Valency Dependent Patterns of Binding of Human L-Selectin toward Sialyl and Sulfated Oligosaccharides of Le^a and Le^x Types: Relevance to Anti-Adhesion Therapeutics†

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ABSTRACT: The human L-selectin is known to bind to immobilized 3′-sialyl-Le^x and -Le^a oligosaccharides both under static and physiological flow conditions. Here the reactivities toward 3′-sulfated and 3′-sialyl-Le^a and -Le^x pentasaccharides are compared by *in-vitro* binding and inhibition assays using preparations of human L-selectin-IgG-Fc chimera in which the selectin is predominantly in di- and tetrameric form (paucivalent) or in the form of a complex with anti-IgG (multivalent). Affinity for the sulfated ligands is marginally greater than for the sialyl ligands, as judged by concentrations required to give 50% inhibition of the multivalent selectin binding to the immobilized sulfated and sialyl ligands. There is a striking difference, however, in the avidities of binding of the two L-selectin forms toward the sulfated and sialyl ligands when these are immobilized in the clustered state: the paucivalent selectin gives detectable binding only to the sulfated ligands when these are immobilized as neoglycolipids on plastic microwells (up to 100 pmol immobilized per well) whereas the multivalent L-selectin binds well to both classes of ligand. Moreover, binding of the paucivalent selectin form is effectively inhibited only by the sulfated ligand, although binding of the multivalent selectin is inhibitable by both the sulfated and sialyl ligands. Such striking valency-dependent differences in ligand binding avidity and inhibitability may be manifest *in vivo* with the membrane-bound L-selectin, as marked variations occur in its density of expression on leukocytes. Thus, for the purpose of selecting inhibitors for development of therapeutic anti-inflammatory compounds, experimental designs based on the paucivalent L-selectin would more clearly single out compounds with broad spectrum anti-adhesive activities toward the both the high- and low-avidity interactions of the cell adhesion protein.

L-selectin is a carbohydrate-binding protein on leukocytes which, through interactions with carbohydrate ligands on postcapillary venules, promotes tethering and rolling adhesions of leukocytes under physiologic flow conditions (Ley et al., 1991) and has a key role in the initial steps of leukocyte homing into peripheral lymph nodes (Gallatin et al., 1983). The protein also participates in leukocyte entry into acute and chronic inflammatory sites (Rosen, 1993; Tedder et al., 1995). The interactions of L-selectin with its carbohydrate ligands are therefore potential targets for therapeutic intervention in disorders of inflammation.

Most of the information available on the specificity of L-selectin toward structurally defined oligosaccharide ligands[1] is in rodent systems. Blood group-related oligosaccharides of the Le^a and Le^x series, 3′-sulfated or 3′-sialylated at the non-reducing end galactose, are recognized both by the rat and the murine forms of L-selectin, with an overall preference of the sialyl-Le^a over sialyl-Le^x (Berg et al., 1992) and a preference of the sulfo-Le^a and -Le^x over the sialyl forms (Green et al., 1992, 1995; Brandley et al., 1993). Various sulfated, non-fucosylated oligosaccharides are also bound by the rodent-L-selectins notably the glycosaminoglycans (Green et al., 1992, 1995; Needham & Schnaar, 1993; Norgard-Sumnicht et al., 1993, 1995). Four potential counter-receptors[1] have been described for L-selectin on murine lymph nodes: these are the mucin type molecules, GlyCAM-1, CD34 and MAdCAM-1 and Sgp200 (Lasky et al., 1992; Baumheuter et al., 1993; Berg et al., 1993; Hemmerich et al., 1994b). Multiple glycoproteins reactive with the rat L-selectin have also been described (Tamatani et al., 1993). Sulfation and sialylation of GlyCAM-1 have been shown to be important for murine L-selectin recognition (Imai et al., 1993; Hemmerich et al., 1994a), and oligosaccharides containing 3′-sialyl-Le^x capping groups with 6′-sulfation at galactose, with or without 6′-sulfation at *N*-acetylglucosamine have been isolated from this glycoprotein (Hemmerich et

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[1] The term ligand is used here to denote the carbohydrate determinants recognized by the carbohydrate recognition domain, and the macromolecular carriers of the ligands are referred to as counter-receptors as proposed (Feizi, 1993; Crocker & Feizi, 1996).

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al., 1994b, 1995; Hemmerich & Rosen, 1994).

There is less information on the reactivities of the human L-selectin with defined oligosaccharide structures. Here the information is largely on recognition of oligosaccharides terminating in 3′-sialyl-Le^a and -Le^x. As in the rodent systems, a preference of sialyl-Le^a over sialyl-Le^x was observed in static binding experiments using cells transfected to express the human L-selectin, and immobilized oligosaccharides linked to protein (Berg et al., 1992). These sialyl oligosaccharide ligands in lipid-linked form, either as glycolipids or neoglycolipids, were shown also to support human L-selectin-mediated leukocyte tethering and rolling under conditions of physiologic flow (Alon et al., 1995). Thus glycolipids as well as glycoproteins such as the human form of MAd-CAM-1 (Shyjan et al., 1996) are potential counter-receptors for this selectin. Structural information is awaited on the oligosaccharides of the human counter-receptors for L-selectin. In recent experiments, however, preferential binding of human L-selectin to 3′-sialyl-Le^x that is additionally 6′-sulfated at the penultimate galactose has been documented in experiments where the preformed tetrasaccharide was enzymatically transferred to Chinese hamster ovary cells (Tsuboi et al., 1996).

Using a human L-selectin-IgG[2] Fc chimera, here we investigate reactivity with 3′-sulfo-Le^a and 3′-sulfo-Le^x, which are recognized by the human E-selectin (Yuen et al., 1992), as well as the rat L-selectin, and compare these with the 3′-sialyl-Le^a and 3′-sialyl-Le^x. We address the effect of selectin valency on these reactivities using the oligosaccharides in the form of neoglycolipids (Tang et al., 1985) due to the versatility of this class of oligosaccharide probes for studying any desired oligosaccharides and their displays over a wide range of densities (Feizi et al., 1994). We observe striking differences between the paucivalent and multivalent forms of human L-selectin not only with respect to their binding intensities but also to susceptibilities to inhibition with the sulfated and sialyl oligosaccharide ligands. We propose that such differences may be manifest *in vivo* as the membrane-associated form of the selectin is expressed at varying levels, and possibly varying densities, at the surface of leukocytes, and that these are important considerations for the design of experiments to identify for therapeutic purposes "broad spectrum" inhibitors of selectin-mediated adhesion.

MATERIALS AND METHODS

Recombinant Soluble L-Selectins. A chimeric soluble form of human L-selectin was made by fusing the extracellular part of the selectin with the Fc domain of human IgG. PCR was performed using the human L-selectin cDNA clone (Siegelman et al., 1989) as a template with the sense primer 5′ atattgcggccgccaatgggctgcagaagaac; and the antisense primer 5′ atttatctagattgacatattggactaggatttg. The resulting 1 kb PCR product as well as the vector pED.Fc (Sako et al., 1995) was digested with *Not*I and *Xba*I. The two components were ligated resulting in construct pED.L-selFc, confirmed by DNA sequencing. The L-selectin segment includes 41 base pairs of 5′UTR and is fused to the Fc sequence after the Gln 317 codon of the second complement-like domain. The pED.L-selFc construct was expressed in COS cells and the L-selectin-IgG chimera was purified from serum-free conditioned medium by affinity chromatography using protein A sepharose (Pharmacia). Rat L-selectin fused to human IgG Fc domain was produced in silkworms (Tamatani et al., 1993) and was a gift from M. Miyasaka.

Table 1: Saccharides Investigated in the Present Study

designations	saccharide moieties
Su-Le^a	Galß1-3GlcNAcß1-3Galß1-4Glc | 3 | 1,4 HSO_3 Fucα
Su-Le^x	Galß1-4GlcNAcß1-3Galß1-4Glc | 3 | 1,3 HSO_3 Fucα
S-Le^a	Galß1-3GlcNAcß1-3Galß1-4Glc |2,3 | 1,4 NeuAcα Fucα
S-Le^x	Galß1-4GlcNAcß1-3Galß1-4Glc |2,3 | 1,3 NeuAcα Fucα
Le^a	Galß1-3GlcNAcß1-3Galß1-4Glc | 1,4 Fucα
Le^x	Galß1-4GlcNAcß1-3Galß1-4Glc | 1,3 Fucα
LNT	Galß1-3GlcNAcß1-3Galß1-4Glc

Oligosaccharides. The oligosaccharides investigated in the present study are given in Table 1. The following oligosaccharides were synthesized chemically: 3′-sialyl-Le^x pentasaccharide,[3] S-Le^x (Hasegawa et al., 1992); 3′-sulfated Le^a pentasaccharide, Su-Le^a (Lubineau et al., 1994); 3′-sulfated Le^x pentasaccharide, Su-Le^x (Augé et al 1997); these were purified on a TSK amide-80 column (4.6 × 250 mm; Anachem, U.K.) by gradient elution using acetonitrile/water in 10 mM NH_4OAc buffer, pH 5 (elution profile: 32%−38% water in 30 min). Lacto-*N*-tetraose (LNT) was purchased from Dextra, and 3′-sialyl-Le^a pentasaccharide, S-Le^a, isolated from human milk was from The Institute of Organoelement Compounds, Moscow.

(Neo)glycolipids. Neoglycolipids (Tang et al., 1985) were prepared by conjugation of oligosaccharides to the amino-phospholipid L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) and purified as previously described (Feizi et al., 1994). Each neoglycolipid gave a single band on TLC and the presence of sulfate, and the monosaccharide sequence in each neoglycolipid was corroborated by liquid secondary ion mass spectrometry (Lawson et al., 1990). In previous investigations (Yuen et al., 1992) S-Le^x neoglycolipid and S-Le^x glycosphingolipid were shown to have equivalent reactivities with E-selectin expressing cells; and in the course

[2] Abbreviations: BSA, bovine serum albumin; DHPE, L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; HRP, horseradish peroxidase; IgG, immunoglobulin G; LNT, lacto-*N*-tetraose; OPD, *O*-phenylenediamine hydrochloride; S-Le^a, 3′-sialyl-Le^a pentasaccharide; S-Le^x, 3′-sialyl-Le^x pentasaccharide; Su-Le^a, 3′-sulfated-Le^a pentasaccharide; Su-Le^x, 3′-sulfated-Le^x pentasaccharide; TBS, 10 mM Tris buffer containing 150 mM NaCl, pH 7.4.

[3] For ease of comparison of the sialylated-Le^a and -Le^x oligosaccharides with the sulfated oligosaccharides, the term pentasaccharide is used to denote the fucosylated tetrasaccharide backbone that they share.

of the present investigation, the two lipid-linked forms gave indistinguishable binding curves with human L-selectin (not shown). The lipid-linked oligosaccharides were incorporated into liposomes (Feizi et al., 1979) consisting of cholesterol, egg lecithin, and lipid-linked oligosaccharide at ratios of 0.4: 4:1 by weight.

Microwell Binding Assays. L-selectin binding to lipid-linked oligosaccharides immobilized on plastic microwells (Falcon 3912) was detected essentially as described (Green et al., 1995) in a total reaction volume of 50 μL. In brief, lipid-linked oligosaccharides in the presence of carrier lipids were evaporated in the microwells. The wells were washed 3 times with 10 mM Tris buffer containing 150 mM NaCl pH 7.4 (TBS) followed by a 2 h blocking with a 1% w/v casein (Pierce) in TBS containing 2 mM Ca^{2+} or 10 mM EGTA, and these diluents were used as appropriate for the subsequent incubation and washing steps. L-selectin was used at 2 μg per mL (100 ng per well) unless otherwise stated; binding was detected using biotinylated goat antibodies to human IgG heavy and light chains (Vector Laboratories) followed by biotinylated horseradish peroxidase (HRP) complexed with streptavidin from Pierce, and colorimetric measurement in a Dynatech MRX plate reader with linear readout up to 3.5 OD using *O*-phenylenediamine hydrochloride (OPD) from Sigma as substrate. The proportions of lipid-linked oligosaccharides retained in the microwells after the washing and blocking steps were determined for Su-Le^a, S-Le^a, and LNT as described previously (Childs et al., 1992) and were found in each case to be approximately 30% of the amount added. Adjustments were not made, therefore, in plotting the binding curves. In experiments to investigate effects of L-selectin clustering on carbohydrate-binding specificity, the selectin (1 vol of a 2 mg per mL of solution) was pre-incubated for 1 h with 6 vol of a 1 mg per mL solution of biotinylated goat-anti-IgG heavy and light chains (the selectin:anti-Ig ratio used, 1:3 by weight, was based on earlier experience[4] with other IgG-Fc chimeras). The selectin thus clustered was diluted to give 2 μg of selectin per mL for binding experiments. In assays investigating inhibition of L-selectin binding, plates were coated with either Su-Le^a or S-Le^a neoglycolipids, 100 pmol per well, blocked with 3% (w/v) bovine serum albumin (BSA) in TBS containing 2 mM Ca^{2+} and L-selectin was diluted to 0.2 μg per mL (10 ng per well) with 0.1% (w/v) BSA in TBS containing 2 mM Ca^{2+}. Having observed in separate experiments (not shown) that lipid-linked oligosaccharide ligands on liposomes are approximately ten thousand fold more potent as inhibitors of the binding than the free oligosaccharides, we performed the inhibition experiments described here using the oligosaccharides as neoglycolipids incorporated into liposomes (Feizi et al., 1979) and suspended in TBS. As a test of the specificity of L-selectin binding to the lipid-linked oligosaccharides immobilized on microtiter wells, DREG 56 antibody directed to L-selectin (Pharmingen) was included as an inhibitor of the binding of 100 ng of L-selectin to 100 pmol of Su-Le^a neoglycolipid. Complete inhibition of binding was achieved at an antibody concentration of 3 μg/mL (75 ng/well).

Chromatogram Binding Assays. L-selectin binding to lipid-linked oligosaccharides separated by TLC in chloroform/methanol/water, 60/35/8 (by volume), was assayed as described previously (Green et al., 1995) with the following modifications. The silica gel chromatography plates were plastic backed HC grade from Camlab; the diluent used was TBS containing 50 mM $CaCl_2$; blocking of reactive sites on the chromatograms was with 5% (w/v) bovine hemoglobin, and L-selectin was used at 2 μg per mL. Duplicate chromatograms were chemically stained with Primulin (Feizi et al., 1994).

[4] R. A. Childs, unpublished.

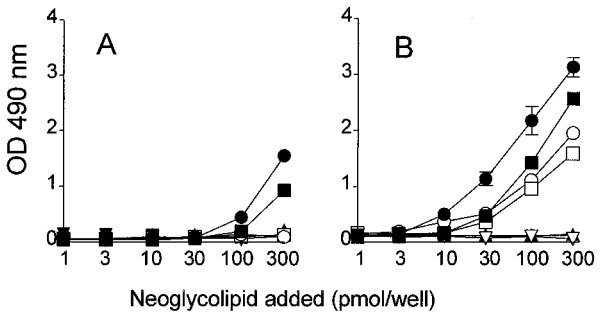

FIGURE 1: Effect of human L-selectin clustering on binding to sulfated and sialyl oligosaccharides presented on microwells. In A and B, quantitative binding assays were performed using varying amounts of lipid-linked oligosaccharides applied onto plastic microwells, and L-selectin-IgG chimera, 100 ng per well, non-complexed (A) or complexed with anti-IgG (B). Binding was detected with a biotin−streptavidin−HRP system as described under Materials and Methods. Results are expressed means in duplicate wells with the range indicated by error bars. Symbols used for the oligosaccharide sequences are as follows: Su-Le^a, ●; Su-Le^x, ■; S-Le^a, ○; S-Le^x, □; Le^a, △; Le^x, ▲; LNT, ▽. The non-acidic oligosaccharides Le^a, Le^x, and LNT gave no binding signals with the two L-selectin forms (symbols for these are overlapping in the base line).

Gel Filtration Analyses of Proteins. L-selectin-IgG chimera goat anti-human IgG heavy and light chains or a soluble mixture of the human selectin and anti-IgG were chromatographed using a Gilson HPLC system equipped with a UV detector reading at 280 nm on a Pharmacia Superose 12 column (1.5 × 30 cm) equilibrated in 500 mM NaCl containing 20 mM Tris buffer, pH 8.0, and 0.02% NaN_3; flow rate was 0.6 mL/min, and signals were expressed in mV.

RESULTS

In initial experiments, the human L-selectin-IgG chimera was found to bind to the sulfo-oligosaccharides Su-Le^a and Su-Le^x but not to the sialyl oligosaccharides S-Le^a and S-Le^x at the highest levels tested, 300 pmol of neoglycolipid applied per well (Figure 1A). At first sight this suggested a difference from rat L-selectin-IgG which was observed to bind to the sialyl- as well as the sulfo-Le^a and -Le^x oligosaccharides under these assay conditions (Green et al., 1995). When the human L-selectin chimera was complexed with anti-IgG, however, not only was there a decrease in the threshold level of the sulfated ligands that elicited a binding signal and an increase in the steepness of the binding curves, but there was also the appearance of new reactivities with the S-Le^a and S-Le^x neoglycolipids at coating levels that gave negative results previously (Figure 1B). Thus the binding now resembled that of the rat L-selectin homolog [cf. Green et al.) (1995)]. All of the binding observed to the acidic Le^a and Le^x oligosaccharides was calcium-dependent as binding signals were not detected in the presence of 10 mM EGTA (not shown).

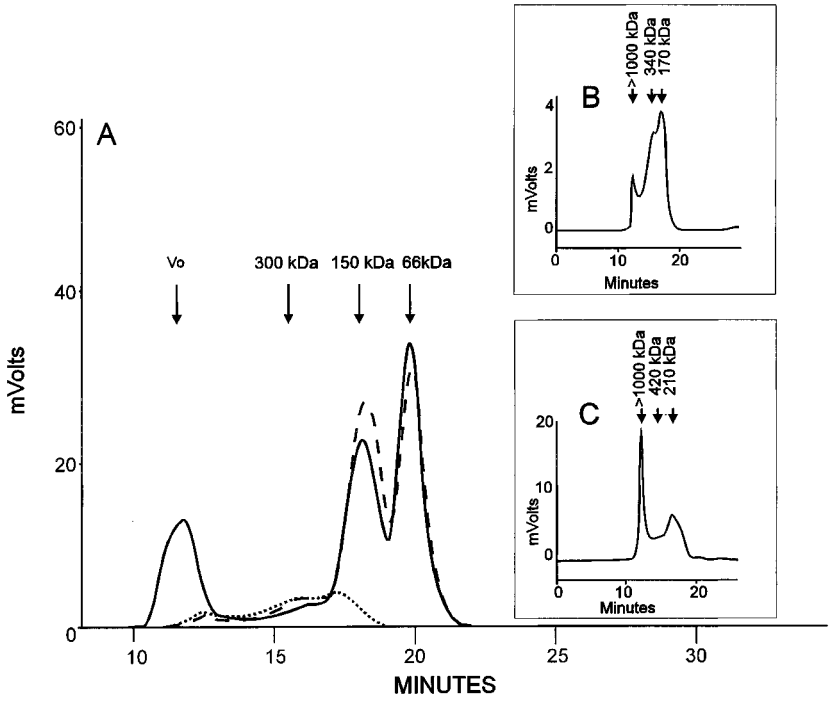

FIGURE 2: Gel filtration of human and rat L-selectin chimeras, and of human L-selectin chimera complexed with anti-IgG. Human L-selectin chimera, 5 μg, goat anti-human IgG, 15 μg, in the presence of BSA were chromatographed on a Pharmacia Superose 12 column separately (dotted and dashed profiles, respectively), and as a mixture after 1 h incubation (solid line profile) as described under Materials and Methods (panel A; see also panel B for the human L-selectin profile with expanded *y* axis). For comparison, the rat L-selectin chimera, 50 μg, was also chromatographed (panel C). The column was calibrated with blue dextran (excluded volume, V_o), rabbit IgG and its dimer (150 and 300 kDa, respectively), and BSA (66 kDa). The proportions of the original human L-selectin chromatographing as a dimer (170 kDa), tetramer (340 kDa), and as multimers in the excluded volume (greater than 1000 kDa) were 48%, 37%, and 15%, respectively, as calculated from peak areas. The respective proportions of the rat L-selectin chromatographing as a dimer (210 kDa), tetramer (420 kDa), and multimers (greater than 1000 kDa) were 40%, 19%, and 41%, respectively. In the L-selectin−antibody mixture, 87% of the L-selectin protein was calculated to be in the multimer (V_o area) by standardizing the "antibody alone" profile to the "antibody plus L-selectin" profile using BSA as an internal standard, and subtracting from the V_o area the amount corresponding to the free antibody (150 and 300 kDa areas).

Gel filtration experiments provided an explanation in terms of valency for the differing binding properties of the preparations of human and rat L-selectin IgG-chimeras. Almost 90% of the preparation of human L-selectin chimera chromatographed as a dimer or tetramer, 170 and 340 kDa, respectively, (Figure 2A,B); only a minor proportion was excluded from the column (greater than 1000 kDa) unless it was intentionally clustered in the presence of the antibody, when almost 90% was calculated to be excluded from the column (see Figure 2A and legend). The two forms of the human L-selectin (original and complexed with antibody) are hereinafter referred to as the paucivalent and multivalent forms, respectively. We now observe that, in contrast to the findings with the human L-selectin chimera, a high proportion of the rat L-selectin chimera (over 40%) is in multivalent form (Figure 2C). Moreover, in separate experiments (not shown) we have observed that the reactivities of this preparation of rat L-selectin with the two sulfo- and two sialyl-Le^a and -Le^x ligands are unaffected in the presence of anti-IgG.

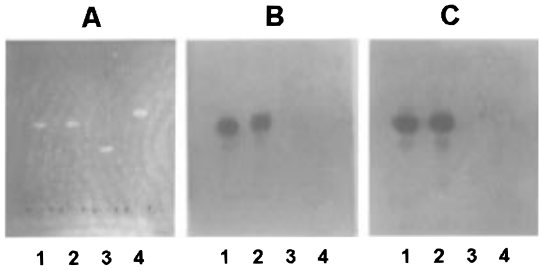

FIGURE 3: Binding of L-selectin to sulfated but not sialyl neoglycolipids presented on chromatograms. 100 pmol of each lipid-linked oligosaccharides, Su-Le^a, Su-Le^x, S-Le^a, and LNT (lanes 1−4, respectively), was chromatographed on silica gel plates and visualized with Primulin stain (A); duplicate plates, (B, C) were overlaid with human L-selectin-IgG chimera (2 μg per mL) which was non-complexed (B) or complexed with anti-IgG (C), and binding was detected with a biotinylated anti-IgG heavy and light chains followed by [^{125}I]streptavidin and autoradiography, as indicated under Materials and Methods. Similar binding patterns were observed (not shown) when 50 or 300 pmol of the lipid-linked oligosaccharides was applied per lane.

The preferential binding of the human L-selectin to the sulfated ligands was more pronounced when the lipid-linked acidic oligosaccharide ligands were presented on chromatograms. In this experimental system, using 50−300 pmol of the lipid-linked ligands, binding was restricted to the two sulfated ligands, irrespective of whether the selectin was in the paucivalent or the multivalent form (Figure 3). An additional difference between the two methods of ligand presentation (on chromatograms and microwells) was that there was no perceptable difference between the strengths of binding to the two sulfated oligosaccharides when these were applied at levels of up to 300 pmol on chromatograms, whereas Su-Le^a was more strongly bound than Su-Le^x on microwells.

Three inhibition experiments were performed with the human L-selectin using Su-Le^a and S-Le^a as representatives of the two classes of ligands. In the first, the L-selectin was in the paucivalent form and the immobilized ligand was Su-

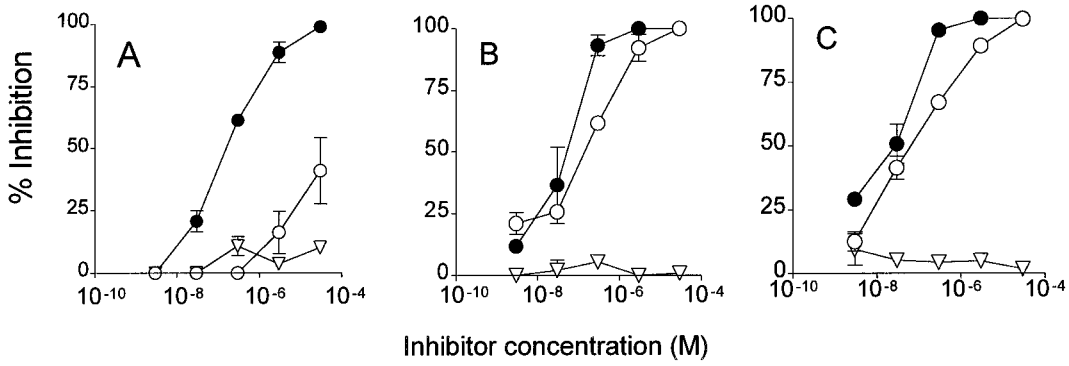

FIGURE 4: Effect of L-selectin valency on the inhibitory activity of S-Le^a toward L-selectin binding. Lipid linked Su-Le^a (A, B) and S-Le^a (C) were coated onto plastic microwells, 100 pmol/well, and inhibition of binding of L-selectin, 10 ng/well, non-complexed (A) or complexed with anti-IgG heavy and light chains (B, C), by the lipid-linked oligosaccharides was assayed as described under Materials and Methods. Under the conditions of the inhibition assay, in the absence of inhibitor, the binding signal (OD at 490 nm) above the background (taken as binding to LNT) was of the order of 0.3−0.8 in the different experiments. Inhibition data are expressed as means in duplicate wells with the range indicated by error bars. Symbols for oligosaccharides are as follows: Su-Le^a, ●; S-Le^a, ○; LNT, ▽.

Table 2: Differing Inhibitabilities of the Binding of the Paucivalent and Multivalent Forms of Human L-Selectin by the Sulfo- and Sialyl-Le^a and -Le^x Pentasaccharide Neoglycolipids

immobilized ligand	inhibitor	IC_{50} (nM)
paucivalent L-selectin		
sulfo-Le^a	sulfo-Le^a	200
	sialyl-Le^a	>30 000
multivalent L-selectin		
sulfo-Le^a	sulfo-Le^a	50
	sialyl-Le^a	200
sialyl-Le^a	sulfo-Le^a	30
	sialyl-Le^a	100

Le^a (Figure 4A); in the second and third experiments, the selectin was in multivalent form and the immobilized ligands were Su-Le^a or S-Le^a, (Figures 4B and C, respectively). In each case the selectin concentration was 0.2 μg per mL, and the immobilized ligand was at 100 pmol per well. There was a striking difference between the inhibitabilities of the binding of the two forms of L-selectin (Figure 3 and Table 2). The binding of the paucivalent form of the selectin could be inhibited effectively only with the sulfated ligand, (IC_{50} value 200 nM), whereas the sialyl ligand showed little inhibitory activity at the highest concentration tested (30 μM). In contrast, the binding of the multivalent selectin could be inhibited both by the sulfated and the sialyl ligands irrespective of whether the immobilized oligosaccharide was Su-Le^a or S-Le^a. The IC_{50} values for the sulfated ligand were 30 and 50 nM when the immobilized oligosaccharides were S-Le^a and Su-Le^a, respectively, and those for the sialyl ligand were three to four times higher, 100 and 200 nM, respectively.

DISCUSSION

Detailed investigations are still awaited of the interactions of the human L-selectin with the wide range of structurally defined oligosaccharides already documented for the rodent systems (Rosen & Bertozzi, 1996; Crocker & Feizi, 1996, and references therein). In the present investigation which is focussed on the interactions of a soluble form of human L-selectin with 3′-sulfated and 3′-sialyl-Le^a and -Le^x oligosaccharides, observations are made that may relate to the interactions of the dynamic membrane-associated form of the protein on the surface of leukocytes, and some principles are established that are applicable to the design of experiments intended for selection of "lead" inhibitors toward the development of effective anti-adhesive, therapeutic substances, as discussed below.

It is clear that there is only a modest difference between the affinities of human L-selectin for the sulfated and the sialyl oligosaccharide ligands, as their IC_{50} values were 30−50 nM for Su-Le^a and 100−200 nM for S-Le^a presented on liposomes. In preliminary experiments, using the free oligosaccharides as inhibitors (not shown), the IC_{50} values were even closer; 160−200 μM for the sulfated ligand and 200−250 μM for the sialyl ligand. This marginal difference in affinities translates, however, into pronounced differences in the avidities of the binding of the paucivalent and multivalent forms of L-selectin as shown by the results of the binding experiments. Thus, when the paucivalent selectin was applied onto the two types of acidic ligand immobilized in microwells, binding signals were elicited with the higher affinity, sulfated ligands but not with the lower affinity, sialyl ligands over the range of ligand concentrations applied (up to 300 pmol per well). Only with the multivalent selectin was binding detectable to the sialyl ligands in addition to the sulfated ligands in this assay system.

An even more pronounced difference in avidities toward the sulfated and sialyl ligands was manifest when the lipid-linked oligosaccharides were presented on chromatograms. Binding was detected to the sulfated but not to the sialyl ligands (50−300 pmol were applied per lane) irrespective of the aggregation status of the L-selectin. Under similar experimental conditions, the rat L-selectin also bound strongly to the sulfated ligands but gave little binding to the sialyl ligands (Green et al., 1995). Although direct comparisons cannot be made with human E-selectin on account of different experimental designs (Larkin et al., 1992; Yuen et al., 1994), there appears to be a marked difference in the relative intensities of binding of the human L- and E-selectins to the sulfated and sialyl ligands. Contrasting with the selective binding of the L-selectin to sulfated Le^a and sulfated Le^x pentasaccharides, Chinese hamster ovary cells expressing E-selectin either at high or at low density, bound to the sialyl-Le^a and -Le^x on chromatograms in addition to sulfated forms (the latter two were tested with the high density selectin only). A further difference between the human E- and L-selectins is that the high-density (but not the low-density) E-selectin cells bound also to the non-sulfated, non-sialylated Le^a and Le^x pentasaccharides (Larkin et al., 1992).

As differences in adhesion to immobilized low-affinity oligosaccharide ligands (Le^a and Le^x pentasaccharides) have been documented in experiments with the Chinese hamster ovary cells that expressed differing levels of membrane-associated E-selectin (Larkin et al 1992), we suggest that the differences observed here in L-selectin binding to the higher and lower affinity ligands (sulfated and sialyl, respectively) are likely to be manifest *in vivo* where the selectin is expressed on the microvilli of leukocyte surface (Picker et al., 1991; Erlandsen et al., 1993). It is known that marked variations occur in the levels of surface expression of the selectin on leukocytes in the process of leukocyte trafficking and infiltration into areas of inflammation: transient increases of L-selectin levels occur under the influence of cytokines such as interferon α and interleukin 2 (Kaldjian & Stoolman, 1995; Palecanda et al., 1992), and diminution of the selectin occurs through shedding in response to stimulation by chemotactic factors and selectin engagement (Griffin et al., 1990; Kishimoto et al., 1989). Thus leukocytes with dense expression of the selectin would be predicted to adhere both to high- and low-affinity oligosaccharide ligands, and the adhesion would tend to be inhibitable by the lower affinity as well as the higher affinity soluble ligands, whereas activated leukocytes with sparse expression of the selectin would adhere preferentially to the higher affinity ligands and would be predicted not to be inhibitable by the lower affinity ligands.

Assay conditions in which the L-selectin is in the form of immune complexes (Brandley et al., 1993; Foxall et al., 1992; Nelson et al., 1993) or is already in a multivalent form, as in the case of the rat L-selectin chimera, would, according to our results, tend to conceal the differences in ligand affinities, and would favor selectin binding to relatively low affinity ligands such as sialyl-Le^a and -Le^x, as well as to the higher affinity ligands such as the sulfated ligands. Indeed, in microwell assays, binding was observed to S-Le^x glycolipid in all of the above investigations (Brandley et al., 1993; Foxall et al., 1992; Nelson et al., 1993). Moreover, compounds with inhibitory activities under conditions of high-selectin density would not necessarily be effective inhibitors of the interactions of leukocytes on which the L-selectin is sparsely expressed. This phenomenon is well illustrated by our observation that the IC_{50} value for S-Le^a is over 300-fold greater with the paucivalent than with the multivalent L-selectin. Thus, an important practical implication of the present investigation is that in order to identify "broad spectrum" inhibitors that would have anti-adhesive properties irrespective of the degree of clustering of L-selectin, experiments should be designed to incorporate the paucivalent L-selectin.

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REFERENCES

Alon, R., Feizi, T., Yuen, C. T., Fuhlbrigge, R. C., & Springer, T. A. (1995) *J. Immunol. 154*, 5356−5366.

Augé, C., Dagron, F., Lemoine, R., Le Narvor, C., & Lubineau, A. (1997) in *Carbohydrate Mimics: Concepts and Methods* (Chapleur, Y., Ed.), Verlag Chemie, Weinheim, Germany (in press).

Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D., & Lasky, L. A. (1993) *Science 262*, 436−438.

Berg, E. L., Magnani, J., Warnock, R. A., Robinson, M. K., & Butcher, E. C. (1992) *Biochem. Biophys. Res. Commun. 184*, 1048−1055.

Berg, E. L., McEvoy, L. M., Berlin, C., Bargatze, R. F., & Butcher, E. C. (1993) *Nature 366*, 695−698.

Brandley, B. K., Kiso, M., Abbas, S., Nikrad, P., Srivasatava, O., Foxall, C., Oda, Y., & Hasegawa, A. (1993) *Glycobiology 3*, 633−639.

Childs, R. A., Wright, J. R., Ross, G. F., Yuen, C.-T., Lawson, A. M., Chai, W., Drickamer, K., & Feizi, T. (1992) *J. Biol. Chem. 267*, 9972−9979.

Crocker, P. R., & Feizi, T. (1996) *Curr. Opin. Struct. Biol. 6*, 679−691.

Erlandsen, S. L., Hasslin, S. R., & Nelson, R. D. (1993) *J. Histochem. Cytochem. 41*, 327−334.

Evans, S. S., Collea, R. P., Appenheimer, M. M., & Gollnick, S. O. (1993) *J. Cell. Biol. 123*, 1889−1898.

Feizi, T. (1993) *Curr. Opin. Struct. Biol. 3*, 701−710.

Feizi, T., Childs, R. A., Watanabe, K., & Hakamori, S. I. (1979) *J. Exp. Med. 149*, 975−980.

Feizi, T., Stoll, M. S., Yuen, C.-T., Chai, W., & Lawson, A. M. (1994) *Methods Enzymol. 230*, 484−519.

Foxall, C., Watson, S. R., Dowbenko, D., Fennie, C., Lasky, L. A., Kiso, M., Hasegawa, A., Asa, D., & Brandley, B. K. (1992) *J. Cell Biol. 117*, 895−902.

Gallatin, W. M., Weissman, I. L., & Butcher, E. C. (1983) *Nature 304*, 30−34.

Green, P. J., Tamatani, T., Wantabe, T., Miyasaka, M., Hasegawa, A., Kiso, M., Yuen, C. T., Stoll, M. S., & Feizi, T. (1992) *Biochem. Biophys. Res. Commun. 188*, 244−251.

Green, P. J., Yuen, C.-T., Childs, R. A., Chai, W., Miyasaka, M., Lemoine, R., Lubineau, A., Smith, B., Ueno, H., Nicolaou, K. C., & Feizi, T. (1995) *Glycobiology 5*, 29−38.

Griffin, J. D., Spertini, O., Ernst, T. J., Belvin, M. P., Levine, H. B., Kanakura, Y., & Tedder, T. F. (1990) *J. Immunol. 145*, 576−584.

Hasegawa, A., Ando, T., Kameyama, A., & Kiso, M. (1992) *J. Carbohydrate Chem. 11*, 645−658.

Hemmerich, S., & Rosen, S. D. (1994) *Biochemistry, 33*, 4830−4835.

Hemmerich, S., Bertozzi, C. R., Leffler, H., & Rosen, S. D. (1994a) *Biochemistry 33*, 4820−4829.

Hemmerich, S., Butcher, E. C., & Rosen, S. D. (1994b) *J. Exp. Med. 180*, 2219−2226.

Hemmerich, S., Leffler, H., & Rosen, S. D. (1995) *J. Biol. Chem. 270*, 12035−12047.

Imai, Y., Lasky, L. A., & Rosen, S. D. (1993) *361*, 555−557.

Kaldjian, E. P., & Stoolman, L. M. (1995) *J. Immunol. 154*, 4351−4362.

Kishimoto, T. K., Jutila, M. A., Berg, E. L., & Butcher, E. C. (1989) *Science 245*, 1238−1241.

Larkin, M., Ahern, T. J., Stoll, M. S., Shaffer, M., Sako, D., O'Brien, J., Yuen, C.-T., Lawson, A. M., Childs, R. A., Barone, K. M., Langer-Safer, P. R., Hasegawa, A., Kiso, M., Larsen, G. R., & Feizi, T. (1992) *J. Biol. Chem. 267*, 13661−13668.

Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R., & Rosen, S. D. (1992) *Cell, 69*, 927−936.

Lawson, A. M., Chai, W., Cashmore, G. C., Stoll, M. S., Hounsell, E. F., & Feizi, T. (1990) *Carbohydr. Res. 200*, 47−57.

Ley, K., Gaehtgens, P., Fennie, C., Singer, M. S. Lasky, L. A., & Rosen, S. D. (1991) *Blood 77*, 2553−2555.

Lubineau, A., Le-Gallic, J., & Lemoine, R. (1994) *Bioorg. Med. Chem. 2*, 1143−1151.

Needham, L. K., & Schnaar, R. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1359−1363.

Nelson, R. M., Cecconi, O., Roberts, W. G., Aruffo, A., Linhardt, R. J., & Bevilacqua, M. P. (1993) *Blood 82*, 3253−3258.
Norgard-Sumnicht, K. E., & Varki, A. (1995) *J. Biol. Chem. 270*, 12012−12024.
Norgard-Sumnicht, K. E., Varki, N. M., & Varki, A. (1993) *Science 261*, 480−483.
Palecanda, A., Walcheck, B., Bishop, D. K., & Jutila, M. A. (1992) *Eur. J. Immunol. 22*, 1279−1286.
Picker, L. J., Warnock, R. A., Burns, A. R., Doerschuk, C. M., Berg, E. L., & Butcher, E. C. (1991) *Cell 66*, 921−933.
Rosen, S. D. (1993) *Histochemistry 100*, 185−191.
Rosen, S. D., & Bertozzi, C. R. (1996) *Curr. Biol. 6*, 261−264.
Sako, D., Comess, K. M., Barone, K. M., Camphausen, R. T., Cumming, D. A., & Shaw, G. D. *Cell 83*, 323−331.
Shyjan, A. M., Bertagnolli, M., Kenney, C. J., & Briskin, M. J. (1996) *J. Immunol. 156*, 2851−2857.
Siegelman, M. H., & Weissman, I. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5562−5566.
Suzuki, Y., Toda, Y., Tamatani, T., Watanbe, T., Suzuki, T., Nakao, T., Murase, K., Kiso, M., Hasegawa, A., Tadano-Aritomi, K., Ishizuka, I., & Miyasaka, M. (1993) *Biochem. Biophys. Res. Commun. 190*, 426−434.
Tamatani, T., Kuida, K., Watanabe, T., Koike, S., & Miyasaka, M. (1993) *J. Immunol. 150*, 1735−45.
Tang, P. W., Gooi, H. C., Hardy, M., Lee, Y. C., & Feizi, T. (1985) *Biochem. Biophys. Res. Commun. 132*, 474−480.
Tedder, T. F., Steeber, D. A., & Pizcueta, P. (1995) *J. Exp. Med. 181*, 2259−2264.
Tsuboi, S., Isogai, Y., Hada, N., King, J. K., Hindsgaul, O., & Fukuda, M. (1996) *J. Biol. Chem. 271*, 27213−27216.
Yuen, C.-T., Lawson, A. M., Chai, W., Larkin, M., Stoll, M. S., Stuart, A. C., Sullivan, F. X., Ahern, T. J., & Feizi, T. (1992) *Biochemistry 31*, 9126−9131.
Yuen, C.-T., Bezouska, K., O'Brien, J., Stoll, M., Lemoine, R., Lubineau, A., Kiso, M., Hasegawa, A., Bockovich, N. J., Nicolaou, K. C., & Feizi, T. (1994) *J. Biol. Chem. 269*, 1595−1598.

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