Binding of Sialyl Lewis X to E-Selectin As Measured by Fluorescence Polarization

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ABSTRACT: Fluorescence polarization has been used to directly measure the binding of the tetrasaccharide sialyl Lewis^x (sLe^x[Glc], or NeuAcα2−3Galβ1−4[Fucα1−3]Glc) to a soluble form of E-selectin, a member of the class of adhesion molecules that plays an important role in immune-cell response to inflammation. The experiments utilized a fluorescent derivative of sLe^x[Glc] with fluorescein attached directly to the glucose residue through a β-glycosidic linkage. The resulting fluorescent sLe^x was shown to inhibit binding of HL60 cells to immobilized E-selectin and exhibited fluorescence polarization enhancement in the presence of a monovalent form of a recombinant soluble E-selectin−F_c chimera. Thermodynamic dissociation constants of 107 ± 26 and 120 ± 31 μM were obtained for the fluorescent sLe^x[Glc] and the free sLe^x[Glc] sugars, respectively. These results demonstrate that E-selectin interacts weakly with the minimal carbohydrate recognition determinant sLe^x. Additional binding interactions through the action of the authentic coreceptor or via clustering of the ligand and E-selectin molecules on the respective neutrophil and endothelial cell surfaces may also play a role in the overall cellular binding strength. However, the basic interaction between carbohydrate and protein appears weak, consistent with other carbohydrate−protein interactions studied to date.

It is now well established that members of the leukocyte and lymphocyte class of white blood cells can interact with the endothelium under physiologic instances such as local inflammation and subsequently emigrate from the blood stream into the surrounding tissue. This has led to a novel approach to the development of anti-inflammatory agents aimed at interfering with the inflammatory cascade at the basic level of recruitment of these cells. One group of adhesion molecules involved in this process, the selectins, are mammalian lectins which are distinguishable by their ability to recognize unique carbohydrate determinants on their coreceptors [reviewed in Bevilacqua and Nelson (1993), Lasky (1992), Varki (1992), and Welply et al. (1994a)]. The group is presently comprised of three members, E-, P-, and L-selectin, all three of which possess an amino-terminal lectin domain homologous to that found in other C-type lectins. It is this motif that is responsible for the carbohydrate-binding capacity of these molecules. In the case of E- and P-selectin, the minimal carbohydrate epitope that is needed for selectin binding is the branched oligosaccharide sialyl Lewis^x, NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAc (Phillips et al., 1990; Polley et al., 1991; Tiemeyer et al., 1991) or its stereoisomer sialyl-Lewis^a, NeuAcα2−3Galβ1−3(Fucα1−4)GlcNAc (Berg et al., 1991; Handa et al., 1991; Tyrrell et al., 1991).

Current screening efforts to identify antagonists of E- and P-selectin-mediated leukocyte−endothelial adhesion have generally employed assays that utilize essentially multivalent forms of sLe^x,[1] presented in the form of either the intact cells—neutrophils or HL60 cells—or as a glycolipid monolayer deposited on some surface such as plastic. These types of assays do not permit the determination of the true binding constant of a small molecule, such as sLe^x, to E-selectin. Rather, they have been utilized to compare the relative binding properties of various molecules by measuring the concentration, IC_{50}, needed to produce a 50% inhibition of the basic interaction. So, for example, we have used HL60 binding to immobilized E-selectin and neutrophil-rolling on activated endothelial cell monolayers to measure the relative effectiveness of free sLe^x sugars and sLe^x−BSA conjugates (Welply et al., 1994b). One of the issues that has been a basic concern is that assays of this type may be inherently biased against small-molecule antagonists since they must essentially compete against a highly multivalent carbohydrate surface such as is provided by an HL60 cell. A variety of assays, such as the use of an sLe^x−glycolipid, either

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[1] Abbreviations: BSA, bovine serum albumin; C-type, calcium-type; E1, monovalent E-selectin human IgG F_c fusion protein; E2, bivalent E-selectin human IgG F_c fusion protein; FITC, fluorescein isothiocyanate; Fuc, α-L-fucose; FTase, α3/4-fucosyltransferase V; Gal, β-D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; HBSS, Hanks balanced salt solution; HPLC, high-performance liquid chromatography; HP-TLC, high-performance thin-layer chromatography; PAGE, polyacrylamide gel electrophoresis; lactosamine, Galβ1−4GlcNAc; NeuAc, *N*-acetyl-D-neuraminic acid; sialyllactosamine, NeuAcα2−3Galβ1−4GlcNAc; sialyllactosamine-Fl_s, NeuAcα2−3Galβ1−4GlcNAc-OCH_2-CH_2NHC=SNH-fluorescein; α2,3-sialyllactose, NeuAcα2−3Galβ1−4Glc; sialyllactose-Fl_o, NeuAcα2−3Galβ1−4Glc-O-fluorescein; sLe^a, NeuAcα2−3Galβ1−3(Fucα1−4)GlcNAc; sLe^x[Glc], NeuAcα2−3Galβ1−4(Fucα1−3)Glc; sLe^x[GlcNAc], NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAc; sLe^x[Glc]-Fl_o, NeuAcα2−3Galβ1−4(Fucα1−3)Glc-O-fluorescein; sLe^x[GlcNAc]-Fl_s, NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAc-OCH_2CH_2NHC=SNH-fluorescein. SL-ethylamine, NeuAcα2−3Galβ1−4GlcNAc-$OCH_2CH_2NH_2$; sLe^x-ethylamine, NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAc-$OCH_2CH_2NH_2$. With the exception of fucose, all sugars are in the D-configuration.

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presented on a surface such as plastic (Tiemeyer et al., 1991; Yuen et al., 1992, 1994) or in the form of a liposome (Phillips et al., 1990; Polley et al., 1991), have been developed by a number of groups. The fundamental design of these assays, however, still remains multivalent in nature and is subject to the same potential bias as described above for the HL60 cell-based assay.

Utilizing assays of the type just described, the measured IC_{50} of the free oligosaccharide sLe^x(GlcNAc)—the minimal naturally-occurring carbohydrate determinant recognized by E-selectin—has generally been reported to be in the range of 0.5−1 mM (Foxall et al., 1992; Nelson et al., 1993; Welply et al., 1994b). It should be noted, however, that this measured IC_{50} value may be substantially higher than the true thermodynamic dissociation constant, K_d, the result of an inherent bias of the above-described immobilization assays against small-molecule antagonists. Consequently, an evaluation of the true K_d for the E-selectin−sLe^x system would not only provide important information on the strength of the basic carbohydrate−lectin interaction but could also establish whether more sensitive assays might be developed for detecting anti-E-selectin antagonists from chemical and natural product libraries. These libraries typically have compound concentrations in the low micromolar range, too low to antagonize the E-selectin−carbohydrate interaction in the above-described assays.

A technique that has the potential for high sensitivity, and is capable of directly measuring the interaction between protein and ligand without utilizing multivalency, is fluorescence polarization (McClure & Edelman, 1966; Weinhold & Knowles, 1992). The method permits direct measurement of the binding of the fluorescently tagged ligand to the protein by monitoring the level of fluorescence polarization and has been used to explore the binding of sialosides to influenza hemagglutinin (Weinhold & Knowles, 1992). We report here on the successful synthesis of a fluorescent derivative of sLe^x-(Glc) that gives fluoresence polarization in the presence of a soluble form of E-selectin. Using this fluorophore, we have measured the equilibrium dissociation constant for the sLe^x-E−selectin complex.

EXPERIMENTAL PROCEDURES

Materials. UDP-galactose, bovine milk β1,4-galactosyltransferase, calf intestinal alkaline phosphatase, alkaline phosphatase-conjugated goat anti-human IgG(Fc), *Clostridium perfringens* neuraminidase (type X), GDP-fucose, and fluorescein mono-β-D-glucopyranoside were purchased from Sigma (St. Louis, MO). α2,3-Sialyllactose was isolated from bovine colostrum as previously described (Veh et al., 1981) and used as the sodium salt. Truncated, recombinant α3/4-fucosyltransferase V (FTase) lacking the putative transmembrane region was produced in BHK-1 cells. The cDNA for FTase was generated by PCR from human genomic DNA (Clontech) based on the sequence reported by Weston et al. (1992), and the construct was ligated into mammalian expression system pMON3359 (Hippenmeyer & Highkin, 1993). The enzyme was partially purified from conditioned medium by ammonium sulfate precipitation and SP-Sephadex chromatography (Prieels et al., 1981). *Trypanosoma cruzi* β-galactoside-α2,3−*trans*-sialidase was isolated by immunoaffinity chromatography and immobilized on Concanavalin A-Sepharaose as previously described (Scudder et al., 1993).

Synthesis of sLe^x Oligosaccharides and Fluoresceinated Derivatives. The oligosaccharides and fluoresceinated derivatives used in this study were synthesized by a combination of chemical and enzymatic procedures (see Figure 1 for structures of these compounds). Manuscripts detailing the synthesis of the 2-aminoethylglycosides of sialyllactosamine (SL-ethylamine) and of sLe^x[GlcNAc] (sLe^x-ethylamine) are presently in preparation. The fluoresceinated conjugates sialyllactosamine-Fl$_s$ and sLe^x[GlcNAc]-Fl$_s$ were obtained from SL-ethylamine and sLe^x-ethylamine, respectively, by the following method: 5.7 μmol of SL-ethylamine (or sLe^x-ethylamine) in 0.5 mL of water was treated with 46 μmol of FITC (isomer II) in 0.5 mL of dimethylformamide containing 0.1 mL triethylamine at 25 °C for 1 h. The solvents were removed by evaporation under reduced pressure, and the resulting fluorescein derivative sialyllactosamine-Fl$_s$ (or sLe^x-[GlcNAc]-Fl$_s$) was purified by column chromatography on Silica-Gel (2 × 20 cm, Merck 200−300 mesh, 60 Å) using a mixture of chloroform/methanol/water (65/35/8, by vol.). The fluorescein derivatives sialyllactose-Fl$_o$ and sLe^x[Glc]-Fl$_o$ were enzymatically synthesized from fluorescein mono-β-D-glucopyranoside by the following method: 5 μmol of fluorescein mono-β-D-glucopyranoside was first converted to Galβ1−4Glcβ1-*O*-fluoroscein by incubation at 22 °C for 16 h with 1 mL of 50 mM Tris-HCl buffer, pH 7.9, containing 10 μmol of UDP-galactose, 10 μmol of $MnCl_2$, 0.5 mg of α-lactalbumin, 1 unit of alkaline phosphatase, and 1.5 units of galactosyltransferase. The reaction mixture was applied to a 1 mL Sep-Pak C_{18} cartridge that was washed with 10 mL of water before eluting Galβ1−4Glcβ1-*O*-fluoroscein with methanol. Galβ1−4Glcβ1-*O*-fluoroscein was converted to sialyllactose-Fl$_o$ by incubation for 16 h at 22 °C in 2 mL of 50 mM cacodylate/HCl buffer, pH 6.9, containing 70 μmol of α2,3-sialyllactose, 2 μmol of $CaCl_2$, and 0.25 mL of concanavalin A-Sepharose-*trans*-sialidase [0.18 units/mL of gel as assayed against 10 mM α2,3-sialyllactose and 4 mM [^{14}C]*N*-acetyllactosamine (Scudder et al., 1993)]. Sialyllactose-Fl$_o$ (approximately 95% yield) and unreacted Galβ1−4Glcβ1-*O*-fluoroscein were isolated as above using a Sep-Pak C_{18} cartridge. Compound sLe^x-[Glc]-Fl$_o$ was produced from sialyllactose-Fl$_o$ by incubating the latter for 16 h at 22 °C in 0.75 mL of 50 mM cacodylate/HCl buffer, pH 6.9, containing 0.04 unit (assayed against 8 mM lactosamine) of FTase, 7.5 μmol of $MnCl_2$, 5 μmol of GDP-fucose, 0.375 mmol of NaCl, and 1 unit of alkaline phosphatase. The reaction was judged by HP-TLC to have proceeded 50%. Compound sLe^x[Glc]-Fl$_o$ and unreacted sialyllactose-Fl$_o$ were isolated on Sep-Pak C_{18} as above, and they were further purified by preparative TLC on Silica-Gel 60 plates (20 × 20 cm, 0.5 mm thickness) using a solvent system of chloroform/methanol/water (65/35/5, by vol.). The purified derivatives sialyllactose-Fl$_o$ and sLe^x[Glc]-Fl$_o$ were quantified by measuring the sialic acid released following treatment with *C. perfringens* neuraminidase (Welply et al., 1994b). By negative ion electrospray mass spectrometry, the compounds appeared pure and yielded $(M-H)^{1-}$ ions at *m/z* of 960.5 and 1106.6, respectively, consistent with the structures indicated in Figure 1.

Soluble E-Selectin. An E-selectin−F$_c$ expression vector (supplied by Brian Seed, Massachusetts General Hospital, Boston, MA), which encodes a fusion protein containing the extracellular portion of E-selectin fused to an IgG F$_c$ fragment, was transfected into BHK cells, and the condi-

tioned media from these cells was utilized as the source of soluble E-selectin. This form of E-selectin, designated E2 (containing two carbohydrate recognition domains per molecule), was purified from media by affinity chromatography on a column containing murine anti-human E-selectin antibody A3 conjugated to CNBr-activated Sepharose 4B (Pharmacia) according to manufacturer's directions. A3 and M4 anti-E-selectin antibodies were generated by immunization of mice with IL-1 activated human umbilical vein endothelial cells. To generate the monomeric form of soluble E-selectin, designated E1, purified E2 was treated with papain (Boehringer Mannheim) using an E2/papain ratio of 100:1 (w/w) in 154 mM NaCl and 25 mM $CaCl_2$, at 37 °C, for 18 h to cleave the IgG_1 F_c portion from the E-selectin. The IgG_1 F_c portion and intact E2 were removed by chromatography over protein A-Sepharose (Pharmacia), and the papain was removed by chromatography over a Sephadex G150 column (2.5 × 48 cm) equilibrated in Dulbecco's PBS containing 1 mM calcium chloride and 1 mM magnesium chloride, pH 7.4. Calculations of E1 concentrations used for the fluorescence polarization experiments were made using a molecular weight of 80 000. This value was based on data from a reducing SDS−PAGE experiment and also agrees with a recently published value for a soluble E-selectin monomer obtained from Chinese hamster ovary cells (Hensley et al., 1994).

Anti-E-Selectin Antibodies. The A3 and M4 anti-E-selectin monoclonal antibodies used for affinity-chromatographic purification of soluble E-selectin were purified from cell culture fluid by ammonium sulfate precipitation or from ascites fluid by caprylic acid precipitation followed by ammonium sulfate precipitation of the immunoglobulin, according to the method of Reik et al. (1987). $F(ab')_2$ were prepared by digesting the purified immunoglobulins overnight with activated papain in 0.1 M sodium acetate, pH 5.5, and 3 mM EDTA, using a 20:1 (w/w) ratio of immunoglobulin to enzyme, similar to the method of Parham et al. (1982). The reaction was stopped by the addition of 30 mM iodoacetamide. Digested antibodies were chromatographed on DEAE-cellulose equilibrated in 5 mM Tris-HCl, pH 7.5, to remove the F_c. Intact IgG_1 was removed by protein A-Sepharose column chromatography. $F(ab')_2$ fragments were separated from other reaction products by chromatography over Sephadex G75 (2.5 × 46 cm) equilibrated in Dulbecco's phosphate-buffered saline without divalent cations.

HL60 Binding Assays. HL60 cell binding assays were performed with E2 immobilized on Immulon-2 microtitre plates (Dynatech, Chantilly, VA), using a modification of the method of Welply et al. (1994b). Purified E2 (0.19 μg) was added to each well in a final volume of 50 μL of PBS, and the plates were incubated overnight at 4 °C. Nonspecific binding sites were blocked by a 1 h incubation with binding buffer [25 mM HEPES and 2% BSA (w/v) in HBSS, pH 7.0] at room temperature. Compounds were added to the wells in 150 μL of binding buffer and incubated at room temperature for 20 min. To this, 50 μL of fixed HL60 cells (2×10^5cells) was added to each well. The plate was centrifuged at 33*g* for 2 min, and the wells were carefully filled with binding buffer (approximately 100 μL) to minimize artifacts due to air bubble entrapment. Each plate was subsequently sealed with acetate sealing tape being careful to displace trapped air bubbles. Nonadherent HL60 cells were eliminated by inverting the plate for 10 min, removing the acetate film and aspirating the binding buffer. Bound cells were examined using optical microscopy.

Fluorescence Spectroscopy Measurements. Absorption and fluorescence spectra were recorded on Perkin-Elmer Lambda 3B and Spex Fluorolog II instruments, respectively. For the fluorescence assays, 400 μL samples at room temperature were held in glass semi-micro cells (NSG Precision cells type 52) with the 1 cm path length along the excitation direction. Emission from the fluorescein moiety of the ligand was elicited with 465 nm excitation, and the fluorescence spectrum was recorded from 480 to 650 nm under the four polarization conditions V_V V_H, H_H, and H_V, reflecting the polarization of exciting/monitoring light (e.g., H_V denotes horizontal excitation and vertical detection). Glan Taylor prisms in the excitation and emission paths were used to control the polarization.

Measurements of the fluorescence anisotropy (r) were made for the E1−sLe^x[Glc]-Fl_o (or E2−sLe^x[Glc]-Fl_o) protein-ligand complex in a 50 mM Tris, pH 7.6, 10 mM $CaCl_2$ buffer using two concentrations of ligand, 0.2 and 1.0 μM, and varying the concentration of protein from 15 to 173 μM. The V_V, V_H, H_H, and H_V spectra were individually corrected for background scattering at each of the four respective polarization conditions using samples containing appropriate concentrations of either E1 or E2. Values for r were calculated at 5 nm intervals across the 480−650 nm region according to the formula

$$r = (V_V - GV_H)/(V_V + 2GV_H) \quad (1)$$

where $G = H_V/H_H$. The average r value was then calculated. The value of r was found to be constant for any given sample throughout the 480−650 nm region examined, and in all cases the same r value within experimental error was measured for the two ligand concentrations for a given E-selectin concentration.

The value of K_d was determined using the fluorescence anisotropy data from the intercept and slope of a double-reciprocal plot based on the formula

$$\frac{1}{r - r_F} = \left(\frac{K_d}{r_B - r_F}\right)\frac{1}{P_T} + \left(\frac{1}{r_B - r_F}\right) \quad (2)$$

where r_B and r_F are the fluorescence anisotropies of the fluorescent ligand sLe^x[Glc]-Fl_o in the E-selectin-bound and free state, respectively. Equation 2 is derived under the assumption that P_T the (total) concentration of protein is much greater than the (total) concentration of ligand, so that the condition that $P_T \sim P_F$ is satisfied, where P_F is the concentration of free (i.e., uncomplexed) protein. This assumption is valid under the conditions employed and is supported by the observation that the value of r was essentially identical for the two titrations with sLe^x[Glc]-Fl_o at 0.2 and 1.0 μM, respectively (Table 1).

The competitive titration analysis of the nonfluorescent sLe^x(Glc) ligand was performed using 166 μM E1 plus 1.0 μM sLe^x[Glc]-Fl_o in 50 mM Tris, pH 7.6, 10 mM $CaCl_2$ buffer and measuring r as a function of increasing sLe^x(Glc). The value of the dissociation constant for the nonfluorescent ligand was determined by fitting the fluorescence anisotropy

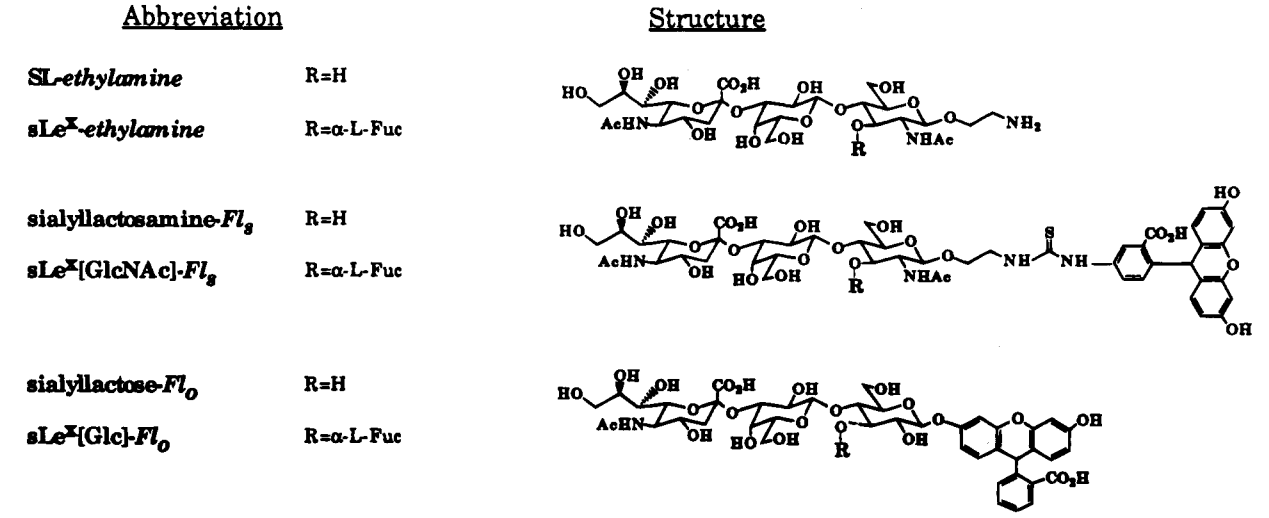

FIGURE 1: Structures and abbreviations of fluoresceinated oligosaccharides and precursor glycoconjugates used in this study.

data (see Figure 4) to the following formula taken from Weinhold and Knowles (1992).

$$\frac{1}{L_T} = \frac{K_d}{K_I}\left[P_T\left(\frac{r_B - r}{r - r_F}\right) - L_T\left(\frac{r_B - r}{r_B - r_F}\right)\right] - K_d \qquad (3)$$

where L_T is the concentration of the nonfluorescent sLeˣ-(Glc), K_d is the dissociation constant of E1−sLeˣ[Glc]-Fl$_o$ determined previously, and K_I is the E1−nonfluorescent sLeˣ-(Glc) dissociation constant. An 8% cumulative dilution of the protein sample by the end of the titration was disregarded in this analysis.

RESULTS

The structures of the fluorescent oligosaccharides used in this study are shown in Figure 1. Sialyllactosamine-Fl$_s$ and sLeˣ[GlcNAc]-Fl$_s$ contain fluorescein moieties attached to the GlcNAc residues via a thiourea bridge. The compounds were generated by a total chemical synthesis, culminating in the conversion of the respective precursor 2-aminoethylglycosides to their fluorescent forms by treatment with FITC. Sialyllactose-Fl$_o$, and sLeˣ[Glc]-Fl$_o$ were generated from fluorescein mono-β-D-glucopyranoside as the starting material utilizing sequential enzymatic additions and contain fluorescein directly attached through a β-glycosidic linkage to glucose.

Both sLeˣ[GlcNAc]-Fl$_s$ and sLeˣ[Glc]-Fl$_o$ were found to inhibit binding of HL60 cells to an immobilized E-selectin−F$_c$ chimera, E2 (Figure 2), confirming that addition of the fluorophore to sLeˣ did not interfere with binding of the sLeˣ part of the molecule to E-selectin. sLeˣ[Glc]-Fl$_o$ displayed an affinity similar to, or slightly more potent than, its corresponding nonfluorescent sugar, sLeˣ(Glc). sLeˣ[GlcNAc]-Fl$_s$ appeared to be a slightly better antagonist than sLeˣ-ethylamine, the aminoethyl aglycon of sLeˣ(GlcNAc). In contrast, the non-fucosylated fluorophore sugars sialyllactosamine-Fl$_s$ and sialyllactose-Fl$_o$ (not shown) as well as α2,3-sialyllactose had no effect on HL60 binding to E-selectin, confirming the need for an sLeˣ motif in these experiments to block HL60 binding. The IC$_{50}$ values for sLeˣ[GlcNAc]-Fl$_s$ and sLeˣ[Glc]-Fl$_o$ and the corresponding nonfluorescent sugars ranged from 0.2 to 0.7 mM. These values are similar to published values for nonfluorescent

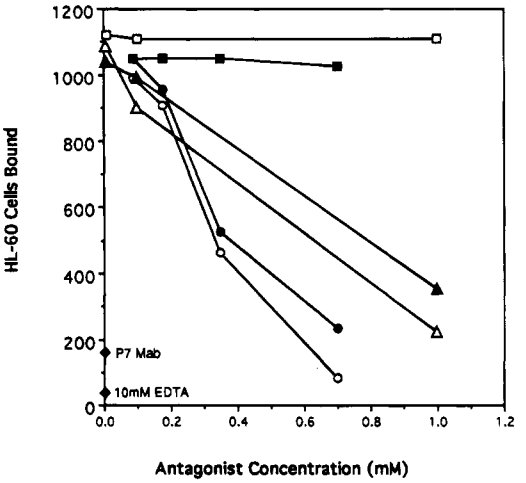

FIGURE 2: Effect of fluoroscent and nonfluorescent derivatives of sLeˣ, siallylactose, and sialyllactosamine on binding of HL60 cells to immobilized E-selectin. Increasing concentrations of the nonfluorescent sugars sLeˣ(Glc) (●), sLeˣ-ethylamine(▲), and α2,3-sialyllactose (■), and of the fluorescent sugars sLeˣ[Glc]-Fl$_o$ (○), sLeˣ[GlcNAc]-Fl$_s$ (△), and sialyllactosamine-Fl$_s$ (□) were added to incubation mixtures, and binding was measured as described in Experimental Procedures. Figure 2 also shows residual binding of HL60 cells in the presence of either a P7 anti-E-selectin blocking Mab (◆) or 10 mM EDTA (◆).

forms of sLeˣ (Brandley et al., 1993; Foxall et al., 1992; Nelson et al., 1993; Welply et al., 1994b).

Fluorescence polarization experiments on sialyllactosamine-Fl$_s$, sLeˣ[GlcNAc]-Fl$_s$, sialyllactose-Fl$_o$, and sLeˣ[Glc]-Fl$_o$ were performed in the presence of either E2, which contains two carbohydrate recognition domains per molecule, or a papain-generated monomeric form of E-selectin (E1) (see Experimental Procedures for preparation of E1 from E2). Although the HL60 binding data indicated that sLeˣ-[GlcNAc]-Fl$_s$ was an antagonist of HL60 binding, no fluorescence anisotropy enhancement was observed with this fluorophore in the presence of E2 (data not shown). A possible explanation for this result is that the linker arm involved in the attachment of fluorescein to sLeˣ allows unhindered rotation of the fluorescein moiety. This explanation was borne out by subsequent experiments utilizing sLeˣ-[Glc]-Fl$_o$, which has fluorescein attached directly to the glucose residue through a glycosidic bond, and which successfully produced fluorescence anisotropy enhancement

Table 1: Fluorescence Anisotropy Values, r, for sLe^x[Glc]-Fl$_o$ with Varying Concentrations of E-Selectin

sample				r	
				sLe^x[Glc]-Fl$_o$	
E1[a] (μM)	E2 (μM)	EGTA (mM)	M4 (mM)	1.0 μM	0.2 μM
173				0.172[b]	0.184[b]
140				0.159[b]	0.164[b]
53				0.130[b]	0.133[b]
41				0.121[b]	0.120[b]
34				0.115[b]	0.115[b]
25				0.108[b]	0.110[b]
23				0.105[b]	
15				0.095[b]	0.095[b]
140		20[c]		0.080	
53		20		0.084	
41		20		0.082	
23		20		0.083	
15		20		0.091	
	22			0.124	
	22		40	0.085[d]	

[a] Calculation of [E1] was made using a molecular weight of 80 000. [b] These values were used to generate the data points displayed in Figure 3, using the average of the r values observed at the two ligand concentrations (except for the single value of r at E1 = 23 μM), and using an r_F = 0.075 in the calculation. [c] At higher concentrations of EGTA a precipitate was sometimes observed in the samples and r could not be measured reliably. [d] In a separate series of experiments, an average value of r = 0.085 was found for 20 mM EGTA solutions of E2−sLe^x[Glc]-Fl$_o$, demonstrating that addition of M4 to the E2−sLe^x[Glc]-Fl$_o$ complex drops the value of r to a background level.

in the presence of either E2 or E1 (Table 1). Further evidence that the enhanced anisotropies observed in the presence of E1 and E2 were attributable to binding of the oligosaccharide portion of the sLe^x-fluorophore to the carbohydrate recognition domains was obtained by adding EGTA to the E1−sLe^x[Glc]-Fl$_o$ and E2−sLe^x[Glc]-Fl$_o$ mixtures. Because binding of sLe^x to E-selectin is Ca^{2+} dependent (Tiemeyer et al., 1991), addition of EGTA would be expected to eliminate any contribution to the anisotropy that was due to interaction of sLe^x[Glc]-Fl$_o$ with the carbohydrate recognition domain. Addition of EGTA to either the E2−sLe^x[Glc]-Fl$_o$ mixture or E1−sLe^x[Glc]-Fl$_o$ mixture (Table 1) reduced the fluorescence anisotropy to essentially the background level observed for sLe^x[Glc]-Fl$_o$ in the absence of E1 and E2. A plot of $1/(r - r_F)$ against 1/[E1], where r is the measured fluorescence anisotropy, r_F is the fluorescence anistropy of free sLe^x[Glc]-Fl$_o$ (taken to be 0.075; see Discussion), and [E1] is the concentration of monomeric form of soluble E-selectin gave a straight line (Figure 3). Using eq 2, a fit to the data of Figure 3 gave a dissociation constant, K_d, for the E1−sLe^x[Glc]-Fl$_o$ complex of 107 ± 26 μM.

To obtain the dissociation constant for sLe^x(Glc), a competition experiment was performed in which the fluorescence anisotropy obtained with sLe^x[Glc]-Fl$_o$ bound to E1 was monitored in the presence of increasing amounts of the nonfluorescent sLe^x(Glc). Because the fluorescent and nonfluorescent ligands compete for the same binding site, increasing concentrations of nonfluorescent sugar should produce a diminution in the polarization signal, as was indeed found to be the case (Figure 4). A fit of this anisotropy curve to eq 3 yields a dissociation constant, K_d, for the E1−sLe^x(Glc) complex of 120 ± 31 μM (for details of calculation, see Experimental Procedures).

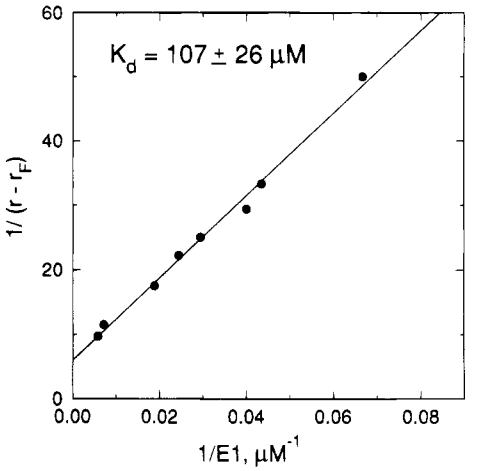

FIGURE 3: Double-reciprocal plot of the titration data of Table 1, using r_F = 0.075 (see Discussion for explanation of determination of r_F). The r value for each concentration of E1 was the result of averaging the values obtained with 0.2 and 1.0 μM ligand concentrations, respectively (with exception of r value for E1 at 23 μM). The straight line is a linear regression fit of the data to eq 2, giving a K_d of 107 ± 26 μM for the E1−sLe^x[Glc]-Fl$_o$ complex.

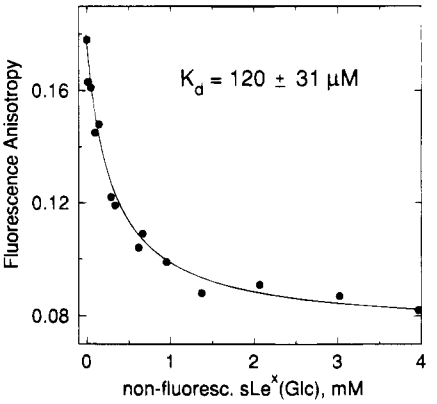

FIGURE 4: Fluorescence polarization competition experiment. To a solution containing 1.0 μM of the fluorophore sLe^x[Glc]-Fl$_o$ and 166 μM of the monovalent E-selectin E1, the nonfluorescent sugar sLe^x(Glc) was added in increasing amounts and fluorescence polarization measurements taken. The solid curve is a nonlinear least-squares fit of the data to eq 3, giving a K_d for the nonfluorescent E1−sLe^x(Glc) complex of 120 ± 31 μM.

A fluorescence experiment was also performed to establish whether an anti-E-selectin monoclonal antibody, M4, previously shown to block binding of HL-60 cells to E-selectin (Ulich et al., 1994), could affect the anisotropy enhancement obtained when sLe^x[Glc]-Fl$_o$ binds to E2. If the binding sites for sLe^x[Glc]-Fl$_o$ and M4 were mutually exclusive and nonoverlapping, it would be expected that addition of M4 should have no effect on the ratio of free to bound sLe^x-[Glc]-Fl$_o$. Hence, the fluorescence anisotopy observed for the E2−sLe^x[Glc]-Fl$_o$ mixture would not be reduced by addition of M4. (In fact, assuming that an E2−M4−sLe^x-[Glc]-Fl$_o$ ternary complex could exist, the resulting longer rotational correlation time of this complex should theoretically lead to an increase in the anisotropy value.) On the other hand, in the event that the binding sites for sLe^x[Glc]-Fl$_o$ and M4 do indeed overlap, addition of M4 to a sample already containing sLe^x[Glc]-Fl$_o$ and E2 should reduce the initial fluorescence polarization to the background level typically observed for sLe^x[Glc]-Fl$_o$ in the absence of E2. This latter situation was indeed found to be the case,

Table 2: Rate and Equilibrium Constants for Typical Carbohydrate−Protein Interactions

protein[a]	ligand[b]	K_d (M)	IC_{50} (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
hemagglutinin	α-methyl-SA	2.8×10^{-3} [c]	2.5×10^{-3} [d]		
	α2,3-SL	3.2×10^{-3} [c]			
MBP	RM		1.3×10^{-3} [e]		
ABP	arabinose	1.0×10^{-7} [f]		2.4×10^{7} [f]	1.5 [f]
concanavalin	*p*NP-Man	1.1×10^{-4} [g]		5.4×10^{4} [g]	6.2 [g]
	M_2N_2-PA	2.5×10^{-5} [h]			
E-selectin	sLe^x(Glc)	1.2×10^{-4}	3.3×10^{-4}		
			1.0×10^{-4} [i]		
	sLe^x(GlcNAc)		$\sim 1 \times 10^{-3}$ [j]		
M6P-receptor	Man-6-PO_4	7.0×10^{-6} [k]	6.4×10^{-5} [l]		

[a] Protein abbreviations: ABP, arabinose-binding protein; MBP, mannose binding protein; M6P-receptor, mannose 6-phosphate receptor. [b] Ligand abbreviations: α-methyl-SA, α-methylsialic acid; RM, $Man_6GlcNAc_2$-Asn; *p*NP-Man, *p*-nitrophenyl-mannopyranoside; M_2N_2-PA, Manβ1,6-Manβ1,4-GlcNAcβ1,4-GlcNAc-pyridylamine. [c] Sauter et al. (1989). [d] Glick et al. (1991). [e] Lee et al. (1991). [f] Miller et al. (1983). [g] Lewis et al. (1976). [h] Mega et al. (1992). [i] Brandley et al. (1993). [j] Nelson et al. (1993) and Welply et al. (1994b). [k] Tong et al. (1989). [l] Sahagian et al. (1981).

illustrated by the drop in the r value from 0.124 to 0.085 with addition of M4 (Table 1). Thus sLe^x[Glc]-Fl_o and M4 both compete for the same binding site in the carbohydrate-recognition domain of E2.

DISCUSSION

Conformational studies of free sLe^x using NMR techniques indicate that basically one stable conformer of sLe^x exists in aqueous solution (Wormold et al., 1991; Ball et al., 1992; Lin et al., 1992; Ichikawa et al., 1992) and that this conformer displays restricted flexibility (Rutherford et al., 1994) resembling the relatively rigid behavior observed earlier for certain blood group oligosaccharides (Lemieux et al., 1980; Cagas & Bush, 1990; Yan & Bush, 1990). Although the conformation of sLe^x in the bound state is unknown, it is likely that the restricted flexibility observed in solution would be unaltered by interaction of sLe^x with the carbohydrate recognition domain of E-selectin. Because the sialic acid and fucose residues of sLe^x are generally believed to be key contact points for this sLe^x interaction (Brandley et al., 1993), we attempted to generate fluorescent forms of sLe^x with the fluorophore placed somewhere on the Galβ1−4GlcNAc part of the molecule in the hope that this would not perturb the basic E-selectin−sLe^x interaction. We anticipated that the observed overall rigidity of the sLe^x molecule would minimize any attenuation of the polarization signal due to placement of the fluorophore away from the key residues. Consequently, we generated fluorescent derivatives of sLe^x containing fluorescein attached directly to the reducing end of the molecule (on either the Glc or GlcNAc residues of sLe^x[Glc] or sLe^x[GlcNAc], respectively).

The fluorescein derivatives synthesized for this study contained either a somewhat extended thiourea linkage (sialyllactosamine-Fl_s and sLe^x[GlcNAc]-Fl_s) or a direct O-glycosidic linkage of the fluorophore to the sugar (sialyllactose-Fl_o and sLe^x[Glc]-Fl_o). Both sLe^x[GlcNAc]-Fl_s and sLe^x[Glc]-Fl_o gave inhibition curves similar to their parent sugars in HL60 binding assays (Figure 2), indicating that addition of the fluorophore did not dramatically perturb the basic E-selectin−sLe^x interaction. However, only the O-glycosidically linked compound sLe^x[Glc]-Fl_o showed fluorescence anisotropy enhancement in the presence of E-selectin. We interpret the lack of enhanced fluorescence polarization with sLe^x[GlcNAc]-Fl_s to be the result of the high level of flexibility afforded by the thiourea linkage, counteracting the large increase in the rotational correlation time expected when free sLe^x binds to E-selectin.

The approximate IC_{50} values obtained for these fluorescent sLe^x derivatives and their corresponding free sLe^x sugars in the HL60 binding assays are similar to values reported for sLe^x derivatives by other groups (Foxall et al., 1992; Nelson et al., 1993) and are also in accordance with earlier reports by Tyrrell et al. (1991) and Brandley et al. (1993) that sLe^x-(Glc) is a more potent antagonist of E-selectin-mediated interactions than sLe^x(GlcNAc). The IC_{50} value for sLe^x-(GlcNAc) generally is reported to be in the range of 0.5−1 mM. In contrast, the sLe^x(Glc) values determined from the dose−response curves of our HL60 cell assay (Figure 2) and also using a soluble E2-immobilized-glycolipid assay (data not shown) were about 0.33 and 0.2 mM, respectively. This is in reasonable agreement with a value of 0.1 mM recently reported for sLe^x(Glc) by Brandley et al. (1993).

The value for the dissociation constant K_d of the tetrasaccharide sLe^x(Glc)−E-selectin monomer (E1) complex was found by fluorescence polarization to be 120 ± 31 μM, only marginally lower than the typical IC_{50} values (0.5−1 mM) observed for sLe^x(Glc) and sLe^x(GlcNAc) in standard binding assays. The weak binding of the sLe^x tetrasaccharide for E-selectin is also consistent, in general, with those reported for other simple carbohydrate-protein interactions (Table 2). Recently, Yuen et al. (1994), utilizing an E-selectin binding assay which monitors binding of E-selectin-transfected Chinese hamster ovary cells to immobilized glycolipids, reported IC_{50} values for sLe^x considerably below the above reported values. The numbers range from 7.5×10^{-7} M, using an immobilized sLe^x pentasaccharide, to 1.9×10^{-5} M with an immobilized sulfated Le^a pentasaccharide. At present, we have no explanation for what would appear to be a contradiction between the weak interaction we are measuring and the potent antagonism reported for sLe^x in their binding assays.

The largest source of uncertainty in the determination of the K_d for the E-selectin−sLe^x[Glc]-Fl_o interaction relates to the value of r_F used in eq 2 to fit the titration data for the E1−sLe^x[Glc]-Fl_o complex of Figure 3. Several measurements were performed to settle on a value of $r_F = 0.075$. SLe^x[Glc]-Fl_o alone in 50 mM Tris, pH 7.6, 10 mM $CaCl_2$ buffer (no protein present) gave $r = 0.065 \pm 0.005$. When sialyllactose-Fl_o, which does not bind to E-selectin in an HL60 cell binding assay, was tested for fluorescence polarization in the presence of E2, a value for $r = 0.072 \pm 0.05$ was obtained. Additionally, r values ranging from 0.080 to 0.085 were typically observed following addition of EGTA to E1−sLe^x[Glc]-Fl_o or E2−sLe^x[Glc]-Fl_o solutions (see

Table 1). The r value of 0.065 observed for sLe^x[Glc]-Fl$_o$ in buffer alone was not used in fitting the data of Figure 4 because it did not include the contribution of E1 to sample viscosity. (Any increase in viscosity leads to an increase in the fluorescence anisotropy, as exemplified by the value obtained with sialyllactose-Fl$_o$ in the presence of E2, and the value of $r = 0.29$ obtained for sLe^x[Glc]-Fl$_o$ in 75% glycerol.) Also, the r values of 0.80−0.85 observed for solutions of E1−sLe^x[Glc]-Fl$_o$ and E2−sLe^x[Glc]-Fl$_o$ containing 20 mM EGTA were discounted as being too high due to the likelyhood that dissociation of the complex was not 100%. It is interesting to note that a fit of the data of Figure 3 using two extreme values of r_F (0.065 and 0.085) gave K_ds of 52 and 200 μM, respectively, indicating that the choice of r_F used in the calculation did not alter the basic finding that the E-selectin−sLe^x interaction is weak. An r_F value of 0.075 was additionally obtained from a fit of the data of Figure 4 to eq 3, with both r_F and K_I allowed to act as free parameters.

The tightest binding system observed for simple sugar−protein interactions is probably the bacterial sugar-transporting proteins such as the *Escherichia coli* L-arabinose binding protein (ABP). The crystal structure (Quiocho & Vyas, 1984) and sugar-binding properties (Miller et al., 1983; Fukada et al., 1983) of this protein provide detailed information on this carbohydrate−protein interaction. ABP is one of a group of proteins that contain two distinct globular domains attached through a hinge which permits them to close around their target molecules. To date, the bound arabinose is the most buried in any of the sugar-transporting proteins studied, participating in an extensive number of hydrogen bonds and van der Waals contacts within the binding pocket (Quocho, 1986); yet the measured K_d of 10^{-7} for this complex is still relatively weak (Table 2). In constrast, the binding pocket for C-type lectins such as the mannose-binding protein (MBP) (Weis et al., 1992) and E-selectin (Graves et al., 1994) is shallow, and the proteins clearly do not completely engulf their counter receptor sugars. The binding of $Man_6GlcNAc_2Asn$ to MBP has been shown to involve only the terminal mannose residue (Lee et al., 1991; Weis et al., 1992) and to involve primarily two hydrogen bonds formed between a Ca^{2+} and the 3- and 4-hydroxyl groups of this monosaccharide. There are comparatively few van der Waals interactions and no significant aromatic stacking interactions. This, combined with the shallow nature of the carbohydrate-binding site, is reflected in the weak K_i reported for the $Man_6GlcNAc_2Asn$−MBP interaction (Table 2). The recently reported crystal structure for E-selectin reveals that although there are some significant differences from that of MBP—acknowledging that these two C-type lectins are distantly related (~30% identity between the two sequences of the carbohydrate-recognition domain) — the overall topology of the lectin domains shows considerable similarity. Docking of sLe^x to the E-selectin surface is thought to involve fucose in a configuration relative to the Ca^{2+} site similar to that of mannose bound to MBP (Graves et al., 1994). However, the specific interactions of the rest of the molecule, in particular contact interactions involving the sialic acid, have not been identified. The implications of the crystal-structure studies of C-type lectins are that the basic interaction involving the sugar and protein are weak in nature, in agreement with the value we measure for the tetrasaccharide sLe^x and E-selectin.

Of course, the overall strength of the interaction between E-selectin and its counter receptor in vivo may be greater than the value measured here due to additional interactions involving the extended oligosaccharide moiety and the protein itself. Moreover, adhesion of leukocytes to activated endothelial cells clearly involves a high degree of cooperativity, the result of a large number of individual molecular interactions between the cell surfaces. We have separately explored the potential of multivalent forms of sLe^x to act as potent antagonists of leukocyte−endothelial cell adhesion (Welply et al., 1994b), with the result that a multivalent $(sLe^x)_{17}$−BSA conjugate produced a 3 order-of-magnitude affinity enhancement over that of free sLe^x in an E-selectin binding assay.

The weak sLe^x−E-selectin interaction in combination with molecular cooperativity may very well be the key requirement needed to facilitate transient cell−cell interactions such as that observed for rolling of leukocytes on activated endothelium. Leukocyte rolling, occurring at speeds as high as 150 μm/s, is typically thought to result from a balance between two competing forces: (1) the static-interaction strength between the leukocyte and the activated endothelial cell, and (2) sheer stress produced by blood flow through the venule. At the molecular level, the rolling event is believed to derive from a high number of cell-surface interactions involving selectins and their appropriate co-receptors. The measured weak affinity of sLe^x for E-selectin is likely the result of the high dissociation rate constant (k_{off}) for the protein−carbohydrate complex, since the measured association rate constants (k_{on}) for protein−protein and protein−carbohydrate interactions do not vary greatly. In contrast, the k_{off} values show a tremendous range—varying from about ~10^{-4} (s^{-1}) for antibody−protein interactions up to 19 (s^{-1}) for carbohydrate−Concanavalin A (van der Merwe & Barclay, 1994). Fast off rates (high k_{off} values) are typically the case for protein−carbohydrate interactions, and it may be that the high k_{off} is the reason that selectins are particularly useful where disengagement of transiently interacting cells is an important requirement of a biological system.

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