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An investigation of the interactions of E-selectin with fuco-oligosaccharides of the blood group family

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This investigation is concerned with assignments of Lewis[a] (Le[a]) and Le[x] analogs on linear and branched di- to hexasaccharide backbones as components of the recognition motifs for E-selectin. The influence of the location of fucose residue(s) was investigated using 14 structurally defined and variously fucosylated oligosaccharides in biotinylated form or as neoglycolipids in static binding assays, in microwells, and on thin-layer chromatograms. Results of the two assay systems were in agreement overall and showed that the recognition motifs for E-selectin include 4-fucosyl-lacto (Le[a]) and 3-fucosyl-*neo*-lacto (Le[x]) sequences strictly at capping positions and not Le[x] at an internal position as a part of VIM-2 antigen sequence. There is greater potency of the Le[a] over the Le[x] series. Additional fucose residues α1-2-linked to neighboring galactoses or α1-3-linked to inner *N*-acetylglucosamines or to reducing-terminal glucose residues of the tetrasaccharide backbone had little or no effect on the selectin binding. E-selectin binding to the Le[a] or Le[x] capping motif on a 3-linked branch was equivalent to the binding on the corresponding linear backbone. A lack of E-selectin binding to the Le[x] motif capping a 6-linked branch and to the Le[x] trisaccharide linked to biotin via a nine-carbon spacer indicates that the -GlcNAcβ1-3Gal- sequence on the oligosaccharide backbone adjoining the Le[x] is a part of recognition motif for E-selectin. These findings contribute to understanding the molecular basis of E-selectin recognition and could influence future designs of selectin antagonists as possible therapeutic substances.

Key words: biotinylated oligosaccharides/E-selectin ligands/Lewis[a]/Lewis[x]/neoglycolipids

Introduction

E-selectin is a cell adhesion molecule on postcapillary venules whose expression is induced by inflammatory cytokines (Harlan and Liu, 1992; Ley, 2001). E-selectin binds to granulocytes, monocytes, and subsets of lymphocytes and has an important role at the initial stages of the recruitment and emigration of leukocytes from the blood vascular compartment into sites of infection, injury, and inflammation. This adhesion molecule has also been implicated in interactions with tumor cells and their bloodborne metastasis (Nemoto *et al.*, 1998; Ley, 2001; Numahata *et al.*, 2002).

E-selectin, as with the two related cell adhesion molecules, P- and L-selectin, adheres to cells via a lectin module at its membrane-distal end, which recognizes carbohydrate sequences related to the Lewis[x] (Le[x]) and Le[a] blood group antigens, particularly to their 3′-sialyl and 3′-sulfated forms (Brandley *et al.*, 1990; Bevilacqua and Nelson, 1993; Feizi, 1993). There are differences in details of the specificities of the three selectins such that certain variant carbohydrate sequences of this family are preferentially bound by one or other of the three proteins (Rosen and Bertozzi, 1996; Feizi and Galustian, 1999). A notable feature of E-selectin is the ability to bind to the nonacidic Le[a] and Le[x] sequences (Berg *et al.*, 1991; Larkin *et al.*, 1992), albeit less strongly than to the sialyl and sulfated fuco-oligosaccharides analogs. Binding to the isomeric Le[x] sequence has been consistently less than to Le[a]. There have been suggestions, however, that internal Le[x] motifs on poly-*N*-acetyllactosamine sequences, that is, that fucose 1-3-linked to internal rather than subterminal *N*-acetylglucosamine, constitutes a recognition motif for E-selectin such that 3′-sialyl-poly-*N*-acetyllactosamine sequences lacking the outer Le[x] motif but having exclusively inner Le[x] motifs of VIM-2 antigen type (Macher *et al.*, 1988) are strongly bound (Tiemeyer *et al.*, 1991; Stroud *et al.*, 1996a,b). But as discussed elsewhere (Crocker and Feizi, 1996; Feizi, 2001), the presence of minor components with different fucosylation patterns were not always ruled out.

A knowledge base of details of the specificities of carbohydrate-recognizing receptors in the body is important for understanding the molecular bases of their interactions and in considering future designs of antagonists as possible therapeutic substances. Oligosaccharides with defined sequences linked to biotin (Toomre and Varki, 1994; Leteux *et al.*, 1998, 1999) or to lipid (Feizi *et al.*, 1994) are powerful probes for such detailed assignments on account of their ease of immobilization for direct binding experiments. Human milk and urine are invaluable sources of free oligosaccharides of the blood group family that are analogs of those found in epithelial and endothelial cells (Kobata, 2000; Lundblad, 1980). Here we capitalize on the binding of E-selectin to nonacidic Le[a] and Le[x] sequences, and we use biotinylated forms and neoglycolipid (NGL) derivatives of a series of such fuco-oligosaccharides to address questions of E-selectin specificity: (1) recognition of

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the internal Le^x motif, that is, 1-3-linked fucose at an inner *N*-acetylglucosamine or glucose; (2) recognition of the Le^a and Le^x motifs on branched lactosaminyl and *neo*-lactosaminyl backbones; and (3) the influence of 1-2-linked fucose at outer or inner galactoses of Le^a and Le^x oligosaccharides on recognition by E-selectin.

Results

The results of microwell-binding experiments with the biotinylated oligosaccharides and of thin-layer chromatography (TLC) plate-binding experiments with the NGLs are shown in Figures 1 and 2, respectively, and summarized in Table I. They are in overall agreement and corroborate the greater potency of the Le^a series over the Le^x. Thus, in the microwell assays the oligosaccharide structures 2, 3, 4, 5, and 12 based on a linear backbone, Galβ1-3GlcNAcβ1-3Galβ1-4Glc, and containing the Le^a sequence (4′-fucosyl-lacto) at the capping position were the most strongly bound by E-selectin (Figure 1A and C). For reference, binding signals are shown in Figure 1A with the 3′-sialyl and 3′-sulfo Le^a analogs, previously recorded under comparable experimental conditions (Leteux *et al.*, 1999). The enhanced binding conferred by sialylation and sulfation at position 3 of galactose is clearly apparent at 1–3 pmol of ligand loading.

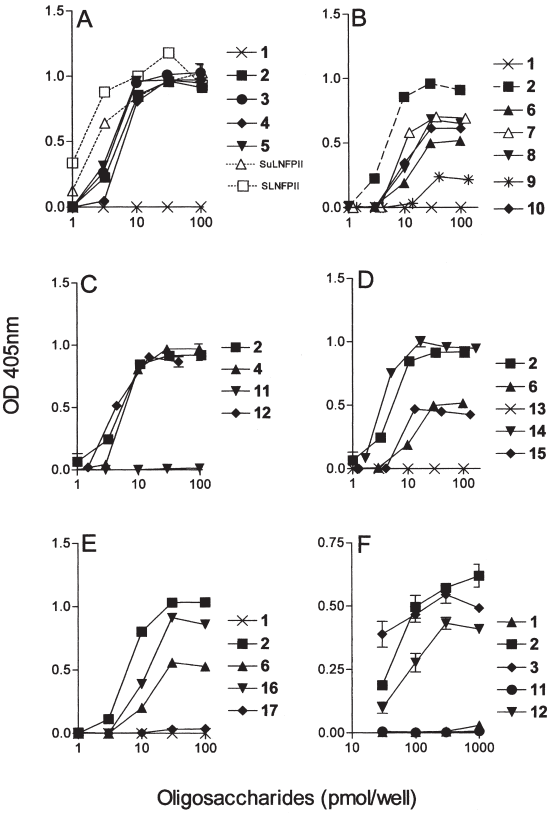

Fig. 1. Microwell-binding experiments with recombinant soluble E-selectin and biotinylated oligosaccharides (**A**–**E**) and neoglycolipids (**F**) immobilized in microwells. The numerical designations of the oligosaccharides are defined in Table I. In panel **A**, the dotted binding curves are for analogs of structure 2, that are sialylated or sulfated at position 3 of galactose, SLNFP II and SuLNFP II, respectively (from Leteux *et al.*, 1999).

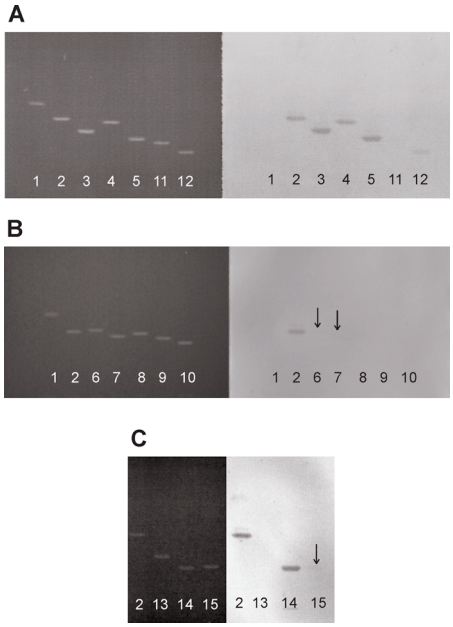

Fig. 2. Binding experiments with recombinant soluble E-selectin and neoglycolipids run on thin-layer chromatographic plates. On the left of each panel, the neoglycolipid bands have been revealed with primulin stain, before overlaying with E-selectin (on the left). Chromatography was upward; arrows indicate weakly stained bands. The numerical designations of the oligosaccharides are defined in Table I.

As in the microwell assays, oligosaccharides 2, 3, 4, and 5 based on a linear backbone and containing the 4′-fucosyl-lacto sequence were strongly bound in the TLC binding assay; oligosaccharide 12 was an exception and was bound less strongly (Figure 2A). These results show that the presence of a 1-2-linked fucose on galactose either on the reducing or the nonreducing side of the Le^a, as in structures 3 and 5, has little or no effect on the E-selectin binding signal. Binding to oligosaccharide structure 6, based on the linear backbone Galβ1-4GlcNAcβ1-3Galβ1-4Glc, and containing the Le^x sequence (3′-fucosyl-*neo*-lacto) at a capping position, was considerably less than to the Le^a series; only in the microwell assays were clear signals observed (Figures 1B and 2B). Here also a 1-2-linked fucose in structures 7 and 10 had little or no effect, but in structure 9 the E-selectin binding was impaired (Figure 1B).

Structure 11, having exclusively an internal Le^x sequence, elicited no detectable binding with E-selectin (Figures 1C and 2A); the binding to structure 12, having both a capping Le^a and an internal Le^x sequence, was not greater than to the Le^a sequence structure 2 (Figures 1C and 2A). Where there was an internal Le^x-like 3′-fucosyl-lactose, as in structures 4 and 8, binding was equivalent to that with their respective Le^a and Le^x analogs, structures 2 and 6 (Figures 1A, 1B, 2A). Collectively, these results, together with those of additional binding experiments with NGLs immobilized on microwells (Figure 3F), show that E-selectin does not recognize the internal Le^x and Le^x-like motifs on these oligosaccharides.

On the branched *N*-acetyllactosamine backbone, there was differential E-selectin recognition of the Le^x sequence on the 6-linked and the 3-linked branches (Figure 1D). The former, as on structure 13, was not bound, and the binding signal with structure 15 was equivalent to that with the linear structure 6. Thus, the Le^x on the 3-linked branch and the

Table I. Oligosaccharide sequences investigated

Designations		Sequences	Assay System: Microwell	Assay System: Chromatogram
1	LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	–*	–
2	LNFP II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc | Fucα1-4	+++	+++
3	LNDFH I	Galβ1-3GlcNAcβ1-3Galβ1-4Glc | | Fucα1-2 Fucα1-4	+++	+++
4	LNDFH II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc | | Fucα1-4 Fucα1-3	+++	+++
5	LNTFH I	Galβ1-3GlcNAcβ1-3Galβ1-4Glc | | | Fucα1-2 Fucα1-4 Fucα1-2	+++	+++
6	LNFP III	Galβ1-4GlcNAcβ1-3Galβ1-4Glc | Fucα1-3	++	±
7	LNnDFH I	Galβ1-4GlcNAcβ1-3Galβ1-4Glc | | Fucα1-2 Fucα1-3	++	±
8	LNnDFH II	Galβ1-4GlcNAcβ1-3Galβ1-4Glc | | Fucα1-3 Fucα1-3	++	–
9	LNnDFH V	Galβ1-4GlcNAcβ1-3Galβ1-4Glc | | Fucα1-3 Fuc1-2	+	–
10	LNnTFH I	Galβ1-4GlcNAcβ1-3Galβ1-4Glc | | | Fucα1-2 Fucα1-3 Fucα1-2	++	–
11	MFpLNH IV (pLNFH IV)	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc | Fucα1-3	–	–
12	DFpLNH II	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc | | Fucα1-4 Fucα1-3	+++	+
13	MFLNH III	Fucα1-3 | Galβ1-4GlcNAcβ1-6 \ Galβ1-4Glc Galβ1-3GlcNAcβ1-3 /	–	–
14	DFLNH (b)	Fucα1-3 | Galβ1-4GlcNAcβ1-6 \ Galβ1-4Glc Galβ1-3GlcNAcβ1- 3/ | Fucα1-4	+++	+++
15	DFLNnH	Fucα1-3 | Galβ1-4GlcNAcβ1-6 \ Galβ1-4Glc Galβ1-4GlcNAcβ1-3 / | Fucα1-3	++	±
16	Le^a(-sp)	Galβ1-3GlcNAc | Fucα1-4	+++	nt
17	Le^x(-sp)	Galβ1-4GlcNAc | Fucα1-3	–	nt

*Binding intensity is scored as strong, equivalent to that with LNFP II (+++), moderate (++), weak (+), trace (±), or negative (–); nt, not tested.

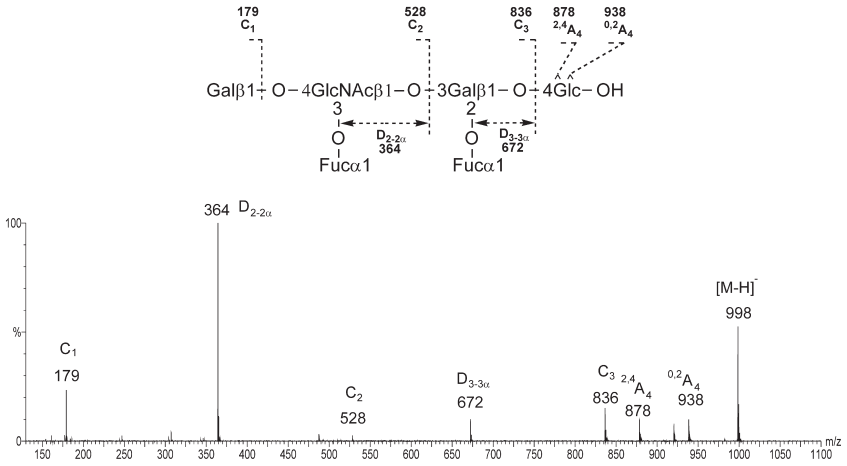

Fig. 3. Negative-ion electrospray CID-MS/MS spectrum of LNnDFH V (oligosaccharide 9).

capping Le^x on the linear tetrasaccharide backbone are equivalent with respect to recognition by E-selectin.

With the branched Le^x/Le^a structure 14, the E-selectin binding signal was similar to that with the linear Le^a sequences (Figures 1D and 2C). Taking into account the lack of recognition of the Le^x sequence on the 6-linked branch, the results indicate that Le^a on the 3-linked branch and on the corresponding linear sequence are equivalent with respect to E-selectin recognition. The Le^a sequence on a 6-linked branch was not available for evaluation.

Further insight into the backbone region recognized by E-selectin was gained from experiments with the Le^a and Le^x trisaccharide-spacer (sp)–biotin derivatives, structures 16 and 17, respectively, that are joined to biotin via a nine-carbon spacer. Although the binding to the Le^a-sp-biotin was almost equal to binding to structure 3, there was a lack of binding to Le^x-sp-biotin (Figure 1E). Thus, the nine-carbon spacer on the sp-biotin contributes sufficiently to the accessibility of the Le^a determinant on the disaccharide backbone, as was shown previously for six or nine ethylene units as spacer groups attached to the sialyl-Le^x tetrasaccharide (Gege *et al.*, 2000). But for the weak ligand Le^x, the nine-carbon spacer is insufficient for eliciting detectable E-selectin binding. Our results further point to the -GlcNAcβ1-3Gal- backbone region adjoining the Le^x motif as being a part of the E-selectin recognition motif.

Discussion

These experiments complement enzymological and physiological approaches by focusing on a series of interrelated and well-characterized oligosaccharides that would be difficult to assemble from cells and tissues. The salient conclusions are, first, that among the sequences investigated, E-selectin recognizes strictly the Le^a and Le^x sequences that are located at capping positions. Second, there is equivalent E-selectin recognition of the Le^a capping motif on the linear backbone and on a 3-linked branch; the same can be said for the capping Le^x motif on a 3-linked branch and on a linear backbone. Third, the Le^x motif capping a 6-linked branch is not recognized. Fourth, the -GlcNAcβ1-3Gal- backbone sequence adjoining the Le^x motif is a part of the recognition motif for E-selectin. Fifth, additional fucose residues 2-linked to neighboring galactoses or 3-linked to inner *N*-acetylglucosamines or to glucose residues at the reducing end of the tetrasaccharide backbone have little or no effect on the selectin binding.

Our results with this set of fuco-oligosaccharides, highlighting the importance of fucose 3′- or 4′-linked to *N*-acetylglucosamine in the capping region, corroborate two earlier TLC binding experiments. These showed a lack E-selectin binding (1) to an NGL analog derived from oligosaccharide structure 11 (Larkin *et al.*, 1992) and (2) to the NGL of a chemically synthesized sialyl-hexasaccharide with fucose linked to the inner *N*-acetylglucosamine, NeuAcα1-3Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAc, which is a part of the VIM-2 antigen sequence (cited by Feizi, 1993). These results are consistent with biosynthetic data: the fucosyltransferase (FucTVII) that creates a capping sialyl Le^x structures has a major role in the generation of selectin ligands (Maly *et al.*, 1996). They are also consistent with the crystal structure of the E-selectin carbohydrate-recognition domain in a complex with 3′-sialyl Le^x "cap," NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc (Somers *et al.*, 2000). In the crystal structure, an intimate network of extended interactions has been observed of the protein with the fucose mediated by Ca^{2+} coordination and with the nearby *N*-acetylneuraminic acid. Although structural data would be required to establish conclusively whether on a sialyl poly-*N*-acetyllactosamine sequence a fucose residue at an inner *N*-acetylglucosamine could adopt a position or orientation analogous to that of the fucose in the capping position, the binding data with the asialo fuco-oligosaccharide indicate that this is unlikely.

Our finding that Le^x on a 3- rather than a 6-linked branch is recognized by E-selectin suggests that the Le^x in the context of the 3-linked branch already adopts in solution a "bound" conformation. In contrast, the Le^x in the context of the 6-linked branch may not adopt such a favorable conformation. This may seem rather surprising at first sight in the light of the large body of evidence that at the branched core of *O*-glycans, the enzyme that forms a -GlcNAcβ1-6GalNAc branch (core 2 β1-6-N-acetyl-glucosaminyltransferase I) is important in regulating the biosynthesis of selectin ligands under physiological conditions. The core 2 enzyme activity is essential for generating P-selectin ligands and, although not absolutely essential for the generation of E- and L-selectin ligands, nevertheless contributes

significantly to their ligand functions (Li *et al.*, 1996; Kumar *et al.*, 1996; Snapp *et al.*, 2001; Sperandio *et al.*, 2001; Yeh *et al.*, 2001). It should be noted, however, that E-selectin binding to a short, *N*-acetyllactosamine-based sialyl-Le^x cap at *O*-glycan core 2 branch has not been directly examined, and to our knowledge, there is no evidence that it is bound. However, with L-selectin, there is strong evidence (Yeh *et al.*, 2001) that a lack of the *O*-glycan core 2 branch can be compensated for by overexpression of the unbranched core 1 (Galβ1-3GalNAc). We acknowledge that our binding assays may not necessarily mimic the binding of cell-surface E-selectin to ligands on leukocytes or tumor cells under shear and that the conformations of the oligosaccharides in the neoglycoconjugates investigated here in relation to those on sialyl *O*-glycans are not yet known. Nevertheless, the observations of Yeh *et al.* (2001)), together with our evidence for the involvement in E-selectin recognition of the -GlcNAcβ1-3Gal- sequence on the linear backbone, lead us to propose that the partial contribution of the core 2 enzyme to the generation of physiological E-selectin ligands may be related to biantennary selectin ligand expression as a result of the biosynthesis of linear poly-*N*-acetyllactosamine chains both at core 2 and core 1, rather than the presence of the ligand cap at core 2. Work with sialyl oligosaccharides in this series is required to critically examine this possibility.

Materials and methods

Oligosaccharides

The sequences and abbreviations of the oligosaccharides investigated and their numerical designations are in Table I. Lacto-*N*-tetraose, lacto-*N*-fucopentaose II/III, lacto-*N*-difucohexaose I/II, monofucosyl-para-lacto-*N*-hexaose IV, and monofucosyllacto-*N*-hexaose III were from Dextra Laboratories (Reading, United Kingdom). Lacto-*N*-trifucohexaose I (Messeter *et al.*, 1984) and lacto-*N*-*neo*difucohexaose (LNnDFH I) (Hallgren and Lundblad, 1977a,b) were isolated from pooled urine (collected during the 30th week of pregnancy and 1 week postpartum) of a healthy, 25-year-old woman of blood group BLe^b. The novel oligosaccharide LNnDFH V (see later discussion) was isolated from this source by serial chromatographies: gel filtration, normal-phase high-performance liquid chromatography (HPLC) on a Spherisorb amino column, followed by reverse-phase HPLC on a Spherisorb C18. LNnDFH II (Hallgren and Lundblad, 1977a,b; Donald and Feeney, 1988), lacto-*N*-*neo*trifucohexaose I (Hallgren and Lundblad, 1977b), difucosyl-para-lacto-*N*-hexaose II (Yamashita *et al.*, 1977; Sabharwal *et al.*, 1988), difucosyllacto-*N*-hexaose b (Sabharwal *et al.*, 1988; Kobata and Ginsburg, 1972; Dua *et al.*, 1985), and difucosyllacto-*N*-*neo*-hexaose (b) (Kobata and Ginsburg, 1972; Dua *et al.*, 1985) were isolated from pooled human milk.

Structure determination of LNnDFH V

The structure of oligosaccharide 9 (Table I) designated LNnDFH V was determined by electrospray mass spectrometry (ESMS) using collision-induced dissociation (CID) and product ion scanning and by 500 MHz ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. Monosaccharide composition was deduced as $dHex_2.Hex_3.HexNAc$ from the molecular ion $[M - H]^-$ at *m/z* 998 (Figure 3). The location of the two fucose residues was deduced, and assignment of the capping sequence as being of Le^x type was made by CID-ESMS/MS (Chai *et al.*, 2001, 2002). The molecular ion *m/z* 998 and the C-type ions *m/z* 179, 528, and 836 (Figure 3) are consistent with a backbone sequence of Gal-GlcNAc-Gal-Glc. The mass difference of 349 Da between C_1 and C_2 indicates the presence of one fucose at *N*-acetylglucosamine, and the mass difference of 308 Da between C_2 and C_3 suggests that the second fucose is at the internal galactose. The D-type ion at *m/z* 364 is characteristic of a terminal Le^x determinant (Chai *et al.*, 2001). The 4-linkage of the glucose was deduced by the presence of ^{2,4}A4 and ^{0,2}A4 ions resulting from the saccharide ring fragmentation. The identities and anomeric configurations of the monosaccharide residues were determined by ^{1}H-NMR: Galβ1- (terminal), δ4.530 (H-1); GlcNAcβ1-, δ4.785 (H-1), δ2.061 (CH_3, NAc); Galβ1- (internal), δ4.456 (H-1), δ4.189 (H-4); Glcα/β1-, δ5.213/4.615 (H-1); L-Fucα1-3(GlcNAc), δ5.126 (H-1), δ4.818 (H-5), δ1.163 (H-6) and L-Fucα1-2(Gal, internal), δ5.057 (H-1), δ4.293/4.258 (H-5), δ1.207 (H-6).

Biotinylated oligosaccharides

Oligosaccharides were conjugated to 6-(biotinyl)-aminocaproyl hydrazide (BACH; Sigma, Poole, United Kingdom) as previously described (Leteux *et al.*, 1998). In brief, oligosaccharides (100 nmol) were mixed with BACH (500 nmol) in 25 µl methanol/water/acetic acid, 95:4:1 (by volume); the reaction mixture was incubated at 60°C for 16 h and evaporated to dryness. Purification of the oligosaccharide conjugates was by HPLC as described (Leteux *et al.*, 1998) and their purity and integrity corroborated by ES-MS. The Le^a and Le^x trisaccharides, Galβ1-3(Fucα1-4)GlcNAc and Galβ1-4(Fucα1-3)GlcNAc, respectively, linked to biotin via a nine-carbon spacer, glycosides of 1-hydroxypropyl-3-amino-[BACH], and referred to as sp-biotin derivatives, were purchased from Syntesome (Munich, Germany).

NGLs

NGLs were prepared essentially as described (Feizi *et al.*, 1994). In brief, to the freeze-dried oligosaccharide (e.g., 50 nmol) were added H_2O (3 µl) 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (50 µl, 5 mg/ml $CHCl_3$/MeOH 1:1, by volume) and freshly prepared $NaBH_3CN$ solution (1.5 µl, 10 mg/ml MeOH). The reaction mixture was incubated at 60°C for 24 h. For TLC, NGLs were applied onto aluminium-backed high performance TLC plates (5 µm silica, Merck, Poole, United Kingdom) as 5-mm bands and developed in a $CHCl_3$/MeOH/H_2O (60/35/8, by volume) solvent system. Visualization and quantitation of the NGLs were based on primulin staining and carried out as described (Feizi *et al.*, 1994), and corroboration of their sequences was by *in situ* TLC/liquid secondary-ion mass spectrometry (LSIMS).

MS

For the native and biotinylated oligosaccharides, ESMS and CID tandem MS (Chai *et al.*, 2001, 2002) were carried out on a Q-TOF mass spectrometer (Micromass, Manchester, United Kingdom). Oligosaccharides and biotin derivatives were dissolved in acetonitrile/water 1:1 and 5 µl of sample solution was loop-injected. Solvent (acetonitrile/water 1:1) was delivered by a syringe pump at a flow rate of 5 µl/min. *In situ* TLC/LSIMS of NGLs (Chai *et al.*, 1991) was carried out on a VG Analytical ZAB-2E mass spectrometer equipped with a cesium ion gun operated at 25 keV with an emission current of 0.5 µA. A

mixture of diethanolamine/tetramethylurea/*m*-nitrobenzyl alcohol (2:2:1, by volume) was used as the liquid matrix.

E-selectin binding experiments

A recombinant soluble form of E-selectin was used; fused to CH2, CH3, and CH4 domains of human immunoglobulin M (Fcμ); and expressed in culture supernatants of transfected COS-7 cells (Smith *et al.*, 1996), kindly provided by Dr. J. B. Lowe. Binding assays with biotinylated oligosaccharides were performed as described (Leteux *et al.*, 1999). In brief, the biotinylated oligosaccharides were dissolved in phosphate buffered saline (10 mM phosphate buffer, 2.7 mM potassium chloride, and 137 mM sodium chloride, pH 7.4), and added to high-capacity, streptavidin-coated microtiter wells (Roche Diagnostics, Mannheim, Germany) at the levels shown in Figure 1; reaction volumes were 200 μl. Thereafter, the wells were incubated with 3% (w/v) bovine serum albumin in a blocking step, and the binding of E-selectin Fcμ (1/50 dilution of culture supernatant) was assayed using anti-human IgM followed by protein-A-peroxidase and 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid). Optical density was measured at 405 nm. In previous experiments it has been established that under these conditions, the uptake of the biotinylated oligosaccharides onto the wells is greater than 90% (Leteux *et al.*, 1998).

Binding assays with NGLs on TLC plates were carried essentially as described (Galustian *et al.*, 1997). The NGLs (200 pmol each) were applied on high-performance TLC plates and developed in a solvent system of $CHCl_3$/MeOH/H_2O (60/35/8, by volume). The plates were soaked in Tris buffered saline (TBS) (10 mM Tris–HCl, 150 mM NaCl, pH 8.0, containing 2 mM $CaCl_2$), blocked with 1% (w/v) casein in TBS (blocking buffer), and incubated with E-selectin Fcμ culture supernatant (1/20 of culture supernatant) in blocking buffer. Selectin binding was detected by incubation with biotin-labeled rabbit anti-human IgM (Dako, Denmark) at 1:500 dilution in blocking buffer followed by streptavidin-peroxidase and FAST™-3,3′-diaminobenzidine (Sigma). Binding assays with NGLs immobilized in plastic microwells were carried out as described (Galustian *et al.*, 2002).

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Abbreviations

BACH, 6-(biotinyl)-aminocaproyl hydrazide; CID, collision-induced dissociation; ESMS, electrospray mass spectrometry; Le, Lewis; LNnDFH, lacto-*N*-*neo*difucohexaose; LSIMS, liquid secondary-ion mass spectrometry; MFpLNH, monofucosyl-para-lacto-*N*-hexaose; NGL, neoglycolipid; NMR, nuclear magnetic resonance; sp-biotin, spacer-biotin; TBS, Tris buffered saline; TLC, thin-layer chromatography.

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