De Novo Expression of Endothelial Sialyl Lewisα and Sialyl Lewisβ during Cardiac Transplant Rejection: Superior Capacity of a Tetravalent Sialyl Lewisα Oligosaccharide in Inhibiting L-Selectin-dependent Lymphocyte Adhesion

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Summary

Acute organ transplant rejection is characterized by a heavy lymphocyte infiltration. We have previously shown that alterations in the graft endothelium lead to increased lymphocyte traffic into the graft. Here, we demonstrate that lymphocytes adhere to the endothelium of rejecting cardiac transplants, but not to the endothelium of syngeneic grafts or normal hearts analyzed with the in vitro Stamper-Woodruff binding assay. Concomitant with the enhanced lymphocyte adhesion, the cardiac endothelium begins to de novo express sialyl Lewisα and sialyl Lewisβ (sLea and sLex) epitopes, which have been shown to be sequences of L-selectin counterreceptors. The endothelium of allografts, but not that of syngeneic grafts or normal controls, also reacted with the L-selectin-immunoglobulin G fusion protein, giving further proof of inducible L-selectin counterreceptors. The lymphocyte adhesion to endothelium could be significantly decreased either by treating the lymphocytes with anti-L-selectin antibody HRL-1, or by treating the tissue sections with sialidase or anti-sLea or anti-sLex monoclonal antibodies. Finally, we synthesized enzymatically several members of the sLex family oligosaccharides and analyzed their ability to block lymphocyte adhesion to cardiac endothelium. The monovalent sLex (a tetramer), divalent sLex (a decamer), and tetravalent sLex (a 22-mer) could all significantly reduce lymphocyte binding, but the inhibition by the tetravalent sLex-construct was clearly superior to other members of the sLex family. The crucial control oligosaccharides, sialyl lactosamines lacking fucose but being otherwise similar to the members of sLex family, had no effect on lymphocyte binding.

Lymphocyte extravasation is initiated by the interaction of members of the selectin family and their oligosaccharide-containing counterreceptors (1–5). L-selectin is solely expressed on leukocytes and recognizes an endothelial counterreceptor that is at least α2,3-sialylated, α1,3/4-fucosylated, and contains sulfate group(s) (6–10). Three mucin-like heavily O-glycosylated proteins, GlyCAM-1, CD34, and MAdCAM-1, which participate in the L-selectin-dependent adhesion, have been cloned (11–14). High endothelial cells in peripheral lymph nodes express sialyl Lewisα and sialyl Lewisβ (sLea and sLex, respectively) epitopes (15–17), which are parts of the L-selectin counterreceptor. The endothelial cells in several other locations are sLea and sLex negative, but inflammatory stimuli can induce previously negative endothelium to express these oligosaccharide structures de novo (18). We have also shown that cultured endothelial cells possess the machinery to generate at least sLex, since they have several functional α2,3 sialyl- and α1,3 fucosyltransferases, enzymes involved in generating sLex from (poly)lactosamines (19).

Heart transplant rejection is characterized by a heavy infiltration of lymphocytes into the graft (20–22). Here, we show that rat cardiac endothelium, which does not express sLea and sLex in normal animals, can be induced to express these oligosaccharide epitopes during transplant rejection episodes. This de novo expression of sialylated Lewis oli-
gosaccharides leads to enhanced lymphocyte adhesion to endothelium in a sLea-, sLex-, and L-selectin–dependent manner. The endothelium of rejected heart grafts, but not that of normal hearts or syngeneic grafts, stains directly with the L-selectin–IgG fusion protein. We have synthesized mono-, di-, and tetravalent sLex oligosaccharides and their corresponding sialyl lactosamine (sLN) constructs lacking fucose. Our results show that the tetravalent sLex (a 22-mer oligosaccharide) is superior in inhibiting the L-selectin–dependent lymphocyte adhesion to heart endothelium compared to di- or monovalent sLex oligosaccharides (a deca- and tetramer, respectively). On the contrary, the sLN oligosaccharides did not have any effect on lymphocyte adhesion. Taken together, these data suggest that the upregulation of the endothelial expression of sLea and sLex is of crucial importance in the generation of L-selectin–dependent lymphocyte inflammation in the rejecting cardiac allograft, and that soluble oligosaccharides can inhibit this process.

Material and Methods

Rats and Transplantations. Inbred WF (RT1^b) and DA (RT1^a) rat strains were carried in our own colony and regularly tested for intrastrain acceptance of cardiac and renal transplants, as well as for the absence of intrastrain mixed lymphocyte culture. 10–12-wk-old rats were used for the transplantations, and a modified microvascular technique was used (22). The DA hearts that were transplanted into WF recipients were allografts, WF grafts to WF and DA grafts to DA served as syngeneic controls, and normal nontransplanted hearts served as controls.

Stamper-Woodruff Binding Assay. Syngeneic (DA to DA and WF to WF) and allogeneic (DA to WF) transplants were removed on day 3 after the transplantations. Small pieces of the removed hearts were mounted in tissue Tek medium (Lab-Tek Productions, Naperville, IL) and snap-frozen in liquid nitrogen. 8-μm-thick frozen sections were prepared within 1 h before the use of the sections in the lymphocyte–endothelium binding assay (23).

Single-cell suspensions of mesenteric lymph node lymphocytes were made by mechanical disaggregation in RPMI 1640 medium (GIBCO BRL, Bethesda, MD) supplemented with Hepes (25 mM) and 0.5% FCS and the cells were passed through a 50-μm pore size mesh. Lymphocyte purity was >99%, and the population consisted of 80–90% CD3-positive T cells, 50–60% CD4-positive T cells, 25–35% CD8-positive T cells, and 10–20% CD19-positive B cells, as analyzed by flow cytometric analyses and immunoperoxidase stainings from cytocentrifuge preparations. 3 x 10^6 cells in 100 μl of the medium were plated on top of the tissue sections using a wax pen circle to avoid escape of the fluid. The sections were rotated horizontally on a shaker at 60 rpm for 30 min at +4°C. After incubation, the medium was gently tapped away by an absorbent paper and the slides were fixed in 1.5% cold glutaraldehyde overnight. The slides were stained with thionine for 30 min. The excess thionine was gently washed away using PBS, and the slides were mounted with PBS-glycerol (1:1) or Aquamount Mountant (BDH Ltd., Poole, UK). The number of lymphocytes bound to various heart structures was determined from these preparations.

mAbs and Fusion Proteins. The characterization of two hamster anti-rat L-selectin mAbs, one blocking (HRL1) and the other one not blocking adhesion (HRL2), has been published (24, 25). The generation of PVR-Ig and L-selectin–IgG fusion proteins has also been documented (24, 25). Anti–MHC class II (I-303, IgG1) antibody was from Sera-Lab Ltd. (Crawley Down, Sussex, UK). Anti-sLex mAb (CSLEX-1, IgM) was obtained from Dr. Paul Terasaki (University of California at Los Angeles, Los Angeles, CA), anti-sLea (CA19-9, IgG1) from CIS Bio International (Gif-Sur-Yvette, France), anti-Lewis^x^ (CD15, IgM) mAb from Immunotech (Marseille, France), and anti-Lewis^x^ (IgM) from Ortho Diagnostic Systems (Raritan, NJ). Anti–E-selectin antibody (7A9, IgG1) was a kind gift from Walter Newman (Otsuka America Pharmaceutical Inc., Rockville, MD), and it was used as a negative control. Broad spectrum Vibrio cholerae sialidase acting on α2,3,6,8–linked sialic acid was purchased from Oxford GlycoSystems Ltd. (Abingdon, UK).

Immunohistochemical Stainings. The immunoperoxidase stainings for frozen sections were performed with routine specimens after acetone fixation for 10 min at 4°C. The stainings with fusion proteins were performed as previously described (24, 25). In short, frozen sections were fixed in 1% paraformaldehyde, pH 7.3, for 20 min at 4°C, followed by incubation in 100% methanol with 0.3% H_2O_2 to eliminate endogenous peroxidase for 20 min at 4°C. The sections were then washed in PBS and were incubated for 60 min at 4°C with 1–10 μg of either PVR-Ig or L-selectin–IgG fusion proteins diluted in PBS containing 5% FCS. Then the sections were washed and incubated with biotinylated goat anti–human IgG (Zymed Laboratories, San Francisco, CA) in PBS containing 5% FCS for 30 min at 21°C. The sections were incubated with 3-amin-9-ethyl carbazole and H_2O_2 for 10 min, washed, counterstained with hematoxylin, and mounted with Aquamount mountant (BDH Ltd.) for light microscopic analyses.

Enzymatic Synthesis of sLex Oligosaccharides. Synthesis of the Lex trisaccharide has been described previously (26). For construction of the monovalent sLex tetrasaccharide, the divalent sLex decasaccharide, and the tetravalent sLex 22-saccharide N-acetyllactosamine, the hexasaccharide Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ1-3Galβ1-4GalNAc (27) and the tetradecasaccharide Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-28) were used, respectively, as primers. The acceptors were first α2,3-sialylated by incubating them exhaustively with CMP-NeuNAc and α2,3-sialyltransferase from human placenta. The isolated, fully sialylated saccharides were then α1,3-fucosylated exhaustively with GDP-Fuc and a partially purified preparation of human milk α1,3-fucosyltransferase(s) (26). The sample sizes were estimated by UV absorption against external N-acetylglucosamine. The characterization of the constructs was carried out by ion exchange chromatography, matrix-assisted laser desorption ionization mass spectrometry, and one-dimensional ^1H nuclear magnetic resonance spectroscopy at 500 MHz.

Results

Endothelium in Rejecting Cardiac Transplants Adheres More Lymphocytes than Normal Endothelium. To analyze the role of lymphocyte extravasation during rejection episodes, we performed MHC-incompatible allogeneic transplantations between inbred rat strains DA to WF. Control syngeneic transplantations were performed within DA or WF strains.
Figure 1. Binding of lymphocytes to endothelial structures in normal and transplanted heart tissue in the Stamper-Woodruff assay. The microphotographs have been taken so that the lymphocytes (round and black) are in focus and the underlying (gray) tissue is slightly out of focus. Bound lymphocytes are marked by small black arrows. (a) Only very few lymphocytes are bound to the endocardium of normal hearts. (b) In the syngeneic grafts and in (c) allografts, the number of bound lymphocytes is also at a very low level. The same observation was made in arterioles; these structures in normal hearts (d) practically did not adhere lymphocytes at all, and the binding in syngeneic grafts (e) and allografts (f) was at a very low level. Venules (dashed line) from normal hearts (g) and syngeneic grafts (h) adhered only a few lymphocytes, but on the contrary, the venules in the allografts (i) adhered an increased number of lymphocytes. Intermuscular capillaries adhered some lymphocytes in the normal tissue (j) and in the syngeneic grafts (k). (l) There was a clear enhancement in the lymphocyte adherence to intermuscular capillaries in the allografts. A large number of cross and longitudinal sections of intermuscular capillaries are seen in this panel, and only a few of the lymphocytes adhering to these structures are marked by arrows. Note that in all panels there are also some lymphocytes adhering directly on top of the myocardium that are not lying on top of any endothelial structures (a and l, white arrows).

(from DA to DA or from WF to WF strains), and normal hearts (from DA and WF) were used as negative controls. The hearts were removed on day 3 when the rejection had just started, frozen sections were cut, and the Stamper-Woodruff lymphocyte adhesion assay was performed.

In sections prepared from syngeneic grafts of normal hearts, endothelium in the tissue sections prepared from allografts bound significantly more lymphocytes than to the endothelium (Table 1 and Fig. 1). When the anatomical location was analyzed in more detail, the endothelium was divided into several categories according to the size of the vascular structure: (a) endocardium, (b) arterioles, (c) venules, and (d) intermuscular capillaries. Lymphocyte adhesion was assayed in these various compartments and, it was found to be increased onto the intermuscular capillaries and venules during rejection (Table 1 and Fig. 1). There was a constant low background binding of lymphocytes to the myocardium in all heart specimens.
The specificity of lymphocyte–endothelial adhesion was demonstrated in several ways: (a) it was practically absent in syngeneic grafts or normal nontreated hearts (Table 1); (b) it was not affected by the origin of adherent lymphocytes, i.e., both DA and WF cells adhered equally well to DA to WF grafts (data not shown); and (c) it was inhibited by treating the tissue sections with sialidase before adding lymphocytes (data not shown).

### Table 1. Number of In Vitro–adherent Lymphocytes per One High-power Microscopic Field on Normal Hearts and on Syngeneic or Allogeneic Heart Grafts on Day 3 after Transplantation

|                     | Normal      | Syngraft   | Allograft  |
|---------------------|-------------|------------|------------|
| Total area          | 47.4 ± 4.3  | 71.1 ± 5.6 | 151.6 ± 16.1 |
| Endocardium         | 0.1 ± 0.1   | 2.1 ± 0.7  | 2.8 ± 1.3  |
| Arterioles          | 0.1 ± 0.1   | 2.1 ± 0.1  | 1.6 ± 0.7  |
| Venules             | 5.2 ± 0.4   | 7.6 ± 0.5  | 12.2 ± 1.6 |
| Intermuscular capillaries | 23.8 ± 3.1 | 42.7 ± 3.8 | 119.4 ± 12.4 |
| Myocardium          | 17.3 ± 0.0  | 16.6 ± 1.1 | 15.6 ± 1.9 |

The mean ± SEM of seven independent experiments is presented.

De Novo Endothelial Reactivity with Anti-sLea and -sLex Antibodies and L-selectin-Ig Fusion Protein on Rejecting Cardiac Transplants. The data presented above indicate that the inflammation associated with acute rejection induced proadhesive properties to the graft endothelium. Since the selectin-mediated rolling has been suggested to be the primary event in the cascade leading to leukocyte extravasation, we focused on molecules putatively taking part in the generation of lymphocyte infiltration in the rejected heart grafts.

**Figure 2.** Photomicrograph of immunoperoxidase stainings with antioligosaccharide mAbs and L-selectin-IgG fusion protein. (a) Staining of normal heart tissue with anti-sLex antibody revealed no reactivity. (b) Anti-sLea antibody staining of syngeneic graft remained also negative. (c) Allograft tissue did not react with anti-sLea antibody after sialidase treatment, whereas in longitudinal sections of venules in the allograft on day 3 after transplantation stained positively (d, brown) with anti-sLea mAb. (e) A high power field of a capillary in the allograft reacting strongly with anti-sLex antibody. L-selectin–IgG fusion protein revealed no endothelial reactivity in normal hearts (f) and, similarly, the negative control fusion protein PVR–Ig (g) did not react with the syngeneic graft. (h) On the contrary, capillaries and venules in the allograft stained strongly with the fusion protein. (i) A high power magnification of an endothelial cell staining strongly with the L-selectin–IgG fusion protein lining a venule in an allografted heart. ×100 in a, b, d, f, and h; ×400 in c, e, g, and i.
L-selectin has at least three heavily glycosylated mucin-like protein counterreceptors, GlyCAM-1, CD34, and MAdCAM-1 (11–14). In vitro assays have also pointed out the crucial role of sialylated, fucosylated, and sulfated oligosaccharides in L-selectin–dependent adhesion (6–10). We therefore analyzed whether the endothelium would express sialyl Lewis epitopes containing α2,3-sialylated and α1,3/4-fucosylated oligosaccharides. As shown in Table 2, the endothelium in normal hearts and syngeneic grafts did not react with anti-sLea and -sLex mAbs (Ca19.9, IgG1 and CSLEX-1, IgM respectively). On the contrary, all endothelial structures in allografts undergoing rejection expressed sLea and sLex epitopes; the venules and intermuscular capillaries being most reactive (Table 2 and Fig. 2). Concomitantly, isotype-matched control mAbs against E-selectin (IgG1), as well as Lex and Lea (IgM), were negative in normal hearts, as well as in syngeneic and allogeneic grafts (Table 2 and data not shown).

Furthermore, stainings performed with L-selectin–IgG fusion protein also demonstrate endothelial reactivity in venules and intermuscular capillaries only in allografts, but not in syngeneic grafts or normal hearts (Table 3 and Fig. 2). In clear contrast, stainings with control PVR–Ig fusion protein were negative in all heart tissues. The reactivity with L-selectin–IgG fusion protein and anti-sLea and -sLex antibodies could be removed from the allografts by preincubating the tissue sections with sialidase (Fig. 2 and data not shown).

Anti-L-Selectin as Well as Anti-sLea and -sLex Antibodies Inhibit Lymphocyte Adhesion to the Endothelium of Rejecting Cardiac Transplants. To provide evidence for the direct role of L-selectin in the lymphocyte adhesion to cardiac endothelium, we performed the Stamper-Woodruff binding assay using lymphocytes incubated with anti-L-selectin mAbs before placing them into the binding assay. The lymphocyte binding to syngeneic grafts was only slightly above the control levels (Table 1), and this binding could not be significantly modified by anti-L-selectin, -sLea, or -sLex antibodies (data not shown).

Table 2. Expression of Lex, sLea, and sLex on Various Anatomical Structures on Normal Hearts and on Syngeneic or Allogeneic Heart Grafts on Day 3 after Transplantation

|                | Lewis<sup>a</sup> | Sialyl Lewis<sup>a</sup> | Sialyl Lewis<sup>a</sup> |
|----------------|-------------------|--------------------------|--------------------------|
|                | Normal Syngraft Allograft | Normal Syngraft Allograft | Normal Syngraft Allograft |
| Endocardium    | – – –               | – – +                    | – – +                    |
| Arterioles     | – – –               | – – +                    | – – +                    |
| Venules        | – – –               | – – + + +                | – – ± + +                |
| Intermuscular capillaries | – – –               | – – +                    | – – +                    |
| Myocardium     | – – –               | – – –                    | – – –                    |

Figure 3. Effect of various mAbs on the lymphocyte adhesion to allograft endothelium. Lymphocyte pretreatment with a function-blocking anti-L-selectin mAb (HRL-1), as well as anti-sLea and anti-sLex pretreatment to allograft tissue reduced the lymphocyte adhesion significantly. The mean ± SEM of one representative experiment out of five is presented.

On the contrary, the anti-L-selectin mAb HRL-1, shown to recognize a functional epitope, decreased the lymphocyte adherence to allograft tissue down to 37% compared to the nontreated control (Fig. 3). Concomitantly, HRL-2, which is a non–function-blocking anti–L-selectin antibody, had no effect. To show that the de novo–induced endothelial oligosaccharide expression on allograft endothelium was involved in the increased lymphocyte adhesion, we used anti-sLea and -sLex mAbs as inhibitors of adhesion. When the tissue sections prepared from the allografts were incubated with anti-sLea or -sLex antibodies, the lymphocyte adhesion to allografts decreased down to 24 and 38%, respectively (Fig. 3). When the two approaches were used simultaneously, i.e., lymphocytes were treated with the function-blocking anti-L-selectin HRL-1 and the tissue sections were treated with anti-sLea, the lymphocyte adhesion was decreased to background levels (Fig. 3). As a negative control, the tissue sections were incubated with an isotype-matched anti-MHC class II antibody, which reacted with the endothelium but had no effect on the lymphocyte binding.
Table 3. Reactivity of Various Anatomical Structures on Normal Hearts and on Syngeneic or Allogeneic Heart Grafts on Day 3 after Transplantation Stained with Either the Control PVR-Ig- or the L-section-Ig Fusion Protein

|                          | PVR-Ig fusion protein | L-selectin-Ig fusion protein |
|--------------------------|-----------------------|-----------------------------|
|                          | Normal | Syngraft | Allograft | Normal | Syngraft | Allograft |
| Endocardium              | –      | –        | –         | –      | –        | –         |
| Arterioles               | –      | –        | –         | –      | –        | –         |
| Venules                  | –      | –        | –         | –      | –        | ++        |
| Intermuscular capillaries| –      | –        | –         | –      | –        | + +       |
| Myocardium               | –      | –        | –         | –      | –        | –         |

Monovalent sLN

\[\text{NeuNAc}_2 \text{Gal}_{\beta3} \text{GlcNAc}\]

Divalent sLN

\[\text{NeuNAc}_2 \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}\]

Tetravalent sLN

\[\text{NeuNAc}_2 \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}\]

Monovalent sLex

\[\text{NeuNAc}_2 \text{Gal}_{\beta3} \text{GlcNAc}\]

Divalent sLex

\[\text{NeuNAc}_2 \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}\]

Tetravalent sLex

\[\text{NeuNAc}_2 \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}, \text{Gal}_{\beta3} \text{GlcNAc}\]

Figure 4. Primary structures of the enzymatically synthesized oligosaccharides of the sLN and sLex families, which were tested for their ability to inhibit lymphocyte–endothelial interaction.

Figure 5. Effect of various enzymatically synthesized oligosaccharide constructs on the lymphocyte adhesion to allograft endothelium. While all the oligosaccharides in the sLex family reduced binding, the inhibitory capacity of tetravalent sLex was significantly superior to other sLex oligosaccharides. All the sLNs lacking fucose were without effect. The mean ± SEM of one representative experiment out of three is presented. •—, tetravalent sLN; ○—, divalent sLN; ◦—, monovalent sLN; ○—, tetravalent sLex; □—, divalent sLex; ▼—, monovalent sLex.

All Enzymatically Synthesized sLex-type Oligosaccharides Inhibit Lymphocyte Adhesion to the Endothelium of Rejecting Cardiac Transplants but Tetravalent sLex is Most Potent. Finally, we tested the possibility of inhibiting the lymphocyte adhesion to the cardiac endothelium during acute rejection episodes by oligosaccharides. We generated the following family of oligosaccharides by enzyme-aided synthesis: monovalent sLex tetrasaccharide, divalent sLex deca saccharide, and tetravalent sLex 22-mer oligosaccharide, as well as their nonfucosylated sialyl lactosamine analogues (Fig. 4). The structures of these glycans were verified by chromatography, one-dimensional nuclear magnetic resonance, and mass spectroscopy. The lymphocytes were preincubated for 30 min with various concentrations of the oligosaccharides and used thereafter in the Stampfer-Woodruff binding assay in the incubation media. These oligosaccharides did not significantly alter the lymphocyte binding to syngeneic grafts, which was only slightly above the binding to normal heart tissue (Table 1 and data not shown). On the other hand, all members of the sLex family were effective in inhibiting lymphocyte adhesion to cardiac endothelium, but the tetravalent sLex was clearly superior compared to the
other sLex oligosaccharides (Fig. 5). Concomitantly, the nonfucosylated sLN glycans remained without effect.

Discussion

We show in this paper that normal cardiac endothelium does not express sLea or sLex oligosaccharides present in L-selectin counterreceptors and concomitantly does not support lymphocyte adhesion. On the contrary, during acute graft rejection, the cardiac endothelium began to express both of these sialylated Lewis epitopes and to support lymphocyte adhesion. Furthermore, the cardiac endothelium stains in a sialidase-sensitive manner with the L-selectin–IgG fusion protein during acute rejection episodes. The lymphocyte adhesion to graft endothelium was inhibited by treating the endothelium either with sialidase or anti-sLea or -sLex mAbs. Adhesion was also inhibited by treating the lymphocytes with anti-L-selectin mAb or sLex-bearing oligosaccharide constructs. All this points to the crucial role of L-selectin–sLea-, and sLex-dependent lymphocyte extravasation into cardiac allografts during acute rejection.

Acute organ allograft rejections are characterized by a heavy infiltration of lymphocytes (20, 21). Since the hallmark of rejection is lymphocytosis in the graft, the essential task in preventing rejection would be to prevent the entry of lymphocytes into the grafts. A number of studies have indicated that the level of expression of endothelial adhesion molecules intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin increase during cardiac rejection (29–32). Likewise, the direct use of antibodies against these adhesion molecules or their counterreceptors has demonstrated that they are involved in the lymphocyte infiltration (33). The current concept in lymphocyte extravasation, however, is that the cells must first roll on the endothelium via a selectin–carbohydrate-mediated interaction before they can adhere with integrins (1–5, 34, 35). Since the majority of lymphocytes do not possess counterreceptors for E- or P-selectins (36, 37), we sought evidence for L-selectin being involved in the primary rolling leading to lymphocyte infiltration into the heart transplants.

Endothelial expression of L-selectin counterreceptors is very restricted (38, 39). We and others have shown that the high endothelium in the peripheral but not in the mucosal lymph nodes express sLex and sLea oligosaccharides (15–17). In peripheral lymph nodes, the high endothelium supports L-selectin–dependent lymphocyte adhesion, expresses specific glycoforms of CD34, and secretes GlyCAM-1 (11, 13, 14). The murine GlyCAM-1 glycoprotein from peripheral lymph nodes is decorated by sulfated sLex oligosaccharides (40).

Our assays do not answer whether the sLea and sLex epitopes on the endothelium are on protein or lipid backbones, but they demonstrate that the expression of these epitopes is strongly upregulated during inflammation. How is then the sLea and sLex expression induced to endothelial cells? We have shown that cultured endothelial cells express sLex and have several functional α2,3 sialyl- and α1,3 fucosyltransferases, which are able to sialylate and fucosylate distal N-acetyl lactosamine sequences, thus generating sLex epitopes (19). These endothelial enzyme activities can be enhanced in vitro by inflammatory cytokines such as TNF (19). Thus, our previous results indicate that endothelial cells have the machinery for generating sialylated Lex epitopes in an inflammation-regulated manner. A complementary mechanism could involve passive absorption of sLea and sLex from blood circulation by endothelial cells (16), but even this process should be inducible by inflammation.

The question of rodent tissues expressing sLex type of oligosaccharides is a controversial one. There is evidence that rodent leukocytes do not express sLex (41). Murine GlyCam-1 secreted by lymph node endothelium, however, does bear sulfated sLex epitopes (40) and we have previously shown that peritubular capillary endothelium on rat kidney allografts starts to de novo express sLex during rejection (18). So, it may well be that sLex-type oligosaccharides are not expressed on rodent leukocytes, or that they are modifications that do not react with the presently available anti-sLex mAbs. The sLex-type structures in the endothelium of lymph nodes or inflamed tissues of these animals, however, do react with the same mAbs.

The induction of endothelial sialyl Lewis epitopes at sites of inflammation and their crucial role in L-selectin–dependent lymphocyte extravasation leads to the tempting possibility of blocking the generation of inflammation with oligosaccharides. Some published reports show that monovalent sLex tetrasaccharide is able to block the P-selectin–dependent accumulation of granulocytes in models involving lung inflammation or cardiac reperfusion (42, 43). In vitro data suggests also that oligosaccharide constructs bearing two sLex groups (divalent sLex structures) are five times better inhibitors of E-selectin–dependent adhesion compared to monovalent sLex (44, 45). Although the generation of very complex oligosaccharides has been difficult, enzyme-aided synthesis makes it possible at this time (28). We have constructed enzymatically a family of sLex oligosaccharides in quantities sufficient for in vitro binding assays. We could show that the tetravalent sLex was clearly superior in blocking the lymphocyte adhesion to cardiac endothelium compared to mono- or divalent sLex. Quite small concentrations (50 nM) were needed for the inhibitory effects, suggesting that the amounts of oligosaccharides required in preventing inflammation even in vivo will not be out of reach.

Taken together, the present data show that endothelial sLea and sLex expression are strongly induced in endothelium at sites of inflammation, such as organ allograft rejection. Furthermore, this expression of sialylated Lewis epitopes is correlated to L-selectin–dependent lymphocyte adhesion to endothelium as a prelude to extravasation. We predict that direct in vivo use of anti-sLea, -sLex, or -L-selectin antibodies, or even better, of competing oligosaccharide constructs, will inhibit lymphocyte entry into allografts and prevent rejection.
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References

1. Butcher, E.C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell. 67: 1033–1036.
2. Lasky, L.A. 1992. Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science (Wash. DC). 258:964–969.
3. Hogg, N. 1992. Roll, roll, roll your leukocyte gently down the vein. Immunol. Today. 13:113–115.
4. Shimizu, Y., and S. Shaw. 1993. Mucins in the mainstream. J. Cell Biol. 117:895–902.
5. Springer, T.A. 1994. Traffic signals for leukocyte recirculation and leukocyte emigration: a multistep paradigm. Cell. 76:301–314.
6. Green, P.J., T. Tamatani, T. Watanabe, M. Miyasaka, A. Hasegawa, M. Kiso, C.-T. Yuen, M.S. Stoll, and T. Feizi. 1992. High affinity binding of the leukocyte adhesion molecule L-selectin to 3'-sulfated-Lea and -Lex oligosaccharides and the predominance of the sulphate in this interaction demonstrated by binding studies with a series of lipid-linked oligosaccharide. Biochim. Biophys. Res. Commun. 188:244–251.
7. Imai, Y., L.A. Lasky, and R.D. Rosen. 1992. Further characterization of the interaction between L-selectin and its endothelial ligand. Glycobiology. 2:373–381.
8. Foxall, C., S. Watson, D. Dowbenko, C. Fennie, L. Lasky, M. Kiso, A. Hasekawa, D. Asa, and B. Brandley. 1992. The three members of the selectin receptor family recognize common carbohydrate epitope, the sialyl-Lewis- oligosaccharide. J. Cell Biol. 117:895–902.
9. Suzuki, Y., Y. Toda, T. Tamatani, T. Watanabe, T. Suzuki, T. Nakao, K. Murase, M. Kiso, A. Hasegawa, K. Tadano-Aritomi, I. Ishizuka, and M. Miyasaka. 1993. Sulfated glycolipids are ligands for a lymphocyte homing receptor, L-selectin, binding epitope in sulphated sugar chain. Biochem. Biophys. Res. Commun. 190:426–434.
10. Imai, Y., L.A. Lasky, and R.D. Rosen. 1993. Sulphation requirements for GlyCAM-1, an endothelial ligand for L-selectin. Nature (Lond.). 361:555–557.
11. Lasky, L.A., M.S. Singer, D. Dowbenko, Y. Imai, W.J. Henzel, C. Grimley, C. Fennie, N. Gillett, S.R. Watson, and S.D. Rosen. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell. 69:927–938.
12. Briskin, M.J., L.M. McEvoy, and E.C. Butcher. 1993. MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. Nature (Lond.). 363:461–464.
13. Berg, E.L., L.M. McEvoy, C. Berlin, R.F. Bargatze, and E.C. Butcher. 1993. L-selectin-mediated lymphocyte rolling on MAdCAM-1. Nature (Lond.). 366:695–698.
14. Baumhueter, S., M.S. Singer, W. Henzel, S. Hemmerich, M.R. Renz, S.D. Rosen, and L.A. Lasky. 1993. Binding of L-selectin to vascular sialomucin CD34. Science (Wash. DC). 262:436–438.
15. Paavonen, T., and R. Renkonen. 1992. Selective expression of sialyl-Lewis a and sialyl Lewis b, putative ligands for L-selectin, on peripheral lymph node high endothelial venules. Am. J. Pathol. 141:1259–1264.
16. Munro, J.M., S.K. Lo, C. Corless, M.J. Robertson, N.C. Lee, R.L. Barhull, D.S. Weinberg, and M.P. Bevilacqua. 1992. Expression of sialyl-Lewis a, an E-selectin ligand, in inflammation. immune processes and lymphoid tissues. Am. J. Pathol. 141:1397–1408.
17. Sawada, M., A. Takada, I. Ohwaki, N. Takahashi, H. Tatene, J. Sakamoto, and R. Kannagi. 1993. Specific expression of a complex sialyl Lewis a antigens of high endothelial venules in human lymph nodes: possible candidate for L-selectin ligand. Biochem. Biophys. Res. Commun. 193:337–347.
18. Turunen, J., T. Paavonen, M. Majuri, S. Tiisala, P. Mattila, A. Mennander, C. Gahmberg, P. Häyry, T. Tamatani, M. Miyasaka, and R. Renkonen. 1994. Sialyl-Lewis a and L-selectin-dependent site-specific lymphocyte extravasation into the renal transplants during acute rejection. Eur. J. Immunol. 24:1130–1136.
19. Majuri, M., M. Pinola, R. Niemelä, S. Tiisala, O. Renkonen, and R. Renkonen. 1994. a2,3 sialyl- and a1,3-fucosyltransferase-dependent synthesis of sialyl Lewis a, an essential oligosaccharide present in L-selectin counterreceptor in cultured endothelial cells. Eur. J. Immunol. 24:3205–3210.
20. Renkonen, R., A. Soots, E. von Willebrand, and P. Häyry. 1983. Lymphoid cell subclasses in rejecting renal allograft in the rat. Cell. Immunol. 77:188–195.
21. Häyry, P., E. von Willebrand, E. Parthenais, A. Nenlender, A. Soots, I. Lautenschlager, and R. Renkonen. 1984. Intra-graft rejection mechanisms. Immunol. Rev. 77:85–142.
22. Turunen, J.P., P. Mattila, J. Halttunen, P. Häyry, and R. Renkonen. 1992. Evidence that lymphocyte traffic into rejecting cardiac allograft is CD11a- and CD49d-dependent. Transplantation (Baltimore). 54:1053–1058.
23. Renkonen, R., J. Turunen, J. Rapola, and P. Häyry. 1990. Characterization of high endothelial-like properties of peritubular capillary endothelium during acute allograft rejection. Am. J. Pathol. 137:643–651.
S. Tsurufuji, and M. Miyasaka. 1993. Characterization of rat LECAM-1 (L-selectin) by the use of monoclonal antibodies and evidence for the presence of soluble LECAM-1 in rat sera. Eur. J. Immunol. 23:2181-2188.

25. Tamatani, T., K. Kuida, T. Watanabe, S. Koike, and M. Miyasaka. 1993. Molecular mechanisms underlying lymphocyte recirculation. III. Characterization of the LECAM-1 (L-selectin)-dependent adhesion pathways in rats. J. Immunol. 150: 1735-1745.

26. Natunen, J., R. Niemelä, L. Penttilä, A. Seppo, T. Ruohola, and O. Renkonen. 1994. Enzymatic synthesis of two lacto-N-neohexaose-related Lewis x heptasaccharides and their separation by chromatography on immobilized wheat germ agglutinin. Glycobiology. 4:577-583.

27. Wilkman, A., R. Niemelä, L. Penttilä, J. Helin, A. Leppänen, A. Seppo, H. Maaheimo, S. Lusa, and O. Renkonen. 1993. Human serum β-(1-3)-N-acetyl-D-glucosaminyltransferase elongates both branches of bi-antennary backbones of oligo-N-acetyllactosaminoglycans. Carbohydrate Res. 226:155-174.

28. Seppo, A., L. Penttilä, R. Niemelä, H. Maaheimo, and O. Renkonen. 1995. Enzymatic synthesis of octadecameric saccharides of multiple branched blood group I-type, carrying four distal α1,3-galactose or β1,3-GlcNAc residues. Biochemistry. In press.

29. Briscoe, D.M., F.J. Schoen, G.E. Rice, M.P. Bevilacqua, P. Ganz, and J.S. Pober. 1991. Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allograft. Transplantation (Baltimore). 51:537-547.

30. Pelletier, R.P., R.G. Ohye, A. Vanbuskirk, D.D. Sedmark, P. Kincade, R.M. Ferguson, and C.G. Orosz. 1992. Importance of endothelial VCAM-1 for inflammatory leukocytic infiltration in vivo. J. Immunol. 149:2473-2478.

31. Rosen, S.D., and C.R. Bertozzi. 1994. The selectins and their ligands. Curr. Opin. Cell Biol. 6:663-673.

32. Pelletier, R.P., R.G. Ohye, A. Vanbuskirk, D.D. Sedmark, P. Kincade, R.M. Ferguson, and C.G. Orosz. 1993. Molecular mechanisms underlying lymphocyte recirculation. III. Characterization of the LECAM-1 (L-selectin)-dependent adhesion pathways in rats. J. Immunol. 150: 1735-1745.

33. Rosen, S.D., and C.R. Bertozzi. 1994. The selectins and their ligands. Curr. Opin. Cell Biol. 6:663-673.

34. Carlos, T.M., and J.M. Harlan. 1994. Leukocyte-endothelial adhesion molecules. Blood. 84:2068-2101.

35. Picker, L.J., T.K. Kishimoto, C.W. Smith, R.A. Warnock, and E.C. Butcher. 1991. ELAM-1 is an adhesion molecule for skin-homing T cells. Nature (Lond.). 349:769-771.

36. Shimizu, Y., S. Shaw, N. Graber, T.V. Gopal, K.J. Horgan, G.A. van Seventer, and W. Newman. 1991. Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. Nature (Lond.). 349:799-801.

37. Gallatin, W.M., I.L. Weisman, and E.C. Butcher. 1983. A cell surface molecule involved in organ-specific homing of lymphocytes. Nature (Lond.). 303:30-34.

38. Hemmerich, S., and S.D. Rosen. 1994. 6'-sulfated sialyl Lewis x is a major capping group of GlyCAM-1. Biochemistry. 33:4830-4835.

39. Ito, K., K. Handa, and S. Hakomori. 1994. Species-specific expression of sialosyl Le x on polymorphonuclear leukocytes (PMN) in relation to selectin-dependent PMN response. Glycoconjugate J. 11:232-237.

40. Mulligan, M.S., J.C. Paulson, S. deFrees, Z.-L. Zheng, J.B. Lowe, and P.A. Ward. 1993. Protective effects of oligosaccharides in P-selectin-dependent lung injury. Nature (Lond.). 364:149-151.

41. Mueller, E.R., W. Hsiao, F.A. Gaeta, M.J. Forrest, and A.M. Lefer. 1994. Sialyl Lewis x-containing oligosaccharides in P-selectin-dependent lung injury. J. Clin. Invest. 93:1140-1148.