Synthesis of a tetravalent sialyl Lewis x glycan, a high-affinity inhibitor of L-selectin-mediated lymphocyte binding to endothelium

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Kidney transplant rejection is an inflammatory process characterized by lymphocyte infiltration. Our earlier observations have shown that peritubular capillary endothelium (PTCE) is the site of lymphocyte entry into the rejecting renal allograft. During rejection, PTCE begins to express sialyl Lewis x de novo, and binds lymphocytes by a mechanism largely dependent on L-selectin. Hence, inhibiting the lymphocyte–endothelial interaction with oligosaccharide ligands of L-selectin offers an attractive possibility to prevent the inflammation and rejection. Here, we report enzyme-assisted synthesis of N-acetyllactosamine-based tetra-, deca-, and docosameric saccharides carrying one, two or four distally located sialyl Lewis x groups [NeuNAca2-3Galβ1-4(Fucα1-3)GlcNAc] (sLex), respectively. When tested for their ability to inhibit lymphocyte–endothelial interaction during rat kidney transplant rejection, all sLex-saccharides were inhibitors in the Stamper–Woodruff binding assays; the analogues lacking fucose showed no inhibitory potency. The tetravalent sLex glycan proved to be a high-affinity adhesion inhibitor with an IC50 < 50 nM. While less powerful than the tetravalent glycan, also the divalent sLex saccharide was a much better inhibitor than the monovalent glycan. Hence, increasing multivalency and, possibly, increasing chain length of the polylactosamine backbone, enhances the inhibitory potency of sLex bearing glycans in the lymphocyte-endothelial adhesion assay. This suggests that L-selectin behaves as a “functional oligomer” on lymphocyte surfaces.

Key words: synthesis/oligosaccharide/selectin/adhesion/inhibition

Introduction

The current concept of leukocyte extravasation is based on the consecutive action of several adhesion molecules located on the surface of leukocytes and endothelium. Weak but rapidly established binding between selectins and certain carbohydrate epitopes on mucin-like proteins tethers the leukocytes to the endothelial surface, inducing them to roll along the blood vessel walls. Only the rolling cells can bind to Ig-superfamily molecules such as ICAM-1 via an activated ligand (LFA-1, CD11a/CD18), inducing firm adhesion to the endothelial cells. The tightly bound cells then migrate through the endothelial junctions and extravasate from the circulation (Carlos and Harlan, 1994; Rosen and Bertozzi, 1994; Springer, 1994; Varki, 1994).

Three different selectins together with their oligosaccharide-containing ligands are involved in the initiation of the leukocyte extravasation. Two members of the selectin family, E- and P-selectins, are expressed on stimulated endothelium, whereas the third member, L-selectin is expressed on lymphocytes. All selectins bind to sialyl Lewis x (NeuNAca2-3Galβ1-4(Fucα1-3)GlcNAc) (sLex) and related carbohydrate sequences (Feizi, 1991; Rosen and Bertozzi, 1994; Varki, 1994).

L-Selectin is involved in directing lymphocyte traffic to peripheral lymph nodes (Gallatin et al., 1983). We and others have recently demonstrated a selective expression of sLex saccharides on high endothelium in peripheral lymph nodes (Munro et al., 1992; Paavonen and Renkonen, 1992; Sawada et al., 1993). The natural ligands for L-selectin have not been fully characterized, but a mucin-like glycoprotein GlyCAM-1, carrying 6'-sulfated sLex and oligosaccharides containing 6-sulfated GlcNAc (Hemmerich and Rosen, 1994), and certain glycoforms of CD34 and MAdCAM-1 belong to them (Lasky et al., 1992; Baumhueter et al., 1993; Berlin et al., 1993).

Since lymphocyte infiltration is essential for acute organ transplant rejection, we analyze the regulation of lymphocyte traffic, and thereby try to control acute organ rejection. Since selectins have been shown to be important in the regulation of lymphocyte adhesion to endothelium, we analyzed the expression and function of selectins in the course of acute organ transplant rejection.

Bearing this in mind we synthesized here some unique sLex-containing glycans as well as their non-fucosylated analogues enzymatically and tested them as inhibitors of lymphocyte adhesion to PTCE during transplant rejection. All members of the sLex family of oligosaccharides inhibited lymphocyte adhesion to PTCE, which is for the major part a L-selectin-dependent process (Turunen et al., 1994). The tetravalent sLex glycan containing 22 monosaccharide residues was clearly a more potent inhibitor than the divalent sLex glycan when compared on a “per sLex” basis; the divalent glycan in turn was more potent than the saccharide with only one sLex determinant. On the other hand, all oligosaccharides devoid of fucose, i.e., sialyl lactosamines, were totally unable to inhibit the binding. These data support the notion that increasing multivalency of sLex bearing glycans, and possibly also increasing chain length of the polylactosamine backbone, enhances the inhibitory po-
Table I. Structures of synthetic intermediates and products of the present study

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NeuNACα2-3Galβ1-4GlcNAcβ1</td>
</tr>
<tr>
<td>2 (R1, R2 = H)</td>
<td>R1-3Galβ1-4GlcNAcβ1 R2-3GlcNAcβ1</td>
</tr>
<tr>
<td>3 (R1 = NeuNACα2, R2 = H)</td>
<td>R1-3Galβ1-4GlcNAcβ1 R2-3GlcNAcβ1</td>
</tr>
<tr>
<td>4 (R1 = NeuNACα2, R2 = Fucα1)</td>
<td>R1-3Galβ1-4GlcNAcβ1 R2-3GlcNAcβ1</td>
</tr>
<tr>
<td>5 (R1, R2 = H)</td>
<td>R1-3Galβ1-4GlcNAcβ1 R2-3GlcNAcβ1</td>
</tr>
<tr>
<td>6 (R1 = NeuNACα2, R2 = H)</td>
<td>R1-3Galβ1-4GlcNAcβ1 R2-3GlcNAcβ1</td>
</tr>
<tr>
<td>7 (R1 = NeuNACα2, R2 = Fucα1)</td>
<td>R1-3Galβ1-4GlcNAcβ1 R2-3GlcNAcβ1</td>
</tr>
</tbody>
</table>

LacNAc; Galβ1-4GlcNAc. 6' and 3' refer to 6 and 3 positions of the Gal ring of LacNAc.

Results

Synthesis of sLex glycans

The structures and numbering of key glycans are presented in Table I. Glycan 1 was prepared essentially as described (de Vries et al., 1993). The purified product gave a NMR-spectrum identical to those described previously (data not shown) (de Vries et al., 1993). Glycan 4 was synthesized from glycan 2 (Renkonen et al., 1990) and characterized in the same way as glycan 7 from glycan 5 (see below). 1H-NMR data on 2, 3, and 4 are shown in Table II.

Synthesis of glycan 6

Seventy-five nanomoles of the tetra-antennary glycan 5 was incubated with CMP-NeuNAc and α2,3 sialyltransferase. MonoQ chromatography (Figure 1A) gave a minor product eluting like a trisialo-oligosaccharide marker, while the major product (59 nmol) eluted more slowly. The 1H-NMR-spectrum of the major product confirmed its identity as compound 6 (Table III). In comparison to a spectrum of the acceptor 5, the spectrum of 6 revealed the transfer of four equivalents of NeuNAc in α2,3 linkage.
The appearance of NeuNAc H-3ax signal at 1.803 ppm and H-3eq signal at 2.756 ppm, four equivalents both, shows that the newly added NeuNAc residues indeed were in α2,3-linkage. This notion is supported by the appearance of a four peak pattern at 4.119 ppm which can be assigned to H-3s of the penultimate galactoses. Characteristically, the H-1 signals of the penultimate galactoses were also shifted downfield +0.080 ppm, pinpointing the acceptor sites in the molecule (Machytka et al., 1994).

Synthesis of glycan 7
Glycan 6 was α1,3 fucosylated. HPAE-chromatography of the products (Figure 1B) gave two major peaks eluting earlier than starting material. Peak T1 proved to be the tetrasialo-tetrafuco-glycan 7: in desialylated form it resisted completely a treatment with β-galactosidase (Kobata, 1979) and gave a single peak of m/z = 3183 (calc. 3183 Da) in matrix assisted laser desorption ionization mass spectrometry. About 400 nmol of pure 7 have been synthesized.

1H-NMR-spectrum of T1 saccharide (Figure 2, Table III) confirms that the four α-linked fucoses were present. This can be seen as four Fuc H-1 signals at 5.118 ppm (two equivalents), 5.084 ppm and 5.076 ppm (one equivalent each), respectively, and as a Fuc H-6 signal at 1.167 ppm (12 equivalents). Among the H-1 signals of the galactoses of 6, only those of the penultimate, sialylated residues were shifted upon fucosylation, confirming that fucosylation and sialylation had occurred solely at the distal Galβ1→4GlcNAc units of 5. This confirms and extends our previous data showing that branch forming Galβ1→4GlcNAc residues of
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Fig. 2. Expansions of 500 MHz ¹H-NMR-spectrum of saccharide 7. Intensity scale in the left expansions is twice the intensity scale in the right expansions. Lines in the figure point to the chemical shift of indicated signal multiplet. Signals marked with an asterisk arise from an impurity present in deuterated water. For exact chemical shift values, see Table III.

polylactosamines are not fucosylated under the conditions used (Niemelä et al., 1995a).

### Table III. ¹H-NMR Chemical shifts of structural reporter group signals of synthetic glycans 5–7

<table>
<thead>
<tr>
<th>Residue</th>
<th>Proton</th>
<th>Glycan</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc</td>
<td>H-1</td>
<td>5.207/4.725</td>
<td>5.210/4.725</td>
<td>5.210/4.727</td>
<td></td>
</tr>
<tr>
<td>Gal</td>
<td>H-1</td>
<td>4.459</td>
<td>4.460</td>
<td>4.456</td>
<td></td>
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<td></td>
<td>H-4</td>
<td>4.147</td>
<td>4.140</td>
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<td></td>
<td>H-19</td>
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<td>4.140</td>
<td>4.138</td>
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<tr>
<td></td>
<td>H-22</td>
<td>4.697</td>
<td>4.689</td>
<td>4.695</td>
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<tr>
<td></td>
<td>H-34</td>
<td>N.D.</td>
<td>4.119</td>
<td>4.089</td>
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<td></td>
<td>H-37</td>
<td>4.464</td>
<td>4.544</td>
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<tr>
<td></td>
<td>H-40</td>
<td>N.D.</td>
<td>4.119</td>
<td>4.089</td>
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<tr>
<td>NeuNAc</td>
<td>H-3eq</td>
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<td>2.756</td>
<td>2.762</td>
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<tr>
<td></td>
<td>H-3ax</td>
<td>1.803</td>
<td>1.798</td>
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<tr>
<td></td>
<td>H-10</td>
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<td>Fuc</td>
<td>H-1</td>
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<td></td>
<td>H-6</td>
<td>1.167</td>
<td>1.167</td>
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<tr>
<td></td>
<td>H-13</td>
<td>5.077</td>
<td>5.077</td>
<td>5.077</td>
<td></td>
</tr>
</tbody>
</table>

Chemical shifts are given in ppm scale by reference to internal acetone signal set to 2.225 ppm. If two values for a resonance are given, they refer to α/β anomers of the molecule in question, respectively.

Assignments may have to be exchanged.

¹¹FucH-1 and ¹³FucH-1 signals could not be assigned individually.
N.D., Not determined.

**Multivalent sLex-oligosaccharides are high-affinity inhibitors of lymphocyte adhesion**

Having generated the complex oligosaccharides, we tested their ability to inhibit L-selectin mediated adhesion of lymphocytes to endothelium of rat kidney transplants undergoing acute rejection. All exogenous α2,3-sialic acid- and α1,3-fucose-containing polylactosamines (i.e., mono-, di-, and tetravalent sLex, structures 1, 4, and 7, respectively) inhibited the lymphocyte binding to PTCE significantly (Figure 3). Lymphocyte adhesion decreased 38% in the presence of 0.5 μM monovalent sLex (1), and the IC₅₀ values for di- and tetravalent sLex (4 and 7) were 1.0 μM and < 0.05 μM, respectively. At the most effective concentration used, the glycans 7 prevented 73% of lymphocyte adhesion. None of the fucose-free structures, i.e., sialyllactosamine, and glycans 3 and 6, inhibited the lymphocyte adhesion, indicating the crucial role of fucose in the binding.

A decrease in the inhibitory efficiency of 7 was observed at the highest concentration used (Figure 3). Too few L-selectin molecules may be available for the 5 μM ligand to bind in a multivalent manner. The decrease of binding efficiency at higher concentrations may also be sign of increasing saccharide–saccharide interactions; homotypic Lex–Lex binding has been observed before (Hakomori, 1991).

**Discussion**

Knowing that the sLex sequence is characteristic to oligosaccharides capable of L-selectin recognition, we synthesized here mono- and oligovalent sLex saccharides as well as their fucose-free analogs (sialyl LN) for L-selectin dependent cell adhesion experiments. The sLex sequence is known to be characteristic to oligosaccharides capable of
L-selectin recognition (Rosen and Bertozzi, 1994) The syntheses involved the use of monomeric N-acetyllactosamine and poly-N-acetyllactosamine backbones (Renkonen et al., 1990; Seppo et al., 1995) that were converted into mono-, di-, and tetravalent sLex glycans 1, 4, and 7 (see Table I for structures) by using enzymatic α2,3-sialylation and α1,3-fucosylation. The structures of the products were established by (i) glycosidase treatment followed by chromatography, (ii) NMR-spectroscopy, and (iii) mass spectrometry. The fucose residues were transferred to the sialylated rather than to the proximal and “inner” Galβ1-4GlcNAc residues of glycans 3 and 6 by the human milk enzyme(s) as expected (Niemelä et al., 1995a). To our knowledge, glycan 7, consisting of 22 monosaccharides in a precisely defined array, represents the largest heterosaccharide constructed so far de novo.

All members of the sLex family inhibited lymphocyte adhesion to rejecting kidney transplant PTCE in vitro, while the fucose-free sialyl LN constructs did not. The tetravalent glycan 7 proved to be a high-affinity inhibitor (IC50 < 50 nM). We have previously shown that this assay measures mainly L-selectin dependent adhesion (Turunen et al., 1994).

The particularly high affinity of glycan 7 for L-selectin is probably generated by the multiplicity of sLex epitopes, implying that one molecule of 7 may bind to several carbohydrate recognition domains (CRDs) of L-selectin on lymphocyte surface. The crosslinking of L-selectin CRDs on the cell surface may take place regardless of the monomer-oligomer status of the protein itself. Even monomeric receptors bound to other surface constituents, or cytoskeleton (Kansas et al., 1993), may become crosslinked on cell surface like individual hemagglutinin trimers on intact influenza virus by bivalent sialosides (Glick et al., 1991). Likewise, CD22 on CHO cells is a structurally and functionally multimeric lectin, possessing higher affinity for multiply sialylated compounds over monosialylated ligands (Powell et al., 1995). High affinity binding of multivalent saccharides to various cell surface lectins has been documented also in a number of other reports, including (Lee et al., 1984, 1989; DeFrees et al., 1993, 1995; Litscher et al., 1995; Niemelä et al., 1995b).

Of potential importance for the high affinity is also the length of the saccharide chains linking the sLex determinants in glycan 7. These chains consist of as many as five monosaccharide units; in addition, they contain at least one GlcNAcβ1-6Gal bond, lending them extra length and flexibility. It is conceivable that such spacers between the binding epitopes enhance the possibility of multistrate binding of 7. This notion is supported by our recent data showing that the bi-antennary glycan Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc of two long arms is a high affinity inhibitor of mouse gamete adhesion, while a similar octasaccharide construct, Galα1-3Galβ1-4GlcNAcβ1-3Galα1-3Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc of short arms is not (Litscher et al., 1995; Niemelä et al., 1995b).

Although no poly-N-acetyllactosamine structures have yet been identified from any L-selectin ligand, their presence in the P-selectin binding protein PSGL-1 has been reported. The ligand carries sLex-terminating O-linked poly-N-acetyllactosamine chains on adjacent serine residues (Norgard et al., 1993; Moore et al., 1994). Like PSGL-1, also GlyCAM-1 is cleaved by the O-sialoglycoprotease of Pasteurella hemolytica, an enzyme that recognizes sialylated O-glycans on adjacent or clustered Ser/Thr residues of the glycoprotein ligand (Norgard et al., 1993). Indeed, biologically relevant selectin recognition is mediated often by clustered O-glycans (Varki, 1994). It is remarkable that clustered O-glycans are likely to be involved also in adhesion of mouse sperm to egg (Kinloch et al., 1995), a process that is inhibited by a synthetic polyglycosaminine sharing the same backbone structure with glycan 7 (Litscher et al., 1995). These data suggest that multiply branched polyglycosaminines may prove themselves valuable inhibitors of several different saccharide-dependent recognition processes. It is possible that multiply branched polyglycosaminines act as natural ligands in some of the processes.

The dose range of the monovalent sLex-glycan used in this study was 1000-fold lower than the one reported for inhibition of the binding between soluble recombinant L-selectin and immobilized sLex glycolipids (Foxall et al., 1991). Obviously, the data of the two assays cannot be directly compared (Varki, 1994). Interestingly, Stamper-Woodruff data obtained in binding experiments performed with lymphocytes and rejecting rat heart endothelium (Turunen et al., 1995) with the saccharides 1, 4, and 7 were quite similar to the present ones. The similarity probably reflects decisive interactions between the saccharides and L-selectin on lymphocyte surface in both sets of experiments.

Data from in vivo injection experiments with rats and cats have also demonstrated the value of low concentrations of monovalent sLex in inhibiting short-term P-selectin mediated inflammation (Mulligan et al., 1993a,b; Buerke et al., 1994). Even long-term (48 h) inflammatory responses can be inhibited by continuous infusion of anti-L-selectin mAb to animals. Interestingly, neither these ani-
mals nor L-selectin knockout mice generated alterations in the differential count of peripheral blood leukocytes (Arbones et al., 1994; Pizcueta and Luscinskas, 1994). Our aim is to perform analogous in vivo experiments involving relatively long term infusion of rats with oligovalent sLex saccharides in order to see whether they are able to prevent the allograft transplant rejection.

Materials and methods

Acceptor saccharides

The structures and numbering of the key oligosaccharides used are presented in Table 1. N-acetyllactosamine (Galβ1-4GlcNAc) was purchased from Sigma. The glycans 2 and 5 were synthesized by enzyme-aided in vitro synthesis as described (Renkonen et al., 1995; Seppo et al., 1995).

Glycosyltransferases

Human placental microsomes, containing the α2,3-sialyltransferase activity (van den Eijnden and Schipper, 1981), were prepared as follows: 100 g of fresh human placenta was homogenized in 500 ml of cold 0.1 M Tris-maleate, pH 6.7 (buffer A) at + 4°C. The homogenate was centrifuged at 3400 g at + 4°C for 30 min and the supernatant was further centrifuged at 200,000 g + 4°C for 60 min. The microsome pellet was suspended in 10 ml of buffer A yielding a suspension containing 80 mg/ml protein. α2,3-Sialyltransferase reactions were performed in 100 μl of buffer A containing the oligosaccharide acceptor (100–200 nmol of acceptor sites), 10-fold molar excess CMP-NeuNAc and 2 mg of human placental sialyltransferase inhibitor at 37°C. The reactions were terminated after 12–18 h incubation at 37°C by addition of 100 μl of water and heating in a boiling water bath for 2 min. The precipitating protein was removed, the combined supernatant and washings were lyophilized, and oligosaccharides were isolated by gel filtration on a Superdex 75 HR column.

Glycosidase digestions

For sialidase reactions, 1–4 nmol of oligosaccharide in 16 μl of total sialic acid content was extracted from human milk by use of SP-Sepharose C-50 as described (Eppenberger-Castori et al., 1989). The α1,3/4-fucosyltransferase reactions were performed as described (Palic et al., 1989).

Spectroscopic methods

NMR-spectroscopy was performed as described previously (Seppo et al., 1995). Matrix assisted laser desorption ionisation mass spectroscopy of the underivatised oligosaccharides was performed as described previously (Seppo et al., 1995).

Rats and kidney transplantation

Indbred WF (RT1') and DA (RT1') rat strains were used for kidney transplantations. DA kidneys transplanted into a WF recipient were allografts, WF grafts to WF served as syngeneic controls and normal non-transplanted kidneys as controls. The total leukocyte number from the kidneys was quantitated and the differential cell counts were obtained as described (Turunen et al., 1994).

Stamper–Woodruff binding assay

Small pieces of the transplanted kidneys removed at day 3 were mounted in Tissue Tek medium (Lab-Tek Productions, Naperville, IL), snap-frozen in liquid nitrogen, and sectioned as described previously (Turunen et al., 1994). The lymphocyte binding assay was performed as described (Stamper and Woodruff, 1976). The lymphocytes were preincubated with the oligosaccharides in the binding buffer for 30 min in +4°C and used in the Stamper–Woodruff binding assay without further washings in the presence of the saccharides.

Acknowledgements

This study was supported by grants from the Academy of Finland, The University of Helsinki, the Kidney Foundation, Helsinki, Jenny and Antti Wiurih Foundation and the Technology Development Center, Finland.

Abbreviations

tLex, sialyl Lewis x; sLN, sialyl-α2,3-N-acetyllactosamine; PTC, peritumoral capillary endothelium; HPAEC, high pH anion exchange chromatography; PAD, pulsed amperometric detection; CRD, carbohydrate recognition domain.

References


