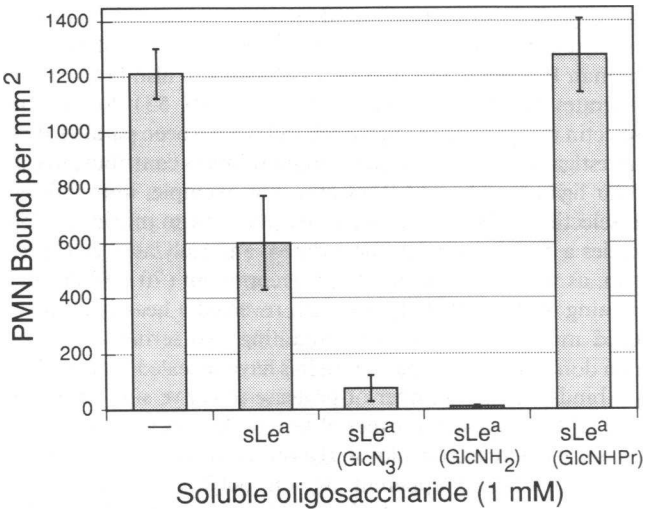

Figure 8. Inhibition of PMN adhesion to immobilized E-selectin-Ig by synthetic oligosaccharide derivatives of sLe^a. Solution-phase sLe^a and its amino-, azido-, and *N*-propionyl-substituted derivatives were tested for their ability to block PMN adhesion to immobilized E-selectin-Ig immobilized on microtiter wells. Oligosaccharide inhibitors were present throughout the adhesion assay, except in the control wells (first bar). Data are expressed as the mean and standard deviation of cells counted in quadruplicate wells. Background adhesion to a nonspecific immunoglobulin fusion protein was subtracted from all values, and was < 5% of adhesion to E-selectin-Ig.

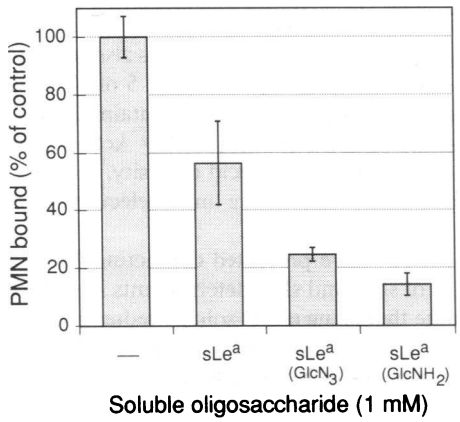

Figure 9. Inhibition of PMN adhesion to cytokine-activated endothelial cells by synthetic oligosaccharide derivatives of sLe^a. Solution-phase sLe^a and its amino-, azido-, and *N*-propionyl-substituted derivatives were tested for their ability to block PMN adhesion to confluent human umbilical vein endothelial cells that had been stimulated for 4 h with TNF-α. Data are expressed as the mean and standard deviation of cells counted in quadruplicate wells. Control wells measured adhesion in the absence of added oligosaccharide (*first bar*).

the oligosaccharide sLe^a, found on colon cancer cells, is bound by E-selectin (39, 68, 69). Recent reports demonstrate that human P-selectin and murine L-selectin can also bind carbohydrates bearing sLe^x and sLe^a (47, 49, 50, 53). Studies on selectin recognition of oligosaccharides have been paralleled by investigations suggesting that certain proteins contribute to cellular ligands of L- and P-selectin. For example, immobilized L-selectin has been used to affinity purify from murine lymph nodes a 50-kD sulfated, fucosylated, and sialylated glycoprotein, as well as a related 90-kD glycoprotein (70). Molecular cloning of the 50-kD glycoprotein revealed a heavily glycosylated mucin-type molecule containing two serine/threonine rich domains (71). Separate studies have revealed that P-selectin binds a small number of protease-sensitive sites on PMN and HL60 cells (46). Further, P-selectin has been used to affinity purify and ligand blot a glycoprotein(s) from leukocyte extracts, with an apparent M_r on polyacrylamide gels of $\sim$ 250,000 under nonreducing conditions and 120,000 under reducing conditions (72). Together these studies suggest that both P- and L-selectin bind with high affinity to a relatively small number of cell surface glycoproteins. The function of the protein in these ligands remains to be determined.

Our approach has been to use synthetic oligosaccharides related to sLe^x and sLe^a to determine fine specificity of selectin recognition and to identify high-affinity ligands for potential use as antiinflammatory therapeutic agents. Toward this end, we have emphasized the measurement of binding interactions between purified macromolecules and their inhibition by solution-phase oligosaccharides. Moreover, we have chosen to focus on oligosaccharides that show inhibitory activity at concentrations $\leq$ 1 mM. Our observations support the hypothesis that E-selectin binding to sLe^a and sLe^x involves the sialic acid, galactose, and fucose residues, in agreement with previous reports (51, 52). First, nonsialylated and nonfucosylated oligosaccharides corresponding to both sLe^a and sLe^x had little or no effect on E-selectin interactions at concentrations up to 1 mM. Second, E-selectin failed to recognize a structural isomer of sLe^a (2-6sLe^a) with the sialic acid linked α2-6 (rather than α2-3) to galactose. Finally, a trisaccharide similar to sLe^x (Fuc1-6sLacNAc) with fucose linked α1-6 (rather than α1-4) had little or no activity in our assays. Our studies also point to the importance of the *N*-acetyl group at carbon 5 of Neu5Ac because the oligosaccharide $KDNLe^x$, which contains a different type of sialic acid lacking this group (KDN = keto-deoxynonulosonic acid; for review of sialic acid diversity, see reference 73), was significantly less active in E-selectin assays than sLe^x.

Prior reports (51, 52) have presented conflicting data on the relative activities of sLe^x and sLe^a determinants in cell adhesion assays; we note that using either soluble reducing tetrasaccharides or 8-methoxycarbonyloctyl glycosides, sLe^a was the more potent blocker of E-selectin-Ig binding (by approximately threefold) and of E-selectin-dependent adhesion. Furthermore, we have demonstrated that replacement of the *N*-acetyl ($-NHCOCH_3$) group of sLe^x or sLe^a with amino ($-NH_2$) or azido ($-N_3$) moieties enhances their interaction with E-selectin by an additional four- to sixfold in the competitive ELISA (Table II). In contrast, replacement of the *N*-acetyl group with an *N*-propionyl group ($-NHCOCH_2CH_3$) decreased the blocking activity of sLe^a and had little or no effect on sLe^x (Fig. 5). It has been reported that a tetrasaccharide related to sLe^x with glucose in place of GlcNAc is a better inhibitor than sLe^x of E-selectin-dependent adhesion (52) and of E-selectin-Ig binding to immobilized sLe^x glycolipid (49). Taken together, these data suggest that replacement of the *N*-acetyl group with a smaller moiety or one capable of participating in ionic bonds can strengthen binding to E-selectin.

The majority of our studies were performed using oligosaccharides synthesized with an aliphatic aglycone attached in β-glycosidic linkage to the reducing sugar. The presence of this structure enhanced the blocking activity of sLe^x and sLe^a approximately twofold. This phenomenon may be due to stabilization of the carbohydrate structure by the aglycone, making it a better ligand, or it may be the result of the aglycone contributing an additional component of binding either outside or inside the carbohydrate binding pocket. Enhanced lectin recognition of carbohydrates derivatized with hydrophobic groups has been observed in other systems. For example, the elderberry bark lectin, which binds Neu5Ac-Gal/GalNAc, shows increased affinity for glycosides with added β-linked nonpolar aglycones (63). More recently, influenza hemagglutinin was shown to display increased affinity for monovalent sialosides after substitution with aglycones terminating in aromatic structures (64). The possibility of producing higher-affinity selectin ligands related to sLe^a or sLe^x by the addition of other nonpolar groups deserves attention.

As noted in the introduction, prior studies have demonstrated that human E- and P-selectin and murine L-selectin can bind sLe^x and sLe^a, suggesting that they have similar or identical oligosaccharide recognition properties. Other investigations have demonstrated differential adhesion of certain cells to E- and P-selectin as well as differential protease sensitivity of putative cellular ligands (47, 48, 51, 74). These results suggest that E- and P-selectin may recognize distinct ligands, but do not reveal whether the differences reside in the oligosaccharide structure itself or in its mode of presentation (e.g., *N*-linked sugar, *O*-linked sugar, glycolipid), or both. In our studies, E- and P-selectin clearly demonstrate differential recognition of oligosaccharides in solution. Both sLe^x and sLe^a demonstrated substantial blocking activity against E-selectin-Ig, whereas their nonsialylated counterparts had little or no effect up to concentrations of 1 mM. By contrast, both the nonsialylated and sialylated structures were found to block P-selectin-Ig, albeit relatively weakly. As assessed in both cell adhesion assays and in the competitive binding ELISA, replacement of the *N*-acetyl group of GlcNAc by amino or azido moieties profoundly enhanced interactions of sLe^a and sLe^x with E-selectin, but had no measurable effect on their interactions with P-selectin. The molecular basis for differential recognition of oligosaccharides by E- and P-selectins remains to be determined.

A recent study has demonstrated that multimeric complexes containing murine L-selectin-Ig bound to immobilized sLe^x-glycolipid (49). This interaction was inhibited by 50% in the presence 5 mM sLe^x. In our studies, human L-selectin-Ig displayed little or no detectable binding to BSA-neoglycoconjugates containing Le^x, sLe^x, Le^a, or sLe^a in the presence of Tween-20, added to minimize the possibility of protein aggregation. These combined observations suggest that multiplicity of binding may be important for L-selectin recognition of sLe^x and sLe^a. Nevertheless, potential interspecies differences in binding specificity and affinity need to be addressed. Consistent with previous observations (56, 57), L- and P-selectin-Ig bound to immobilized sulfatides. In preliminary studies, several oligosaccharides at 500 μM, including sLe^x and sLe^a,

failed to block this interaction (Drs. Nelson and Bevilacqua, unpublished observations).

Leukocyte adhesion to the vessel wall and extravasation are essential for host defense. However, in certain settings such as adult respiratory distress syndrome, ischemic reperfusion injury, and arthritis, leukocytes cause substantial tissue damage. The importance of E-selectin in leukocyte adhesion and inflammation is now widely accepted. In an effort to identify anti-inflammatory therapeutic agents we have chosen to focus on soluble oligosaccharides related to sLe^x, the first described ligand for E-selectin. We have demonstrated that solution-phase sLe^a is a more potent blocker of E-selectin than is sLe^x. Furthermore, addition of an aliphatic aglycone in combination with an amino substitution on the GlcNAc of sLe^a resulted in a compound with 36-fold higher activity than sLe^x, as measured in a competitive binding assay. These studies have also demonstrated differential recognition of oligosaccharides by E- and P-selectin. It is anticipated that high-affinity carbohydrate ligands for E-selectin may prove useful in the treatment of human diseases.

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