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HIGH AFFINITY BINDING OF THE LEUCOCYTE ADHESION MOLECULE L-SELECTIN TO 3'-SULPHATED-Le^a AND -Le^x OLIGOSACCHARIDES AND THE PREDOMINANCE OF SULPHATE IN THIS INTERACTION DEMONSTRATED BY BINDING STUDIES WITH A SERIES OF LIPID-LINKED OLIGOSACCHARIDES

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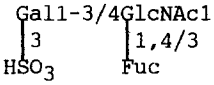
SUMMARY: The binding of the leucocyte adhesion molecule L-selectin has been investigated toward several structurally defined lipid-linked oligosaccharides immobilized on silica gel chromatograms or plastic wells. In both assay systems the 3'-sulphated Le^a/Le^x type tetrasaccharides



were more strongly bound than 3'-sialyl analogues. A considerable binding was observed to the 3'-sulphated oligosaccharide backbone in the absence of fucose but not to a 3'-sialyl analogue or fuco-oligosaccharide analogues lacking sulphate or sialic acid. Affinity for other sulphated saccharides: 3'-sulphoglucuronyl neolactotetraosyl ceramide and glycolipids with sulphate 3'-linked to terminal or sub-terminal galactose or N-acetylgalactosamine was detected in the chromatogram assay only. These studies, together with earlier reports that L-selectin binding to endothelium is inhibited by sulphatide, highlight the relative importance of sulphate in the adhesive specificity of this protein.

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Abbreviations: BSA, bovine serum albumin; HNK-1 glycolipid, 3'-sulphoglucuronyl neolactotetraosyl ceramide; LNFP-II and -III, lacto-N-fucopentaose II and III; LNNT, lacto-N-neotetraose; Lac-Cer, lactosyl-ceramide; Man_5P, pentamannose phosphate; M-6-P, mannose-6-phosphate; PPME, phosphomannosyl ester; 3'S-LNFP-II and -III, 3'-sialyl-lacto-N-fucopentaose II and III; 3'S-LNFT-III, 3'-sialyl-lacto-N-fucotetraose III; 3'S-LNT, 3'-sialyl-lacto-N-tetraose; 3'Su-tetra and 3'Su-tri, 3'-sulphated Lewis^a/Lewis^x-type tetrasaccharides, and the defucosylated analogues; TBS, 10 mM Tris/HCl buffered isotonic saline, pH 7.5; TBS-Ca^{++}, TBS containing 50 mM $CaCl_2$; TBS-EGTA, TBS containing 20 mM EGTA [ethylene-bis (oxyethylenenitrilo) tetraacetic acid]; TLC, thin layer chromatography.

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The adhesion molecule on leucocytes termed L-selectin is known for its key role in the initial steps of lymphocyte extravasation into peripheral lymph nodes (1). This protein which may also be involved in neutrophil influx to inflammatory sites (2) is a member of the Ca^{++}-dependent, 'C-type', lectin family (3-6). Distinct glycoproteins to which L-selectin binds have been identified: a 50 kDa and a 90 kDa glycoprotein containing sulphate, fucose and sialic acid, from murine peripheral and mesenteric lymph nodes (7,8), and a major 90-105 kDa glycoprotein from human tonsils (9). The oligosaccharide ligands for L-selectin on these glycoproteins have not yet been characterized. However, studies using normal mouse lymphocytes or transfected pre-B cells expressing L-selectin (10) have shown that this adhesion protein binds to the 3'-sialyl-Le^a- and 3'-sialyl-Le^x-active hexasaccharides when conjugated to human serum albumin. Studies with a recombinant, soluble form of murine L-selectin (an L-selectin-IgG chimera) have shown that this protein also binds to a chemically synthesized glycolipid containing the sialyl Le^x-active hexasaccharide (11). In the present study, the binding of rat L-selectin-IgG chimera was examined to a series of structurally defined lipid-linked acidic oligosaccharides containing sulphate or sialic acid or phosphate. We report here that the 3'-sulphated analogues of Le^a and Le^x are the most potent L-selectin ligands thus far.

MATERIALS AND METHODS

Rat L-Selectin-IgG Chimera. A soluble fusion protein of rat L-selectin and human IgG1 was produced by the use of the baculovirus system as described elsewhere[1]. In brief, to generate the silkworm transfer vector pBm-rLEC-IgG, a fragment encoding the extracellular domain of rat L-selectin was obtained from rat L-selectin cDNA (12) and inserted into the pBmPVR-IgG plasmid that had been used to produce poliovirus receptor-Ig fusion protein (S. Koike & A. Nomoto; manuscript in preparation). The pBm-rLEC-IgG and the baculovirus DNA (BmNPV T3) were co-transfected into BmN cells (13) to produce recombinant viruses which were then injected into silkworm larvae and haemolymph collected 4 days after the injection (13). The soluble fusion protein was isolated from the haemolymph by Protein A affinity chromatography[1].

Glycosphingolipids (Fig. 1). Lactosylceramide (Lac-Cer) and sulphatide were from Sigma Chemical Co. Poole, UK. The sulphated glycolipids SM3 (ref 14, 15), SM2 and SM1a (ref 15, 16) and SB2 and SB1a (refs 17, 18) were generous gifts of Dr. I. Ishizuka, Teikyo University School of Medicine, Tokyo, Japan. The 3'-sulphoglucuronyl neolactotetraosyl ceramide (HNK-1 glycolipid) was a generous gift from Dr. F.B. Jungalwala, Eunice Kennedy Shriver Centre for Mental Retardation, Waltham, Mass., USA. The 3'-sialyl-lacto-N-fuco-pentaose III (3'S-LNFP-III) and 3'-sialyl-lacto-N-fucotetraose III (3'S-LNFT-III) ceramides were chemically synthesized (19,20).

Neoglycolipids (Fig. 1). Neoglycolipids were prepared (21) by conjugation of the following oligosaccharides to L-1,2-dipalmitoyl-*sn*-glycero-3-phospho-

[1]Tamatani, T., Kuida, K., Watanabe, T., Koike, S., and Miyasaka, M. (1992) Submitted for publication.

ethanolamine: 3'-sialyl-lacto-N-fucopentaose II (3'S-LNFP-II) (Biocarb, AMS Biotechnology Burford, UK); lacto-N-fucopentaose II (LNFP-II), lacto-N-fucopentaose III (LNFP-III), lacto-N-neotetraose (LNNT) and 3'-sialyl-lacto-N-tetraose (3'S-LNT) (ref 22); pentamannose phosphate (Man_5P) obtained by acid hydrolysis from *Hansenula holstii* phosphomannan (ref 23). Neoglycolipids from an equimolar mixture of 3'-sulphated-Le^a and 3'-sulphated-Le^x tetrasaccharides (3'Su-tetra) derived from an ovarian cystadenoma glycoprotein and produced using L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine were those described previously (24). The defucosylated analogues (3'Su-tri) were prepared (24) by acid hydrolysis of the parent neoglycolipids, and further purified by preparative TLC, and shown by liquid secondary ion mass spectrometry to be free of the parent fucosylated compounds.

Chromatogram Binding Assays. Chromatogram overlay assays were performed essentially as described previously (25) with the following modifications. Chromatograms containing neoglycolipids and glycolipids (≈ 1 nmol each) were dipped in 0.1% Plexigum P28 in n-hexane for 60 s, dried, and soaked in 10 mM Tris/HCl buffered isotonic saline, pH 7.5 (TBS) containing 50 mM $CaCl_2$ (TBS-Ca^{++}) and 5% (w/v) bovine haemoglobin (Sigma) at 20°C for 2 h. The chromatograms were rinsed three times in TBS-Ca^{++}, drained and overlaid at 20°C for 2 h with L-selectin-IgG chimera, 30 µg/ml, in TBS-Ca^{++} or TBS containing 20 mM EGTA (TBS-EGTA) and 1% (w/v) haemoglobin. For inhibition assays, fucoidan (Sigma) 10 mg/ml or phosphomannose ester (PPME) derived by acid hydrolysis of *H. holstii* phosphomannan (23), 5 mg/ml, or mannose-6-phosphate (M-6-P) from Sigma, 50 mM, were included in the L-selectin-IgG chimera solution. These solutions were incubated for 1 h prior to overlays. Chromatograms were washed three times in TBS-Ca^{++} or TBS-EGTA, as appropriate. Binding was detected by overlaying the chromatograms with Staphylococcal Protein A, (Sigma) labelled with ^{125}I (26), specific activity 2 µCi/µg; 5 x 10^5 cpm/ml were overlaid at 20°C for 2 h in TBS-Ca^{++} or TBS-EGTA containing 1% (w/v) haemoglobin; chromatograms were washed three times and subjected to autoradiography (96-120 h). In separate experiments cited in ref 24, equivalent E-selectin binding was observed to the 3'S-LNFP-III sequence on ceramide or as a neoglycolipid.

Microwell Binding Assays - Serial dilutions of neoglycolipids and glycolipids were prepared in methanol containing 4 µg/ml cholesterol and 4 µg/ml egg lecithin. Fifty microlitres of each dilution of neoglycolipid or glycolipid were added to Immulon 4 plastic microwells (Dynatech Laboratories Ltd., Billingshurst, UK) in duplicate, and evaporated to dryness at 37°C. As negative controls the carrier lipids only were applied. The wells were washed three times with TBS-Ca^{++} and treated at 20°C for 2 h with 3% (w/v) bovine serum albumin (BSA) from Sigma, in TBS-Ca^{++}, 100 µl/well; the washing step was repeated. Approximately 20% (w/w) of neoglycolipids and sulphatide applied remained in microwells (27 and T.F., A. Herraez, unpublished). L-selectin-IgG, 20 µg/ml, in TBS-Ca^{++} or TBS-EGTA or TBS-Ca^{++} containing 50 mM M-6-P (in each case in the presence of 1% BSA (w/v)) was added to the wells, 50 µl/well, at 20°C for 2 h. The wells were washed three times with either TBS-Ca^{++} or TBS-EGTA, as appropriate, and 50 µl of ^{125}I-labelled Staphylococcal Protein A was added to the wells at 1 x 10^5 cpm/well in TBS-Ca^{++} or TBS-EGTA containing 1% (w/v) BSA and incubated at 20°C for 2 h. The wells were washed in TBS-Ca^{++} or TBS-EGTA, dried and radioactivity counted. Counts bound in the presence of carrier lipids alone (200-600 cpm) were subtracted from counts in the presence of the lipid-linked oligosaccharides.

RESULTS

Among the lipid-linked oligosaccharides tested (Fig. 1), the strongest L-selectin binding was to the 3'-sulphated Le^a/Le^x tetrasaccharides (3'Su-tetra) in both assay systems. The 3'-sialyl-Le^a- and 3'-sialyl-Le^x-

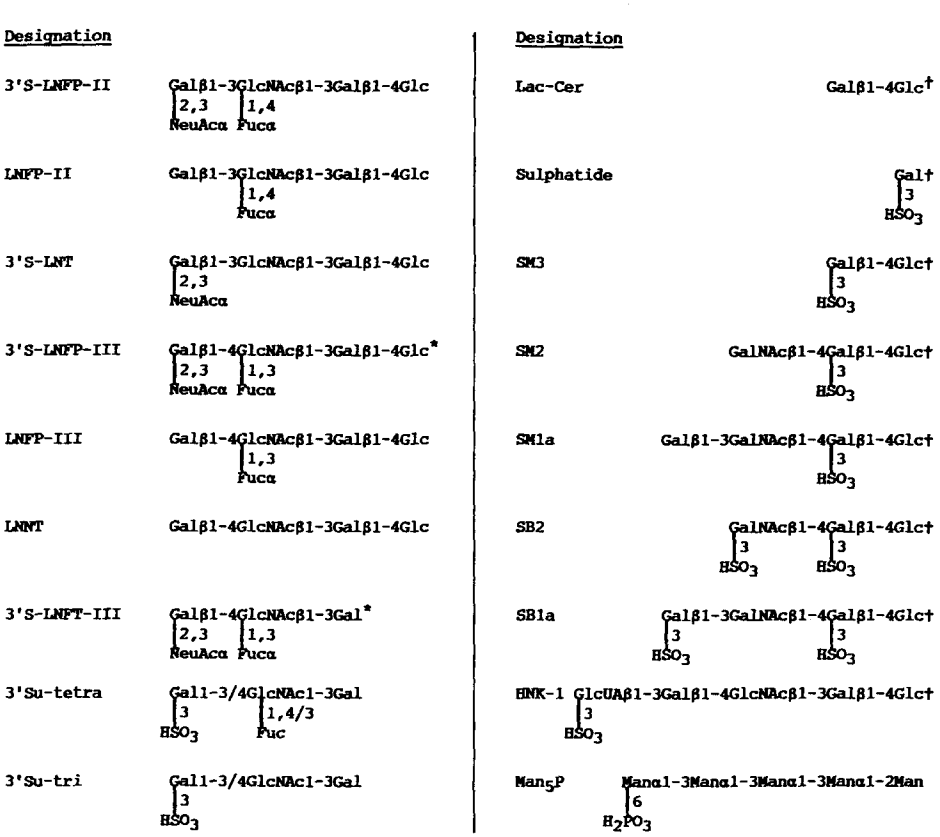
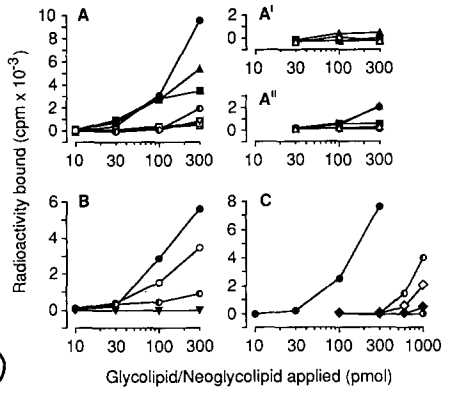


Fig. 1 Lipid-linked oligosaccharides examined for L-selectin binding. Natural and chemically synthesized glycosyl-ceramides are indicated by the symbols † and *, respectively; the remaining oligosaccharides were tested as neoglycolipids.

active oligosaccharides (3'S-LNFP-II and -III) were also bound but with lower intensities than with 3'Su-tetra (Fig. 2A and 3A'). The superior binding to the sulphated fuco-oligosaccharides was particularly evident when the intensities of L-selectin binding to 3'S-LNFT-III and 3'Su-tetra (with trisaccharide backbones) were compared (Fig. 2A). These results, moreover, point to the importance of backbone length as a determinant of the intensity of L-selectin binding to lipid-linked oligosaccharides.

Binding of L-selectin to 3'Su-tri was less than to the fucosylated parent tetrasaccharide (Fig. 2B and 3B'), but was clearly greater than to the 3'-sialyl oligosaccharide 3'S-LNT (Fig. 2A and 2B), again indicating that affinity for the sulphated backbone is greater than for the sialylated equivalent. The affinity of L-selectin for sulphate was further shown by results of binding experiments with sulphatide and the several other sulphated glycolipids. Sulphatide (formerly shown to inhibit binding of L-selectin (6,28)) was bound, albeit weakly, in both assay systems. In the chromatogram assay, binding was observed also to SM3, SM2, SB2, SB1a and to



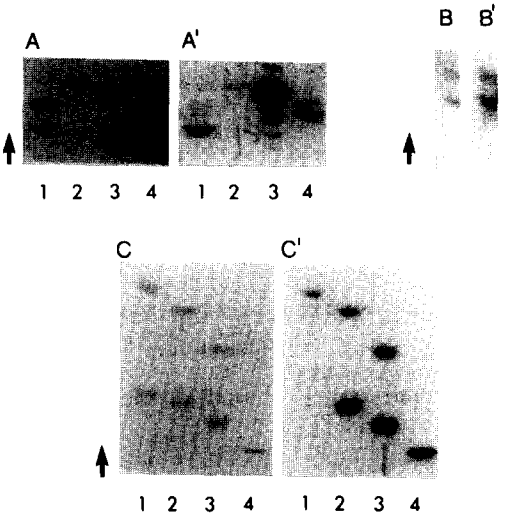


Fig. 2 Binding of L-selectin-IgG chimera to lipid-linked oligosaccharides in microwell assays. Lipid-linked oligosaccharides applied to the microwells at different levels were overlaid with L-selectin-IgG chimera (20 μg/ml); binding was detected with [^{125}I]-Staphylococcal Protein A. Means of duplicate points are shown. Panels A, B and C are separate experiments. Panels A, A' and A" are a single experiment where A' and A" are results in the presence of 20 mM EGTA and 50 mM M-6-P, respectively. Symbols are: ● , 3'Su-tetra; ○ , 3'Su-tri; ▲ , 3'S-LNFP-II; △ , LNFP-II; ■ , 3'S-LNFP-III; □ , 3'S-LNFT-III; ◐ , sulphatide; ▽ , 3'S-LNT; ▼ , LNNT; ◇ , SB2; ◆ , SB1a. In panel C, ◑ , applies to Lac-cer, SM3, SM2, and SM1a which gave no detectable binding in this experiment. In separate experiments binding was not observed to LNFP-III, HNK-1 and Man_5P.

Fig. 3 Binding of L-selectin-IgG chimera to sialylated or sulphated lipid-linked oligosaccharides in chromatogram overlay assays. Lipid-linked oligosaccharides were chromatographed on silica gel plates (solvent system-chloroform/methanol/water, 60/35/8, by volume) and overlaid with L-selectin-IgG chimera (30 μg/ml). Binding was detected by autoradiography, panels A', B', and C' after incubation with [^{125}I]-Staphylococcal Protein A; thereafter, the chromatograms were stained with orcinol, panels A, B and C. In A and A' lane 1 contained LNFP-II (upper band) and 3'S-LNFP-II (lower band); lane 2, LNNT; lane 3, 3'Su-tetra (upper band), Man_5P and trace of 3'S-LNFP-II (lower-most and neighbouring bands); lane 4, LNFP-III (upper band) and 3'S-LNFP-III (lower band). In B and B' the lane contained 3'Su-tri (upper band) and 3'Su-tetra (lower band). In C and C' lane 1 contained sulphatide (upper band) and SM1a (lower band); lane 2, SM3 (upper band) and SB2 (lower band); lane 3, SM2 (upper band) and SB1a (lower band); lane 4, HNK-1 glycolipid.

HNK-1 glycolipid but not to SM1a (Fig. 3C'); thus sulphate on terminal or sub-terminal galactose or N-acetylgalactosamine or glucuronic acid is bound by L-selectin in this assay condition. Of the latter six sulphated glycolipids only SB2 and SB1a showed any detectable binding in the microwell assay (Fig. 2C). Binding was not detected to the Man_5P in either assay system.

The reactivities observed in the microwell assay were inhibited or markedly diminished in the presence of 20 mM EGTA or 50 mM M-6-P (Fig. 2A' and A"). In the chromatogram assay these inhibitors, and PPME (5 mg/ml) and fucoidan (10 mg/ml) gave a diminished intensity of L-selectin binding (not shown).

DISCUSSION

These results establish the 3'-sulphated Le^a/Le^x tetrasaccharides as ligands for L-selectin that are superior to the 3'-sialyl analogues. Although the presence of the fucose residue resulted in enhanced binding of this adhesion molecule to the 3'-sulphated oligosaccharide backbone, the fucosylated backbone in the absence of sulphate or sialic acid did not support detectable binding. Binding to lipid-linked 3'Su-tetra, 3'S-LNFP-II and 3'S-LNFP-III are features shared with the human endothelial adhesion molecule, E-selectin (22,24). However, there are distinct features in the relative importance of the sulphate and fucose residues in the binding of these two adhesion molecules. Only L-selectin can bind to the non-fucosylated oligosaccharides 3'Su-tri (Fig. 2B) and only E-selectin can bind to 3/4-fucosyl oligosaccharides LNFP-II and LNFP-III (22) that lack sialic acid or sulphate. These differences may underlie previous observations (10) that L-selectin binds with a greater intensity than E-selectin to the peripheral lymph node vascular glycoprotein PNAd, and E-selectin but not L-selectin binds to the cutaneous lymphocyte antigen CLA.

L-selectin binding to HNK-1 glycolipid and to the glycolipids SM3, SM2, SB2 and SB1a was detected only in the chromatogram assay, indicating that presentation of L-selectin recognition elements on these lipid-linked oligosaccharides differs on the two artificial matrices used. There may be a relationship between the binding to these acidic glycolipids observed here and the recently reported L-selectin binding to tissue sections of the central nervous system (29) and the kidney[1]. It will be important to determine whether these sulphated saccharides are accessible for L-selectin-mediated adhesion of infiltrating leucocytes in the intact tissues or whether they become accessible upon tissue damage, and to establish whether L-selectin-mediated leucocyte binding is a mechanism for initiating or perpetuating the pathology of demyelinating diseases, such as the encephalitides and multiple sclerosis, and of the nephritides.

L-selectin did not bind to Man_5P, although this adhesion molecule binds to PPME (30), and this binding is diminished in the presence of this polysaccharide and the free monosaccharide, M-6-P (ref 31 and present study). The mannose residues on PPME may have different glycosidic linkages, for example 1-6 rather than 1-3 and 1-2 found on Man_5P (ref 32). These observations suggest that the oligosaccharide backbone sequence has an

influence on L-selectin binding. In a previous study on amyloid P protein, which binds to the various sulphated glycosphingolipids tested here, binding to lipid-linked Man_5P was observed rather than to PPME (23).

There is much current interest in the therapeutic potential of analogues that inhibit the binding of adhesive proteins involved in the pathology of inflammation. From the observations in the present study and earlier studies (10,22-24) there are good prospects for design of analogues that differentially inhibit the binding of the three adhesive molecules, E- and L-selectin and amyloid P protein.

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