Core 2 Oligosaccharide Biosynthesis Distinguishes between Selectin Ligands Essential for Leukocyte Homing and Inflammation

Lesley G. Ellies,* Shigeru Tsuboi,¹ Bronisława Petrońiak,† J ohn B. Lowe,‡ Minoru Fukuda,¹ and J amey D. Marth§

*Howard Hughes Medical Institute
Division of Cellular and Molecular Medicine
University of California San Diego
La Jolla, California 92093
† The Burnham Institute
10901 North Torrey Pines Road
La Jolla, California 92037
‡ Howard Hughes Medical Institute
Department of Pathology
University of Michigan Medical School
Ann Arbor, Michigan 48109

Summary

Mammalian serine/threonine-linked oligosaccharides (O-glycans) are commonly synthesized with the Golgi enzyme core 2 β-1,6-N-acetylgalcosaminyltransferase (C2 GlcNAcT). Core 2 O-glycans have been hypothesized to be essential for mucin production and selectin ligand biosynthesis. We report that mice lacking C2 GlcNAcT exhibit a restricted phenotype with neutrophilia and a partial deficiency of selectin ligands. Loss of core 2 oligosaccharides reduces neutrophil rolling on substrata bearing E-, L-, and P-selectins and neutrophil recruitment to sites of inflammation. However, the diminished presence of L-selectin ligands on lymph node high endothelial venules does not affect lymphocyte homing. These studies indicate that core 2 oligosaccharide biosynthesis segregates the physiologic roles of selectins and reveal a function for the C2 GlcNAcT in myeloid homeostasis and inflammation.

Introduction

Serine/threonine (O)-linked oligosaccharides are diverse structures prevalent on cell surfaces and secreted proteins. In the synthesis of mammalian O-linked oligosaccharides (O-glycans), the core 2 β-1-6 N-acetylgalcosaminyltransferase (C2 GlcNAcT) is a key branching enzyme controlling O-glycan structural diversity (Williams and Schachter, 1980; Bierhuizen and Fukuda, 1992). Widespread expression of C2 GlcNAcT activity among most tissues may explain why the majority of mammalian O-glycans are of the core 2 subtype (Schachter and Brockhausen, 1989; Brockhausen, 1995; Figure 1). Core 2 O-glycans are biantennary and may be diversified by glycosyltransferases that add N-acetylgalcosamine (GlcNAc) and galactose (Gal) monosaccharides in β1-3 and β1-4 linkages, thereby generating lactosamine disaccharide repeats termed polyolactosamines. These can be further modified with sialic acid (Sia) and L-fucose (Fuc) linked at terminal positions. Core 2 oligosaccharides are common components of mucins—glycoproteins with a majority of mass attributable to O-linked oligosaccharides. Mucins are considered to be essential for respiratory epithelium, the gastrointestinal tract, and the immune system, by providing a protective function to cell surfaces and regulating cell-cell interactions (reviewed in Hounsell et al., 1996; Strous and Dekker, 1992). Core 2 O-glycan biosynthesis can also provide the oligosaccharide scaffold used in constructing ligands for the selectin family of leukocyte adhesion molecules (Figure 1; reviewed in Lasky, 1995; Lowe, 1997; Springer, 1995).

Selectins and their oligosaccharide ligands control lymphocyte trafficking to lymph nodes in normal circumstances and neutrophil recruitment to vascular endothelium during inflammation (reviewed in Kansas, 1996; McEver and Cummings, 1997). E- and P-selectin are expressed on activated endothelium and P-selectin is present on activated platelets, while L-selectin is found on leukocytes. Selectin deficiencies result in varying degrees of impaired lymphocyte trafficking, reduced neutrophil recruitment to sites of inflammation, and decreased leukocyte turnover (Mayadas et al., 1993; Arbones et al., 1994; Johanson et al., 1995; Labow et al., 1995). Selectin ligand biosynthesis requires the α-1,3 fucosyltransferase VII (FucT-VII) enzyme and is essential for both lymphocyte homing and neutrophil recruitment in inflammation (Maly et al., 1996). The types of oligosaccharides bearing physiologically relevant selectin ligands are undefined, although a variety of possibilities exist among N-glycans, O-glycans, and glycolipids, as well as proteoglycans (reviewed in Varški, 1997). The major ligand for P-selectin is reported to be a sialylated, fucosylated, core 2 O-glycan clustered with tyrosine sulfate on P-selectin glycoprotein ligand-1 (PSGL-1; Norgard et al., 1993; Li et al., 1996). Sulfated, sialylated O-linked oligosaccharides on the high endothelial venules (HEVs) of secondary lymphoid organs are also ligands for L-selectin. These ligands are present on various selectin counter-receptors including CD34 (Baumhueter et al., 1993), GlyCAM-1 (Lasky et al., 1992), MadCAM-1 (Berg et al., 1993), Sgp200 (Rosen and Bertozzi, 1994), and the podocalyxin-like protein (Sassetti et al., 1998). Other reported selectin counter-receptors in which O-glycosylation does not appear to be essential include E-selectin ligand-1 (ESL-1) (Steegmaier et al., 1995) and CD24 (Aigner et al., 1995), as well as heparin proteoglycans (Norgard-Sumnicht et al., 1993). Moreover, glycolipids contain selectin ligands that function in vitro (Larkin et al., 1992; Alon et al., 1995; Stroud et al., 1996).

Core 2 O-glycan expression has also been reported to regulate lymphoid cell physiology and immune responses (Tsuboi and Fukuda, 1997). In addition, changes in C2 GlcNAcT expression occur during T lymphocyte development and activation (Piller et al., 1988; Baum et al., 1995), Wiskott-Aldrich Syndrome (WAS) (Higgins et al., 1991; Piller et al., 1991), AIDS (Fox et al., 1983), and leukemia (Brockhausen et al., 1991; Saitoh et al., 1991). The physiologic roles ascribed to core 2 oligosaccharides may reflect their potential structural diversity. In
defining the physiologic roles of core 2 oligosaccharides, it was necessary to generate a germline deletion of the C2 GlcNAcT gene. Core 2 oligosaccharide deficiency was well tolerated in mice and revealed an unexpected degree of cell-type-specific function.

Results

Targeted Mutagenesis and Deletion of the C2 GlcNAcT Gene

C2 GlcNAcT is a Golgi localized type II transmembrane glycosyltransferase and is conserved among mammals studied (Bierhuizen and Fukuda, 1992; Sekine et al., 1997). A mouse genomic clone encompassing the C2 GlcNAcT protein-coding exon was used in constructing a gene-targeting vector designed to control exon deletion by Cre-loxP recombination (Figure 2A). Homologous recombination of the targeting vector in embryonic stem (ES) cells incorporated selection markers and 3 loxP sites for the subsequent production of systemic C2 GlcNAcT−/− or conditional C2 GlcNAcT+−/− mutations in vivo (Figures 2B and 2C). These alleles were transmitted into the mouse germline, and offspring homozygous for either the C2 GlcNAcT− or C2 GlcNAcT+ allele were generated. Such offspring were present among 25% of littermates, lacked overt physical or behavioral abnormalities, developed normally, and were fully fertile. Mice homozygous for C2 GlcNAcT− allele were further analyzed.

C2 GlcNAcT Activity and Core 2 O-Glycan Abundance

C2 GlcNAcT enzyme activity is specifically detected by using a substrate analog of the core 1 oligosaccharide (Yousefi et al., 1991). In mice homozygous for the C2 GlcNAcT− allele, tissues normally expressing high C2 GlcNAcT levels were devoid of significant activity, including the spleen, bone marrow, and kidney (Figure 3A). To determine whether loss of C2 GlcNAcT activity resulted in a deficiency of core 2 O-glycans, oligosaccharide structures were analyzed in metabolically labeled splenocytes. O-linked oligosaccharides isolated from splenocytes homozygous for the C2 GlcNAcT− allele lacked Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAc, indicating a deficiency of core 2 O-glycans (Figure 3B, upper panel, peak 3). Desialylation and additional chromatographic analysis further indicated a loss of core 2 O-glycans (Figure 3B, lower panel, peak 3). The majority of core 1 oligosaccharides in C2 GlcNAcT-deficient splenocytes were sialylated, consistent with structures expected in the absence of C2 GlcNAcT activity.

Monoclonal antibodies previously implicated as oligosaccharide-dependent were also applied to characterize C2 GlcNAcT-deficient cells. B lymphocytes specifically express CD22 and the B cell epitope B220, the latter of which is a glycoform of CD45 (Johnson et al., 1989). Splenocytes lacking C2 GlcNAcT activity were devoid of the B220 epitope, while CD22 and CD45 protein levels at the cell surface were unaltered (Figure 3C, left panels). The CD43 glycoprotein is highly expressed on leukocytes as two distinct glycoforms differentially recognized by monoclonal antibodies S7 and 1B11. In mice homozygous for the C2 GlcNAcT− allele, myeloid cells remained positive for S7 binding, while 1B11 antibody binding was distinctly absent (Figure 3C, right panels). These data reveal that homozgyosity at the C2 GlcNAcT− allele results in a deficiency of C2 GlcNAcT activity and core 2 O-glycans.

C2 GlcNAcT Deficiency Results in a Moderate Neutrophilia

Upon histologic examination, no alterations were detected in cellular or organ morphologies within C2 GlcNAcT− deficient mice. The kidney, lungs, intestinal tract, and associated epithelium were unremarkable, and mucin levels in the intestinal goblet cells were indistinguishable from controls (data not shown). Analyses of serum biochemistry indicated normal renal function (data not shown). Hematologic examination disclosed a blood leukocytosis. Total white blood cell counts were elevated 2.4-fold in C2 GlcNAcT null mice. This increase
Core 2 O-Glycans Modulate Selectin Physiology

Figure 2. Deletion of the C2GlcNAcT Gene in Embryonic Stem Cells and Mice

(A) The wild-type C2GlcNAcT genomic locus was used in conjunction with the pfllox vector to construct a targeting vector in which the single exon open reading frame was flanked by loxP sites (C2 GlcNAcT<sub>F[tkneo]</sub>). Restriction enzyme sites indicated are BamHI (B), BglII (Bg), HindIII (H), NotI (N), and XbaI (X).

(B) Transient Cre expression in C2GlcNAcT-targeted ES cells resulted in subclones isolated with a C2 GlcNAcT<sub>D</sub> (systemic-null) or C2 GlcNAcT<sub>F</sub> (conditional-null) mutation.

(C) Southern blot analysis of a BamHI/BglII digest of ES cell DNA probed with a loxP probe confirmed the expected structures. Wild-type RI ES cell DNA showed no hybridization with the loxP probe. Three loxP sites are present in a targeted parental clone (156), one loxP site is present in each of two C2 GlcNAcT<sub>D</sub> subclones (156.24 and 156.29), and two loxP sites are present in the C2 GlcNAcTF subclone (156.34). In the right panel, tail DNA from a heterozygous mating of progeny from a C2 GlcNAcT<sub>D</sub> chimera digested with HindIII and probed with the genomic probe indicates the 6.5 kb wild-type allele and the 3.7 kb mutant allele.

was almost entirely accounted for by a 4.3-fold increase in neutrophils (Figure 4A). Bone marrow progenitor cell numbers were normal in C2 GlcNAcT null mice, implying that the leukocytosis is not a consequence of increased neutrophil production (Figure 4B). In other studies, circulating platelet levels and morphology were also unchanged and no difference in bleeding time was apparent (data not shown). These observations are reminiscent of certain results obtained from mice deficient in selectins or selectin ligands.

C2 GlcNAcT Participates in Selectin Ligand Formation and Leukocyte Rolling

To detect a role for C2 GlcNAcT in selectin ligand biosynthesis, selectin-Ig chimeras were used to examine levels of E-, L-, and P-selectin ligands on cell surfaces. The E- and P-selectin chimeras specifically bind to carbohydrate ligands on myeloid cells in the blood including granulocytes and monocytes (Maly et al., 1996). Using flow cytometry, peripheral blood leukocytes from C2 GlcNAcT null mice were found to be deficient in both E- and P-selectin ligands. The P-selectin chimera did not significantly bind, whereas a low level of E-selectin ligand binding remained (Figure 5A). These results were not due to reduced expression of proteins that carry selectin ligands. Cell surface levels of PSGL-1, L-selectin, and CD24 on C2 GlcNAcT-deficient leukocytes were unaffected, as were levels of various adhesion molecules involved in the firm attachment of leukocytes to the endothelium (CD11a, CD11b, and CD18; Figure 5B).

A parallel plate flow chamber system was used to measure the contribution of core 2 oligosaccharides to functional selectin binding. Immobilized E-, L-, or P-selectin-Ig chimeras served as the adhesion substrate for neutrophil rolling in this system. The binding of FucT-VII null leukocytes was monitored in direct comparisons as they do not appreciably bind selectins at most shear forces (Maly et al., 1996). Leukocytes deficient in core 2 oligosaccharides exhibited reduced but significant rolling activity on E- and P-selectins at all shear forces used, while FucT-VII null leukocytes did not interact appreciably with the immobilized selectin-Ig chimeras (Figure 5C). At the highest shear forces, 50% of C2 GlcNAcT null leukocytes remained bound to the P-selectin substrate and approximately 20% bound to the E-selectin substrate. In contrast, leukocyte rolling on L-selectin appeared especially dependent upon core 2 oligosaccharide biosynthesis as C2 GlcNAcT-deficient leukocytes were unable to bind except at shear forces below 10 dynes/cm².

Core 2 Oligosaccharides Recruit Neutrophils to Sites of Inflammation

Since core 2 oligosaccharides contribute to selectin ligand biosynthesis, it seemed likely that C2 GlcNAcT null mice would exhibit abnormalities in lymphocyte trafficking and neutrophil recruitment to sites of inflammation. During the first 4 hr following an inflammatory stimulus, neutrophil recruitment to sites of inflammation is largely dependent upon selectin function (Mayadas et al., 1993). Peritonitis induced by thioglycollate injection can be used to assess selectin function in a model of acute
inflammation involving quantitation of neutrophil recruitment in vivo. In the absence of core 2 oligosaccharides, a severe deficit in neutrophil recruitment to inflamed peritoneum was apparent with only 20% of control neutrophil numbers recoverable at 4 hr (Table 1). This extent of reduction in neutrophil numbers is similar in FucT-VII-deficient mice that are more deficient in selectin ligand formation (Maly et al., 1996). These data indicate that core 2 oligosaccharides provide an essential function in selectin-mediated neutrophil recruitment during acute inflammation.

**L-Selectin Ligands Produced by C2 GlcNAcT Are Dispensable for Lymphocyte Homing**

L-selectin and FucT-VII are crucial for lymphocyte homing to the lymph nodes (Arbones et al., 1994; Maly et al., 1996). Since L-selectin counter-receptors on HEVs may be sialylated mucins, it seemed likely that C2 GlcNAcT was involved in L-selectin ligand production. However, secondary lymphoid organs from C2 GlcNAcT-deficient mice exhibited normal tissue size and follicular anatomy without alterations in either lymphocyte abundance or subset proportions (Figure 6A). Nevertheless, L-selectin binding to lymph node HEVs was reduced in the absence of C2 GlcNAcT (Figure 6B). This deficit in L-selectin ligand formation was not apparent using higher concentrations of the L-selectin-Ig chimera (data not shown) and was without significant consequence, as lymphocyte homing to C2 GlcNAcT-deficient lymph nodes and spleen was statistically unaltered (Figure 6C; data not shown). These results indicate that C2 GlcNAcT activity in HEV L-selectin biosynthesis is dispensable for lymphocyte homing to secondary lymphoid organs.

**Figure 4. Peripheral Hematology in C2 GlcNAcT-Deficient Mice**

(A and B) Blood was collected from the tail vein of 6- to 8-week-old mice. Automated total white blood cell counts and automated and manual differential counts were carried out using a CELL-DYN 3500 and Wright-Giemsa-stained smears. Counts of 20 mice of each genotype were expressed as cells per ml of whole blood ± SEM.

(C) Colony forming units in the bone marrow were analyzed by in vitro differentiation of nucleated bone marrow cells in methylcellulose in the presence of growth factors. The number of colony-forming units (GM, granulocyte/macrophage; GEMM, granulocyte/erythroblast/macrophage/monocyte; E, erythroblast; B, B cell) was counted at day 10. Data are means ± SEM from six mice of each genotype.
A discussion on the role involving selectin-mediated responses of myeloid cells.

Core 2 Oligosaccharides in the Biosynthesis of Selectin Ligands

C2 GlcNAcT operates differentially in E-, L-, and P-selectin ligand formation. The deficiency of E- and P-selectin binding to core 2 O-glycan-deficient leukocytes observed by flow cytometry was tempered by data from neutrophil rolling assays that revealed significant residual binding on both E- and P-selectin substrates. Additionally, a small amount of L-selectin binding remained in comparison to Fuc-T-VII null leukocytes, but only at the lowest shear forces applied. All three selectins can bind to PSGL-1 and it is possible that PSGL-1 acts as the major selectin counter-receptor in the rolling assay. Residual E- and P-selectin binding by C2 GlcNAcT-deficient leukocytes indicates a possible role for N-glycans in physiologic selectin ligand formation. The CD24 glycoprotein is extensively N-glycosylated on myeloid cell surfaces where it has been reported to mediate binding of monocytes and neutrophils to P-selectin (Aigner et al., 1995). In addition, the E-selectin counter-receptor, ESL-1, contains only N-linked oligosaccharides and has been found on leukocyte microvilli where it may regulate initial cell adhesion events (Steegmaier et al., 1997). Leukocyte L-selectin ligand production appears to be

Table 1. Neutrophil Recruitment to Peritoneal Exudates during Inflammation

<table>
<thead>
<tr>
<th>Hours Poststimulus</th>
<th>C2 GlcNAcT&lt;sup&gt;wt/wt&lt;/sup&gt;</th>
<th>C2 GlcNAcT&lt;sup&gt;Δ/Δ&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4 ± 1 (n = 3)</td>
<td>4 ± 1 (n = 3)</td>
</tr>
<tr>
<td>2</td>
<td>48 ± 6 (n = 10)</td>
<td>5 ± 1 (n = 7)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>499 ± 86 (n = 10)</td>
<td>96 ± 19 (n = 12)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are the means ± SEM of neutrophil numbers (×10<sup>6</sup>) recovered from the peritoneal lavage of thioglycollate-treated mice. The number of mice in each group is indicated in parentheses. An unpaired t test indicated significance of <sup>*</sup>p < 0.001.
greatly dependent upon core 2 oligosaccharide formation, which may be particularly relevant in secondary interactions involving neutrophil-neutrophil binding at sites of inflammation and extravasation (Walcheck et al., 1996). Our studies indicate that although C2 GlcNAcT provides a significant proportion of E-, L-, and P-selectin ligands on leukocytes, it is not as essential as is FucT-VII. Therefore, other oligosaccharide substrates of FucT-VII that are not produced by C2 GlcNAcT are also involved in selectin ligand biosynthesis.

The Physiologic and Cell-Type Specificity of C2 GlcNAcT among Leukocytes

With E- and P-selectin binding evident in shear flow studies of C2 GlcNAcT null neutrophils, the decrease observed in neutrophil recruitment during peritoneal inflammation was surprisingly severe. The extent of the reduction is similar to that observed in the absence of FucT-VII with an 80% decrease in neutrophil recruitment. Not all neutrophil recruitment to inflamed peritoneum requires selectins or FucT-VII. Approximately 20% of neutrophils recruited during the first 4 hr of acute inflammation may be accounted for by the function of the intercellular adhesion molecule-1 (ICAM-1; Kunkel et al., 1996). These findings imply that selectin and FucT-VII involvement in acute inflammation are dependent upon C2 GlcNAcT and core 2 oligosaccharides.

Altered leukocyte homeostasis in C2 GlcNAcT-deficient mice is intermediate in severity in comparison to studies of P-selectin and FucT-VII deficiencies. The major hemodynamic effect in the absence of C2 GlcNAcT was an increase in circulating neutrophil levels. An absence of P-selectin results in a small increase in peripheral neutrophils but no change in total leukocytes (Mayadas et al., 1993), whereas mice deficient in either E- or L-selectin exhibit normal peripheral hematologic profiles (Arbones et al., 1994; Labow et al., 1995). When both P- and E-selectins are missing (Frenette et al., 1996) or in the absence of FucT-VII (Maly et al., 1996), marked increases in leukocytes are observed, above that measured in C2 GlcNAcT-deficient mice. Bone marrow progenitor frequencies were unaltered in C2 GlcNAcT-deficient mice, suggesting that an increase in neutrophil half-life may account for the neutrophilia as was reported with P-selectin deficiency (Johnson et al., 1995). In contrast to results reported in the absence of P-selectin, C2 GlcNAcT-deficient mice exhibited a normal bleeding time (Subramaniam et al., 1996; data not shown).

The selective nature of C2 GlcNAcT function was further evident from studies of lymph node morphology and lymphocyte homing. The partial deficit observed in L-selectin binding to lymph node HEVs appeared inconsequential, as lymphocyte abundance and homing was not affected. Perhaps physiologic L-selectin ligands are normally expressed in overabundance and the quantity remaining in C2 GlcNAcT-deficient mice is sufficient to facilitate normal lymphocyte homing. It is also possible that the HEV glycoproteins modified by C2 GlcNAcT to carry L-selectin ligands do not participate in lymphocyte homing. As L-selectin ligands may exist on separate oligosaccharide classes (N-glycans, O-glycans, glycolipids, etc.), the underlying structures in these classes may influence their presentation and the efficacy of function in binding L-selectin in vivo. L-selectin counter-receptors implicated in lymphocyte homing include sia-lylated mucins such as CD34. Their identity is uncertain and CD34-deficient mice exhibit normal lymphocyte homing (Cheng et al., 1996; Suzuki et al., 1996). A possibility that other oligosaccharide classes are involved is...
consistent with the observation that O-sialoglycoprotease-resistant L-selectin ligands exist on lymph node HEVs (Clark et al., 1998). While the structural features of physiologic L-selectin ligands remain to be fully established, the oligosaccharides involved in lymphocyte homing are dependent upon the function of FucT-VII but do not require C2 GlcNAcT, and thus may not be composed of core 2 oligosaccharides.

C2 GlcNAcT in Oligosaccharide Diversification and Function

The C2 GlcNAcT glycosyltransferase appears essential for generating core 2 O-glycans in the kidney, bone marrow, and splenic leukocytes. Since C2 GlcNAcT can also act on glycolipid substrates (Piller et al., 1988), it is possible that the phenotypes manifested may be due in part to a deficiency of specific glycolipids that are substrates of C2 GlcNAcT in the Golgi. We also cannot rule out the possibility that a distinct gene product encoding a C2 GlcNAcT isozyme is expressed in specific compartments such as lymph node HEVs and may account for functional L-selectin ligand formation. Several studies have suggested the presence of such an isozyme in mucin-producing tissues that is capable of synthesizing both core 2 and core 4 O-glycans (Ropp et al., 1991; Kuhns et al., 1993). Although core 4 O-glycans may partially compensate for C2 GlcNAcT deficiency, C4 GlcNAcT activity is not normally found in myeloid cell types (Schachter and Brockhausen, 1989; Bierhui-zen and Fukuda, 1992), and core 4 activity was not induced in tissues from C2 GlcNAcT-deficient mice (data not shown).

The function of C2 GlcNAcT in neutrophil recruitment during inflammation is consistent with a role in the biosynthesis of selectin ligands. E-, L-, and P-selectin ligand production is only partially C2 GlcNAcT-dependent, yet this partial dependence encompasses a potent and restricted physiologic activity. Inhibition of C2 GlcNAcT activity might provide a selective means to dampen acute inflammatory responses and reperfusion injury (Lowe and Ward, 1997). Our results imply that core 2 O-glycans provide an unexpectedly restricted biological function that may reflect the presence of additional glycosyltransferases with overlapping activities. The unique modulatory roles exerted by glycosyltrans-ferases may arise through differential expression patterns that can affect levels of oligosaccharides and their structural presentation by core and glycoprotein components. Either possibility may explain how the C2 GlcNAcT enzyme selectively regulates myeloid homoeo-stasis and inflammation.

Experimental Procedures

Gene Targeting of the C2 GlcNAcT and Production of Mutant Mice

Isolation of mouse C2 GlcNAcT genomic DNA and construction of a targeting vector bearing Cre loxp recombination signals was accomplished similarly as described (Priel et al., 1997). R1 ES cells (Nagy et al., 1993) were electroporated with 10 μg of the linearized targeting construct and the resulting clones were screened by Southern blotting using the genomic probe. Targeted ES cells were electroporated with 5 μg of Cre expression plasmid, and subclones bearing the C2 GlcNAcT+ and C2 GlcNAcTF alleles were isolated.

C2 GlcNAcT+ and C2 GlcNAcT- chimeric mice were generated using standard techniques (Metzler et al., 1994) and were crossed into the C57BL/6 background for the generation of heterozygous and homozygous offspring. C2 GlcNAcT allele structure was analyzed by Southern blotting and PCR. The wild-type C2 GlcNAcT allele was detected using PCR primers adjacent to the deleted region (W5: 5′-GGGTACCCTGATGACTCCTGTGTC and W3: 5′-CCCTGGAGAACCA GGCACATTGTC-3′) resulting in a 304 bp fragment, while the mutant allele was detected using W5 and a loxp primer (M3: 5′-TCGGATT GATCCCCGGGATC-3′), yielding a 200 bp fragment.

C2 GlcNAcT Enzyme Assays and Oligosaccharide Analysis

The enzyme assay mixture containing 50 mM Mes (pH 7.0), 0.5 μCi of UDP-[3H]GlcNAc in 1 mM UDP-GlcNAc, 0.1 M GlcNAc, 10 mM EDTA, 1 mM acceptor, and 25 μl cell lysate from normal, heterozygous, or homozygous null tissues in a total volume of 50 μl was incubated at 37°C for 1 hr followed by C18 Sep-Pak (Waters) processing (Yousef et al., 1991; Bierhui-zen and Fukuda, 1992). For oligosaccharide analyses, splenocytes from wild-type or C2 GlcNAcT null animals were metabolically labeled with [3H]glucosamine (10 μCi/ml) for 24 hr and processed according to described procedures (Maemura and Fukuda, 1992; Bierhui-zen et al., 1994). O-Linked oligosaccharides were initially analyzed by Bio-Gel P-4 gel filtration as previously reported (Maemura and Fukuda, 1992). Sialylated O-glycans were then desialylated and analyzed by HPLC using an amino-bonded column and standard techniques (Piller et al., 1988).

Flow Cytometry

Single cell suspensions of splenocytes were prepared and erythro- cytocyte enzymes removed by ammonium chloride lysis. Cells were incubated in the presence of antibodies (below) in FACS buffer (2% FCS in PBS) for 20 min at 4°C. For E- or P-selectin binding, cells were treated with 0.5 μg/ml of Fc Block (anti-CD32/16, PharMingen) and then incubated with Gr-1 and either the E- or P-selectin-IgM chimera (Maly et al., 1996), with or without addition of 5 mM EDTA, for 30 min at 4°C. Cells were washed and incubated with a goat anti-human FITC-conjugated secondary antibody (Sigma) as appropriate. Antibodies used were CD11a (M17/4), CD11b (M1/70), CD18 (C71/16), CD22 (M17/4), CD24 (M1/69), CD43 (S7 and 1B11), CD45 (30-F11), CD45R/B220 (RA3-6B2), CD62L (MEL-14), and Gr-1 (RB6-8C5) (PharMingen). The anti-PSGL-1 antibody, 4RA10, was a generous gift from Dr. D. Vestweber. Data were analyzed on a FACScan flow cytometer using CELLQUEST software (Becton Dickinson).

Hematology

Blood from the tail vein of methoxyfluorane anesthetized mice was collected into EDTA-coated polypyrrolene microtubes (Becton Dickinson). Analyses of red blood cells, white blood cells, and platelet cell numbers and morphology were carried out manually and with a CELL-DYN 3500 calibrated with normal mouse blood (UCSD Medical Center, Hillcrest).

Bone Marrow Progenitor Assay

Bone marrow was flushed from the femurs of wild-type or C2 GlcNAcT null mice with 2% FBS in PBS and single cell suspensions prepared by aspirating gently through a 25g needle. 1.5 × 10^6 nucleated cells were plated into 35 mm dishes in triplicate in Methocult M3434 (Stemcell Technologies, Inc.). Dishes were incubated at 37°C, 5% CO2, for 10-12 days and colonies enumerated using light microscopy.

Leukocyte Rolling

Solvant murine E-, P-, and L-selectin IgG chimeric molecules were coated onto polystyrene dishes and assembled in a parallel plate flow chamber (GlycoTech, Rockville, Maryland). Neutrophils from wild-type, C2 GlcNAcT+/-, or FucT-VII-/- mice were prepared at a concentration of 1 × 10^6/ml in rolling medium, 0.2% BSA in HBSS without calcium and magnesium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10 mM HEPES (pH 7.2). The calcium concentration was adjusted to 2 mM immediately prior to infusion of the neutrophils into the flow chamber for 30 sec at 5 dyn/cm² using a syringe pump (KD Scientific Inc., Boston, MA). This infusion
was stopped for 3 min to allow for static adhesion of the neutrophils to the substrate and then restarted at 0.19 dyn/cm². Wall shear forces were doubled every 2 min without interrupting the cell flow. Fields of neutrophils were observed using a 10× objective, and the scene was recorded on VCR tape. Image analysis was performed on 6100/66 Power Macintosh using the Scion version of the public domain NIH Image program (Scion Corporation, Frederick, MD). The fraction of cells remaining adherent under static conditions after 2 min at each specific shear force was determined by manually enumerating the cells and dividing this number by the number of adherent cells observed immediately preceding the initiation of flow (i.e., 100% represents cells found in the observed field after static adherence, prior to initiation of the lowest shear flow rate).

Peritoneal Inflammation
Mice were injected intraperitoneally with 1 ml of 3% thioglycollate (Sigma). At the indicated times, mice were sacrificed and the peritoneal cavities lavaged with 10 ml of ice-cold PBS containing 1% BSA and 0.5 mM EDTA. Red blood cells were removed by hypotonic lysis and leukocytes counted manually using a hemocytometer. Cytospins were stained with Leukostat (Sigma) and neutrophils counted. Peritoneal exudates were also stained with Gr-1 and F4/80 (Caltag) and analyzed by flow cytometry.

Lymphocyte Trafficking
To determine the cellularity of secondary lymphoid organs, tissues were dissected from wild-type and C2 GlcNAcT homozygous null mice. Single cell suspensions of lymphocytes from mesenteric lymph nodes, peripheral (axillary and brachial) lymph nodes, and Peyer's patches were enumerated manually using a hemocytometer. Frozen sections of axillary and brachial lymph nodes were cut at 5 μm, air dried, and fixed in acetone prior to staining with hematoxylin and eosin. In separate experiments, an L-selectin-IgM was applied to frozen sections of peripheral lymph nodes as previously described (Maly et al., 1996; Smith et al., 1996). Serial sections were stained with the peripheral node addressin antibody, MECA 79. Lymphocyte homing assays were carried out as previously described (Maly et al., 1996). In brief, 2.5 × 10⁶ CMFDA (Molecular Probes) labeled wild-type mesenteric lymphocytes were injected into the tail vein of wild-type or C2 GlcNAcT Bioprobes) labeled wild-type mesenteric lymphocytes were injected K.W., Freeze, H.H., Moremen, K.W., Fukuda, M.N., and Marth, J.D.

Analysis of 100,000 CMFDA positive lymphocytes was carried out and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity 1, 247-260.


Jones, A.T., Federspiel, B., Ellies, L.G., Williams, M.J., Burger, A. Should the blood be drawn directly from the tail vein of wild-type or C2 GlcNAcT Bioprobes) labeled wild-type mesenteric lymphocytes were injected K.W., Freeze, H.H., Moremen, K.W., Fukuda, M.N., and Marth, J.D.

References


