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# Structural requirements for the carbohydrate ligand of E-selectin

**(endothelial leukocyte adhesion molecule 1/sialyl Lewis$^x$/inflammation)**

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**ABSTRACT** **The acute inflammatory response requires that circulating leukocytes adhere to, and then migrate through, the vascular wall at the site of injury or infection. Several receptors have been implicated in this adhesion and migration process, including the selectins, a family of carbohydrate-binding proteins. The ligand for one of these proteins, E-selectin (LECAM-2, ELAM-1) has been described by several groups to contain a polylactosamine structure bearing a terminal sialic acid residue and at least one fucose residue. We report here a more detailed investigation into the minimum structural requirements for carbohydrate recognition by E-selectin. Using both direct binding and inhibition studies we demonstrate that the sialyl Lewis$^x$ tetrasaccharides Sia($\alpha$2-3)Gal($\beta$1-4)[Fuc($\alpha$1-3)]GlcNAc, and Sia($\alpha$2-3)Gal($\beta$1-4)[Fuc($\alpha$1-3)]Glc are the smallest oligosaccharides recognized by the lectin. In addition, an oligosaccharide containing the sialyl Lewis$^a$ epitope is also recognized, but less avidly. We propose a structural model of functional groups necessary for recognition by E-selectin, based on these data and additional experiments on modifications of sialic acid and the reducing terminal saccharide.**

The acute inflammatory response requires that circulating leukocytes bind to and penetrate the vascular wall to access the site of injury. Several receptors have been implicated in this interaction, including the selectins [LEC cell adhesion molecules (CAMs)], a family of carbohydrate-binding proteins. These proteins are characterized by the presence of domains with homologies to calcium-dependent lectins (C-lectins), epidermal growth factor, and complement-binding proteins (1–6). Recently, a consensus has been reached regarding the nomenclature of these proteins (28), resulting in the use of "selectin" for this family, and L-selectin, P-selectin, and E-selectin for the proteins LECAM-1 (LAM-1), GMP-140 (PADGEM, LECAM-3), and ELAM-1 (LECAM-2), respectively. Intense interest in these proteins has resulted in several recent publications describing aspects of the carbohydrate ligands for each of the three selectins (7, 8). One member of this family, E-selectin (LECAM-2, ELAM-1) is an adhesion protein transiently expressed on the surface of vascular endothelium and has been implicated in the initial events of neutrophil extravasation. There is general agreement that the ligands for E-selectin include sialylated, polylactosamine oligosaccharides, with a fucose on the first {sialyl-Lewis$^x$ (sLe$^x$) epitope, Sia($\alpha$2-3)Gal($\beta$1-4)[Fuc($\alpha$1-3)]GlcNAc-R} or second {VIM-2 epitope, Sia($\alpha$2-3)Gal($\beta$1-4)GlcNAc($\beta$1-3)Gal($\beta$1-4)[Fuc($\alpha$1-3]GlcNAc-R} *N*-acetylglucosamine from the nonreducing end. Attempts to intervene therapeutically in E-selectin-mediated inflammation may benefit from the definition of the minimum carbohydrate structure necessary for binding. This report describes a series of experiments intended to define the functional groups required for E-selectin recognition of its carbohydrate ligand.

## MATERIALS AND METHODS

**Materials.** The 2-3 sLe$^x$ hexa glycolipid (see Table 1 for nomenclature), the 2-3 sLe$^x$ penta glycolipid, and the 2-6 sLe$^x$ hexa glycolipid were synthesized by previously described methods (9). The 2-3 sLe$^x$ tetra and 2-6 sLe$^x$ tetra were synthesized at Glycomed (Alameda, CA). The glucose derivative of sLe$^x$ tetra [sLe$^x$(Glc) tetra] was purchased from BioCarb (Lund, Sweden) or Oxford Glycosystems (Abingdon, U.K.) or was synthesized at Glycomed. Structures were confirmed by thin-layer chromatography, NMR, mass spectrometry, composition analysis, and linkage analysis. Analytical data supporting the structures of the synthetic glycoconjugates used in this study are provided in Tables 2 and 3. The 2-3 sLe$^a$ hexa as well as the oligosaccharides listed in Table 4 were purchased from BioCarb. Monoclonal antibodies against E-selectin (ELAM-1) and ICAM-1 were obtained from British Bio-technology (Abingdon, U.K.). The CSLEX antibody was purchased from the University of California at Los Angeles Tissue Typing Laboratory, whereas the VIM-2 antibody was purchased from An der Grub Bioresearch (Kaumberg, Austria). All other materials were obtained from standard suppliers and were of the highest quality available.

Polylactosamine glycolipids with *N*-glycolylneuraminic acid were purified from rabbit thymus and then enzymatically fucosylated (11). Human myeloid cell glycolipid extracts containing the VIM-2 epitope were subjected to ion-exchange chromatography (12). Acidic fractions were acetylated and separated on a Florisil column (60–100 mesh, EM Science) (13). After deacetylation, gangliosides were further fractionated by HPLC on a 1 × 50-cm Iatrobead column (Iatron Laboratories, Tokyo) (14). Fractions used in this study reacted strongly with the VIM-2 antibody but were not bound by the CSLEX antibody. Tetrasaccharides were reduced with 100-fold molar excess of $NaBH_4$ (pH 9.0, 55°C, 4 hr), acidified, desalted with AG50 $H^+$ resin, and filtered. Solvent was evaporated with methanol under vacuum. A portion of the 2-3 sLex hexa glycolipid was subjected to mild periodate oxidation (15) to remove C-8 and C-9 of the sialic acid glycerol side chain, then purified by Sep-Pak (Waters) chromatography. The structure of the product (with an aldehyde on C-7) was confirmed by mass spectrometry and monosaccharide composition analysis.

**Carbohydrate Analysis.** Neutral monosaccharide ratios were obtained after hydrolysis of 500 pmol of dried sample glycoconjugate or $G_{M1}$ control (see Table 2). Chromatography was performed on Dionex Carbopac Pa-1 columns and sugars were quantified with the pulsed electrochemical detector (10). Absolute amounts of monosaccharides were corrected for

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Abbreviation: HUVEC, human umbilical vein endothelial cells.

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Table 1. Nomenclature

| Name | Structure |
|---|---|
| 2-3 sLe$^x$ hexa glycolipid | Sia(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc-ceramide |
| 2-6 sLe$^x$ hexa glycolipid | Sia(α2-6)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc-ceramide |
| 2-3 sLe$^x$ penta glycolipid | Sia(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal-ceramide |
| VIM-2 glycolipid | Sia(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)GlcNAc-(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-ceramide |
| 2-3 sLe$^a$ hexa | Sia(α2-3)Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc |
| 2-3 sLe$^x$ tetra | Sia(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc |
| 2-6 sLe$^x$ tetra | Sia(α2-6)Gal(β1-4)[Fuc(α1-3)]GlcNAc |
| 2-3 sLe$^x$(Glc) tetra | Sia(α2-3)Gal(β1-4)[Fuc(α1-3)]Glc |

recoveries achieved for the $G_{M1}$ control. Monosaccharide substituent positions were determined by methylation analysis using 2 nmol of sample glycoconjugates, or $G_{M1}$ standard (see Table 2). The samples were methylated (16) then hydrolyzed as above. The partially methylated monosaccharides were reduced with $NaB^2H_4$ and acetylated (17). The partially methylated alditol acetates were separated by gas chromatography and analyzed with ion-trap mass detection. Negative-ion fast-atom bombardment mass spectra were acquired on a Finnigan MAT 900 spectrometer using a triethanolamine matrix. Proton NMR studies (500 MHz) were used to confirm the structures of the synthetic carbohydrates and to provide data used in deriving conformations of oligosaccharides. Molecular modeling computations were done on a Silicon Graphics 4D85GT system using the cvff force-field parameters in Biosym's INSIGHT/DISCOVER software.

**COS Cell Transfection.** A full-length cDNA encoding E-selectin (ELAM-1) was obtained by the polymerase chain reaction with total RNA extracted from interleukin 1β (Collaborative Research)-stimulated human umbilical vein endothelial cells (HUVECs), as described (11). The plasmid (designated pl-ELAM) used in subsequent experiments was found to contain the published sequence of ELAM-1 (18, 19). These COS cells, transfected with pl-ELAM, express E-selectin on their surfaces as assessed by antibody binding (data not shown).

**Cell Adhesion Assays.** Two cell assays were used to define the interactions of carbohydrate ligands with E-selectin. In the first, glycolipids containing the sLe$^x$ epitope (or analogs) were dissolved in 100% ethanol containing 1 μM phosphatidylcholine and 4 μM cholesterol, and then an equal volume of water was added. These solutions were added to poly(vinyl chloride) microtiter wells (50 μl per well), and the glycolipids were allowed to adsorb for 80 min. Adsorption efficiencies were determined (at input quantities of 100 pmol per well) by extracting 10 wells per glycolipid twice with 1-butanol. The material from the 10 wells was pooled, dried, and subjected to sugar composition analysis as described above. Surfaces with adsorbed glycolipids were blocked with Dulbecco's modified Eagle's medium (DMEM) containing 25 mM Hepes (DMEM-H) and 5% (wt/vol) bovine serum albumin for 30 min and then rinsed with DMEM-H. Adhesion of transfected COS cells (40,000 cells per well in DMEM-H with 0.5% bovine serum albumin) was performed as described (11), using conditions of controlled detachment force (20). Soluble inhibitors, when used, were added to the wells prior to the addition of the cells expressing E-selectin. In all cases, cells were examined microscopically after incubations to ensure that no aggregation or lysis had occurred.

In the second assay, HUVECs (Clonetics, San Diego) were seeded on the bottom of poly(vinyl chloride) wells coated with rat tail collagen type I (6 μg/cm$^2$) and grown to confluence in umbilical vein endothelial cell growth medium containing 2% fetal bovine serum and supplemented with bovine brain extract and heparin (EGM-UV, Clonetics, San Diego). HUVEC monolayers were used for cell assays 2–7 days after plating. The monolayers were stimulated for 4–6 hr with 100 μl of fresh medium containing 10 half-maximal units of recombinant human interleukin 1β (Collaborative Research) per ml. HUVEC monolayers were then washed twice with cold phosphate-buffered saline and placed on ice. Fifty microliters of EGM-UV medium containing 25 mM Hepes (pH 7.4), was added per well. Soluble inhibitors or antibodies, when used, were added to the wells in the same volume of this medium. HL-60 promyelocytic leukemia cells were metabolically radiolabeled overnight with $^{32}P_i$, resuspended in EGM-UV with Hepes, added to the wells (150,000 cells per well in 50 μl of medium), and then incubated on the monolayer for 45 min at 4°C. When antibodies were used, they were preincubated with the HUVEC monolayer for 1 hr prior to the addition of HL-60 cells. The percentage of cells adhering was determined using conditions of controlled detachment force (20).

Table 2. Monosaccharide ratios

| Material | Sia | Fuc | GlcNAc | Gal | Glc |
|---|---|---|---|---|---|
| 2-3 sLe$^x$ hexa glycolipid | 1.4 | 1.2 | 1.0 | 2.0 | 0.9 |
| 2-6 sLe$^x$ hexa glycolipid | | 1.2 | 1.0 | 2.0 | 0.8 |
| 2-3 sLe$^x$ penta glycolipid | 1.0 | 1.1 | 1.0 | 2.0 | 0.2 |
| 2-3 sLe$^x$ tetra | 1.0 | 0.7 | 0.9 | 1.0 | 0.1 |
| 2-6 sLe$^x$ tetra | 1.5 | 1.0 | 0.9 | 1.0 | 0 |
| 2-3 sLe$^x$(Glc) tetra | 0.8 | 1.0 | 0 | 1.0 | 0.9 |

Ratios of neutral monosaccharides were obtained after hydrolysis (2 M trifluoroacetic acid, 100°C, 6 hr) of 500 pmol of dried sample glycoconjugate or $G_{M1}$ control. Samples were dried and dissolved in water prior to injection. Samples for sialic acid (Sia) analysis were hydrolyzed in 0.1 M HCl at 80°C for 4 hr then neutralized with 1 M NaOH. Chromatography was performed on Dionex Carbopac Pa-1 columns with the pulsed electrochemical detector (10). Chromatography of neutral sugars used 15 mM NaOH; for sialic acids, 0.6 M NaOH was used.

Table 3. Substituent positions

| Material | Fuc | GlcNAc | Gal | Glc |
|---|---|---|---|---|
| 2-3 sLe$^x$ hexa glycolipid | Term. | 3,4 | 3 | 4 |
| 2-6 sLe$^x$ hexa glycolipid | Term. | 3,4 | 3 or 6 | 4 |
| 2-3 sLe$^x$ hexa glycoside | Term. | 3,4 | 3 | 4 |
| 2-3 sLe$^x$ penta glycolipid | Term. | 3,4 | 3 | 4 |
| 2-3 sLe$^x$ tetra | Term. | 3,4 or 4 | 3 | — |
| 2-6 sLe$^x$ tetra | Term. | 3,4 | 6 | — |
| 2-3 sLe$^x$(Glc) tetra | Term. | — | 3 | 3,4 |

Monosaccharide substituent positions were determined by methylation analysis on 2 nmol of the sample glycolipids or $G_{M1}$ standard. The 2-3 sLe$^x$ tetra contains a small amount of monosubstituted *N*-acetylglucosamine due to a small percentage of nonfucosylated material in the sample (see Table 2). Residues were determined as follows: 2,3,6-tri-*O*-methylglucitol acetate, 4-substituted glucose; 2,4,6-tri-*O*-methylgalactitol acetate, 3-substituted galactose; 2,3,4-tri-*O*-methylgalactitol acetate, 6-substituted galactose; 3,6-di-*O*-methyl-*N*-methyl-2-acetamido-2-deoxyglucitol acetate, 4-substituted *N*-acetylglucosamine; 6-*O*-methyl-*N*-methyl-2-acetamido-2-deoxyglucitol acetate, 3,4-disubstituted *N*-acetylglucosamine. Term., terminal.

## RESULTS

Previous work (10) has shown that COS cells transfected with pl-ELAM adhere to glycolipids containing the oligosaccharide Sia($\alpha$2-3)Gal($\beta$1-4)GlcNAc($\beta$1-3)Gal($\beta$1-4)[Fuc($\alpha$1-3)]GlcNAc (VIM-2 epitope). Other investigators (21–23) have reported that E-selectin-mediated adhesion can be inhibited by glycoproteins or glycolipids reported to contain Sia($\alpha$2-3)Gal($\beta$1-4)[Fuc($\alpha$1-3)]GlcNAc (sLe$^x$ epitope) or by antibodies against this epitope. To define the minimum carbohydrate structure necessary for recognition by E-selectin, we began by quantifying direct binding of pl-ELAM-transfected COS cells to synthetic 2-3 sLe$^x$ hexa glycolipid or closely related structures. The data (Fig. 1) demonstrate that E-selectin recognizes 2-3 sLex hexa glycolipid in a dose-dependent manner. An otherwise identical glycolipid with sialic acid linked $\alpha$2-6 did not support adhesion of pl-ELAM-transfected COS cells. Less than 10% of COS cells transfected with a control plasmid adhered to 2-3 sLe$^x$ hexa glycolipid (data not shown). The smaller 2-3 sLe$^x$ penta glycolipid was also recognized by pl-ELAM-transfected COS cells, but not as avidly as the 2-3 sLe$^x$ hexa glycolipid. Adsorption efficiencies for the synthetic glycolipids were comparable (16%, 19%, and 21%, respectively, for 2-3 sLe$^x$ penta glycolipid, 2-3 sLe$^x$ hexa glycolipid, and 2-6 sLe$^x$ hexa glycolipid). Glycolipids containing the VIM-2 epitope and lacking the sLe$^x$ epitope (as assessed by mass spectral data and lack of CSLEX antibody binding) were purified from chronic myelogenous leukemia cells and directly compared with synthetic 2-3 sLe$^x$ hexa glycolipid. Fig. 1 shows that the VIM-2 glycolipid is clearly less effective at supporting adhesion of pl-ELAM COS adhesion. Adhesion to sLe$^x$ glycolipids was blocked using monoclonal antibodies against E-selectin (82% inhibition at 3 $\mu$g/ml), but not by antibodies against ICAM-1 (data not shown).

Similar glycolipids lacking fucose and $\alpha$2-3 *N*-glycolylneuraminic acid (instead of *N*-acetylneuraminic acid) were purified from rabbit thymus (86% *N*-glycolyl, 14% *N*-acetyl). These glycolipids, when enzymatically fucosylated (11) to yield an equimolar ratio of fucose to sialic acid, supported E-selectin recognition at levels equal to equivalent amounts of the synthetic 2-3 sLe$^x$ hexa glycolipid (data not shown). Mild periodate treatment of the 2-3 sLe$^x$ hexa glycolipid results in removal of carbons 8 and 9 from the sialic acid glycerol side chain and generates an aldehyde on carbon 7. This treatment, with or without reduction of the aldehyde, had no detectable effect on pl-ELAM COS cell adhesion (data not shown). These data indicate that the glycosidic linkage position of sialic acid is critical for E-selectin recognition, whereas some other aspects of this sugar can be varied without affecting binding. In addition, a pentasaccharide is sufficient for recognition.

Many carbohydrates were tested as potential inhibitors of pl-ELAM-transfected COS cell adhesion to 2-3 sLe$^x$ hexa glycolipid at 37°C (Table 4). The most active oligosaccharide inhibitor was 2-3 sLe$^x$(Glc) tetra {Sia($\alpha$2-3)Gal($\beta$1-4)[Fuc($\alpha$1-3)]Glc}, with an $IC_{50}$ of about 500 $\mu$M. An $IC_{50}$ in the high micromolar range is typical for a monovalent carbohydrate inhibitor of C-type lectin binding (e.g., ref. 24). This tetrasaccharide retained the majority of its inhibitory activity when the glucose was reduced by $NaBH_4$ (Table 4). Surprisingly, 2-3 sLe$^x$ tetra {Sia($\alpha$2-3)Gal($\beta$1-4)[Fuc($\alpha$1-3)]GlcNAc} was much less active than either 2-3 sLe$^x$(Glc) tetra or reduced 2-3 sLex(Glc) tetra. A related hexasaccharide, Sia($\alpha$2-3)Gal($\beta$1-3)[Fuc($\alpha$1-4)]GlcNAc($\beta$1-4)Gal($\beta$1-3)Glc (2-3 sLe$^a$ epitope), showed a small amount of inhibitory activity (21.7% inhibition at 1 mM).

These same compounds were used as potential inhibitors of the adhesion of HL-60 cells to activated endothelial cell monolayers (HUVEC assay) at 4°C. In our hands 75% of the interleukin 1-stimulated adhesion detected could be blocked by monoclonal antibodies against ELAM-1 (at 0.4 $\mu$g/ml), but not by antibodies against ICAM-1 or by preimmune IgG at 100-fold higher concentrations (data not shown). A similar rank order of inhibitors was observed in both assays (Table 4), but all the inhibitors were more effective at blocking HL-60 cell adhesion to HUVECs than pl-ELAM COS adhesion to adsorbed glycolipids. Structures found to be inactive included the following: (*i*) all monosaccharides tested, including fucose and sialic acid; (*ii*) sialylated oligosaccharides with Gal($\beta$1-4)GlcNAc backbones but lacking fucose; (*iii*) fucosylated oligosaccharides with Gal($\beta$1-4)GlcNAc backbones but lacking sialic acid; (*iv*) highly charged polysaccharides that have been reported to inhibit other LEC-CAM-mediated adhesion (25, 26). Results obtained for these inactive structures support the conclusion that fucose and sialic acid together and in appropriate linkages are required for recognition by E-selectin and that anionic charge alone is not a primary mechanism of interaction.

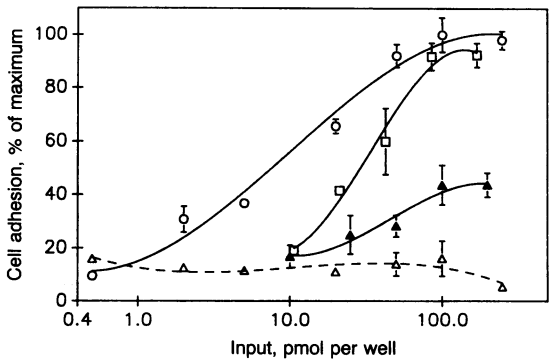


FIG. 1. Adhesion of pl-ELAM-transfected COS cells to synthetic and purified glycolipids. Glycolipids (2-3 sLe$^x$ hexa glycolipid, ○; 2-3 sLe$^x$ penta glycolipid, □; 2-6 sLe$^x$ hexa glycolipid, △; VIM-2 glycolipid, ▲) were adsorbed at the input concentrations indicated, and the pl-ELAM COS adhesion assay was performed. Data from three experiments are presented as percent of maximum adhesion, defined by optimal adhesion to 2-3 sLe$^x$ hexa glycolipid in each experiment (mean ± SEM). Maximum adhesion in these experiments ranged from 21% to 60% of the COS cells added, with backgrounds (adhesion to phosphatidylcholine/cholesterol only) of <5%, and $n \geq 3$ for each lipid concentration within each experiment.

## DISCUSSION

E-selectin has been demonstrated to mediate the adhesion of neutrophils (and other myeloid cells) to activated vascular endothelium in culture. COS cells expressing E-selectin adhere to myeloid cells, as does the purified E-selectin (1, 5). Its *in vivo* localization to sites of chronic inflammation in the skin (27) and the kinetics of its expression following endothelial cell stimulation with interleukin 1, tumor necrosis factor, and lipopolysaccharide (1, 5) suggest a role for E-selectin in the inflammatory response. The ligands described for E-selectin are all sialylated polylactosamine structures with fucose linked to the 3-hydroxyl of at least one *N*-acetylglucosamine residue (7, 8). The fucose and sialic acid residues are required for recognition (11). Here we describe a series of experiments that examined in more detail the minimum carbohydrate structures necessary for E-selectin recognition. The data indicate that (*i*) 2-6 sLe$^x$ glycolipid is not recognized by E-selectin, (*ii*) 2-3 sLe$^x$ tetra blocks E-selectin-mediated cell adhesion, as do closely related structures, and (*iii*) the VIM-2 glycolipid appears to be a weak ligand in comparison to sLe$^x$ glycolipids. These results suggest a working model for the carbohydrate functional characteristics necessary for E-selectin recognition.

Table 4. Soluble inhibitors

| Inhibitor | % inhibition | |
|---|---|---|
| | HUVEC assay (4°C) | pl-ELAM COS assay (37°C) |
| 2-3 sLe$^x$(Glc) tetra | 100.0 ± 2.4 (3) | 66.0 ± 10.5 (3) |
| Reduced 2-3 sLe$^x$(Glc) tetra | ND | 51.4 ± 3.4 (1) |
| 2-3 sLe$^x$ tetra | 86.7 ± 3.5 (2) | 14.0 ± 5.3 (2) |
| 2-3 sLe$^a$ hexa | 75.5 ± 2.7 (2) | 21.7 ± 3.3 (1) |
| 2-6 sLe$^x$ tetra | <10 (2) | <10 (3) |
| Gal(β1-4)GlcNAc(β1-3)-Gal(β1-4)Glc | 21.5 ± 3.4 (1) | <10 (1) |
| Sia(α2-3)Gal(β1-3)GlcNAc-(β1-3)Gal(β1-4)Glc | 20.8 ± 1.6 (1) | <10 (1) |
| Sia(α2-6)Gal(β1-4)GlcNAc-(β1-3)Gal(β1-4)Glc | 16.9 ± 1.6 (2) | <10 (1) |
| Sia(α2-6)Gal(β1-4)GlcNAc-(β1-3)Gal(β1-4)[Fuc(α1-3)]Glc | ND | <10 (1) |
| PPME* (100 μg/ml) | ND | <10 (2) |
| Fucoidan (100 μg/ml) | ND | <10 (2) |
| Heparin (100 μg/ml) | ND | <10 (2) |

Full structures of the first five inhibitors can be found in Table 1. The HUVEC and pl-ELAM COS adhesion assays were performed as described in *Materials and Methods*. Data are reported as the percent inhibition (mean ± SEM), defined as (experimental adhesion − background)/(maximum adhesion − background). Numbers in parentheses indicate the number of experiments in which the inhibitor (1 mM, unless otherwise indicated) was tested. In the HUVEC assay a total of five experiments were performed, with maximum adhesion (no inhibitor) ranging from 3.4% to 12.5% of HL-60 cells added, and background adhesion (to unstimulated HUVEC monolayers) of from 1.2% to 3.0%, with $n \geq 3$ for each experiment. In the pl-ELAM COS assay a total of four experiments were performed, with maximum adhesion (no inhibitor) to input sLe$^x$ hexa glycolipid (30 pmol per well) ranging from 20% to 62%, and background adhesion (to adsorbed phosphatidylcholine/cholesterol) of from 2% to 4%, with $n \geq 3$ for each experiment. Monosaccharides tested (at 10 mM) and found to be noninhibitory were *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, mannose, fucose, *N*-acetylneuraminic acid (sialic acid), *N*-glycolylneuraminic acid, mannose 6-phosphate, and mannose 1-phosphate. Other noninhibitory oligosaccharides tested (at 1 mM) were Sia(α2-6)Gal, Gal(β1-4)GlcNAc, Sia(α2-3)Gal(β1-4)Glc, Sia(α2-6)Gal(β1-4)Glc, Fuc(α1-2)Gal(β1-4)Glc, and Gal(β1-4)[Fuc(α1-3)]Glc. ND, not determined.
*The core phosphomannan polysaccharide from *Hansenula holstii*.

Direct binding of pl-ELAM-transfected cells to chemically synthesized 2-3 sLe$^x$ hexa glycolipid or to glycolipids with the VIM-2 epitope (Fig. 1) confirm earlier indications (7, 8) that sLe$^x$-containing oligosaccharides and related structures are recognized by E-selectin. Analyses of these glycolipids as well as other carbohydrates used in these studies for monosaccharide composition (Table 2) and linkage (Table 3) and by mass spectrometry and NMR (data not shown) are consistent with the structures described. Data comparing the VIM-2 and 2-3 sLe$^x$ glycolipids indicate that all support adhesion, but with different apparent potencies.

Tetrasaccharides with the sLe$^x$ epitope can inhibit the pl-ELAM COS adhesion to adsorbed 2-3 sLe$^x$ hexa glycolipid or the adhesion of HL-60 cells to stimulated vascular endothelium. A comparison of the inhibitory capacity of 2-3 sLex tetra with that of a similar tetrasaccharide, 2-3 sLe$^x$(Glc) tetra, suggests that substitution of a glucose for the *N*-acetylglucosamine at the reducing end of the 2-3 sLe$^x$ tetra generates a more effective inhibitor. These data suggest that the *N*-acetyl group of the reducing sugar residue is not required for recognition by E-selectin. The $IC_{50}$ of 2-3 sLe$^x$(Glc) tetra (in the high micromolar range) is typical of C-type lectin interactions with monovalent oligosaccharide inhibitors (e.g., ref. 24). The difference between 2-3 sLe$^x$ tetra and 2-3 sLe$^x$(Glc) tetra is most pronounced in the pl-ELAM COS adhesion assay, performed at 37°C. Differences between the two assays used in this study may reflect the difficulty of inhibiting adhesion of transfected COS cells (expressing large amounts of E-selectin) to a dense surface of immobilized ligand. Alternatively, the differences may reflect the different temperatures at which the assays are performed. The 2-3 sLe$^x$(Glc) tetra maintains much of its inhibitory capacity after reduction of the glucose with $NaBH_4$, demonstrating that the ring structure of the reducing sugar is not required for recognition by E-selectin. Since both the sialic acid and the fucose are required, it appears that a tetrasaccharide with a reducing terminal glucose is the smallest carbohydrate effectively recognized by E-selectin.

A previous report (11) demonstrated the requirement of sialic acid for E-selectin recognition but did not address the linkage position of the sialic acid to the galactose or which portions of this terminal sugar are involved in binding. Data in Fig. 1 demonstrate that 2-6-linked sialic acid is not recognized. However, the *N*-glycolyl form of sialic acid appears to be as good as the *N*-acetyl form. In addition, removal of carbons 8 and 9 by mild periodate oxidation has little effect on E-selectin binding. This suggests that the glycerol side chain and the *N*-acetyl moiety of the sialic acid may not be involved in E-selectin recognition (see below).

Inhibition data in Table 4 indicate that a hexasaccharide with the 2-3 sLe$^a$ epitope, Sia(α2-3)Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc, can partially inhibit E-selectin recognition at millimolar concentrations even though the linkage positions of the galactose and fucose are reversed compared with those in sLe$^x$. Molecular modeling studies comparing the minimum-energy conformations of sLe$^x$ and sLe$^a$ epitopes show a striking similarity in the relative orientations of sialic acid, galactose, and fucose residues (Fig. 2). In this proposed conformation (detailed results to be presented elsewhere), the three residues *N*-acetylneuraminic acid, galactose, and fucose are stacked one above the other with their functional groups well exposed to the exterior surface of the two molecules.

These models together with NMR data on the conformation of oligosaccharides in solution lead to a hypothesis for the binding face of oligosaccharides interacting with E-selectin. This model incorporates the following conclusions

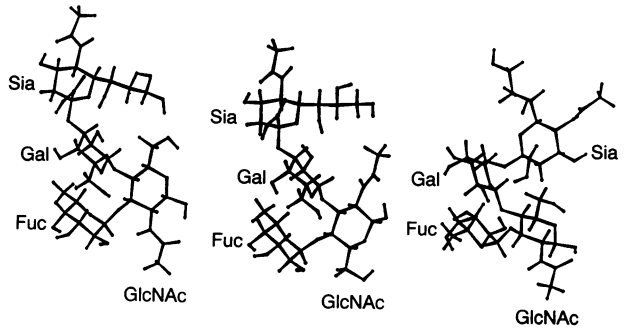


FIG. 2. Minimum-energy conformations of 2-3 sLe$^x$ (*Left*), 2-3 sLe$^a$ (*Center*), and 2-6 sLe$^x$ (*Right*) tetrasaccharides. The striking similarity in three-dimensional structure of the two structures recognized by E-selectin (2-3 sLe$^x$ and 2-3 sLe$^a$) is in contrast to that of the unrecognized 2-6 sLe$^x$.

reached from experiments described here: (*i*) fucose and sialic acid are required for E-selectin binding; (*ii*) 2-6-linked sialic acid is not recognized; (*iii*) removal of carbons 8 and 9 on the sialic acid or substitution of an *N*-glycolyl for the *N*-acetyl of the sialic acid residue do not effect binding; (*iv*) removal of the *N*-acetyl group from the *N*-acetylglucosamine does not reduce binding; (*v*) reduction of 2-3 sLe$^x$(Glc) does not substantially reduce its inhibitory activity. The 2-3 sLe$^x$ and 2-3 sLe$^a$ oligosaccharides, in their minimum-energy conformation, present the same orientation of sialic acid, galactose, and fucose. When linked 2-6, the sialic acid residue assumes a much different conformation in space (Fig. 2). In this model the glycerol side chain (carbons 7, 8, and 9) and the *N*-acetyl group of the sialic acid are oriented away from the recognized face of the oligosaccharide. In addition, opening the reducing terminal sugar results in three minimum-energy configurations, one of which closely matches the original configuration. It is our hypothesis that the carboxyl group of the sialic acid, the 4- and 6-hydroxyls of galactose, and the 2-, 3-, and 4-hydroxyls of fucose are involved in recognition. This hypothesis can be tested by assessing further carbohydrate analogs in direct recognition of E-selectin and as inhibitors. In addition, NMR studies of receptor–ligand interactions in solution may provide additional information about the sites on the E-selectin protein and oligosaccharide that are important in recognition.

**Note Added in Proof.** Since submission of this manuscript Berg *et al.* (29) have also reported that E-selectin recognizes the sLe$^a$ epitope.

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1. Geng, J.-G., Bevilacqua, M. P., Moore, K. L., McIntyre, T. M., Prescott, S. M., Kim, J. M., Bliss, G. A., Zimmerman, G. A. & McEver, R. P. (1990) *Nature (London)* **343**, 757–760.
2. Johnston, G. I., Cook, R. G. & McEver, R. P. (1989) *Cell* **56**, 1033–1044.
3. Geoffroy, J. S. & Rosen, S. D. (1989) *J. Cell Biol.* **109**, 2463–2469.
4. Lasky, L. A., Singer, M. S., Yednock, T. A., Dowbenko, D., Fennie, C., Rodriguez, H., Nguyen, T., Stachel, S. & Rosen, S. D. (1989) *Cell* **56**, 1045–1055.
5. Bevilacqua, M. P., Pober, J. S., Mendrick, D. L., Cotran, R. S. & Gimbrone, M. A., Jr. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9238–9242.
6. Camerini, D., James, S. P., Stamenkovic, I. & Seed, B. (1989) *Nature (London)* **342**, 78–82.
7. Brandley, B. K., Swiedler, S. J. & Robbins, P. W. (1990) *Cell* **63**, 861–863.
8. Moore, K. L., Varki, A. & McEver, R. P. (1991) *J. Cell Biol.* **112**, 491–499.
9. Kameyama, A., Ishida, H., Kiso, M. & Hasegawa, A. (1991) *Carbohydr. Res.* **209**, c1–c4.
10. Hardy, M. R., Townsend, R. R. & Lee, Y. C. (1988) *Anal. Biochem.* **170**, 54–62.
11. Tiemeyer, M., Swiedler, S. J., Ishihara, M., Moreland, M., Schweingruber, H., Hirtzer, P. & Brandley, B. K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1138–1142.
12. Ledeen, R. W., Yu, R. K. & Eng, L. F. (1973) *J. Neurochem.* **21**, 829–839.
13. Saito, T. & Hakomori, S. (1971) *J. Lipid Res.* **12**, 257–259.
14. Macher, B. A., Buehler, J., Scudder, P., Knapp, W. & Feizi, T. (1988) *J. Biol. Chem.* **263**, 10186–10191.
15. Spiegel, S., Ravid, A. & Wilchek, M. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5277–5281.
16. Ciucanu, I. & Kerek, F. (1984) *Carbohydr. Res.* **131**, 209–217.
17. Blakeney, A. B., Harris, P. J., Henry, R. J. & Stone, B. A. (1983) *Carbohydr. Res.* **113**, 291–299.
18. Polte, T., Newman, W. & Gopal, T. V. (1990) *Nucleic Acids Res.* **18**, 1083.
19. Hession, C., Osborn, L., Goff, D., Chi-Rosso, G., Vassallo, C., Pasek, M., Pittach, C., Tizard, R., Goelz, S., McCarthy, K., Hopple, S. & Lobb, R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1673–1677.
20. Blackburn, C. C., Swank-Hill, P. & Schnaar, R. L. (1986) *J. Biol. Chem.* **261**, 2873–2881.
21. Lowe, J. B., Stoolman, L. M., Nair, R. P., Larsen, R. D., Berhand, T. L. & Marks, R. M. (1990) *Cell* **63**, 475–484.
22. Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M. & Seed, B. (1990) *Science* **250**, 1132–1135.
23. Phillips, M. L., Nudelman, E., Gaeta, R. C. A., Perez, M., Singhal, A. K., Hakomori, S. & Paulson, J. C. (1990) *Science* **250**, 1130–1132.
24. Lee, R. T. & Lee, Y. C. (1987) *Glycoconjugate J.* **4**, 317–328.
25. Skinner, M. P., Lucas, C. M., Burns, G. F., Chesterman, C. N. & Berndt, M. C. (1991) *J. Biol. Chem.* **266**, 5371–5374.
26. Stoolman, L. M. & Rosen, S. D. (1983) *J. Cell Biol.* **96**, 722–729.
27. Picker, L. J., Kishimoto, T. K., Smith, C. W., Warnock, R. A. & Butcher, E. C. (1991) *Nature (London)* **349**, 796–799.
28. Bevilacqua, M. P., Butcher, E. C., Furie, B., Furie, B. C., Gallatin, W. M., Gimbrone, M. A., Hanlan, J., Kishimoto, T. K., Lasky, L. A., McEver, R. P., Paulson, J. C., Rosen, S. D., Springer, T. A., Stoolman, L. M., Tedder, T. F., Varki, A., Wagner, D. D., Weissman, I. L. & Zimmerman, G. A. (1991) *Cell* **67**, 233.
29. Berg, E. L., Robinson, M K., Mansson, O., Butcher, E. C. & Magnani, J. L. (1991) *J. Biol. Chem.* **265**, 14869–14872.