Novel Antibodies Reactive with Sialyl Lewis X in Both Humans and Mice Define Its Critical Role in Leukocyte Trafficking and Contact Hypersensitivity Responses*§

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Background: The expression and function of sialyl Lewis X (sLe\(^x\)) in mice are unclear due to the poor reactivity of conventional anti-sLe\(^x\) mAbs with mouse tissues. Here, we developed novel anti-sLe\(^x\) mAbs, termed F1 and F2, which react well with both human and mouse sLe\(^x\), by immunizing fucosyltransferase (FucT)-IV and FucT-VII doubly deficient mice with 6-sulfo-sLe\(^x\)-expressing cells transiently transfected with an expression vector encoding CMP-N-acetylneuraminic acid hydroxylase. F1 and F2 specifically bound both the N-acetyl and the N-glycolyl forms of sLe\(^x\) as well as 6-sulfo-sLe\(^x\), a major ligand for L-selectin expressed in high endothelial venules, and efficiently blocked physiological lymphocyte homing to lymph nodes in mice. Importantly, both of the mAbs inhibited contact hypersensitivity responses not only when administered in the L-selectin-dependent sensitization phase but also when administered in the elicitation phase in mice. When administered in the latter phase, F1 and F2 efficiently blocked rolling of mouse leukocytes along blood vessels expressing P- and E-selectin in the auricular skin in vivo. Consistent with these findings, the mAbs blocked P- and E-selectin-dependent leukocyte rolling in a flow chamber assay. Taken together, these results indicate that novel anti-sLe\(^x\) mAbs reactive with both human and mouse tissues, with the blocking ability against leukocyte trafficking mediated by all three selectins, have been established. These mAbs should be useful in determining the role of sLe\(^x\) antigen under physiological and pathological conditions.

Sialyl Lewis X (sLe\(^x\)) antigen functions as a common carbohydrate determinant recognized by all three members of the selectin family. However, its expression and function in mice remain undefined due to the poor reactivity of conventional anti-sLe\(^x\) monoclonal antibodies (mAbs) with mouse tissues. Here, we developed novel anti-sLe\(^x\) mAbs, termed F1 and F2, which react well with both human and mouse sLe\(^x\), by immunizing fucosyltransferase (FucT)-IV and FucT-VII doubly deficient mice with 6-sulfo-sLe\(^x\)-expressing cells transiently transfected with an expression vector encoding CMP-N-acetylneuraminic acid hydroxylase. F1 and F2 specifically bound both the N-acetyl and the N-glycolyl forms of sLe\(^x\) as well as 6-sulfo-sLe\(^x\), a major ligand for L-selectin expressed in high endothelial venules, and efficiently blocked physiological lymphocyte homing to lymph nodes in mice. Importantly, both of the mAbs inhibited contact hypersensitivity responses not only when administered in the L-selectin-dependent sensitization phase but also when administered in the elicitation phase in mice. When administered in the latter phase, F1 and F2 efficiently blocked rolling of mouse leukocytes along blood vessels expressing P- and E-selectin in the auricular skin in vivo. Consistent with these findings, the mAbs blocked P- and E-selectin-dependent leukocyte rolling in a flow chamber assay. Taken together, these results indicate that novel anti-sLe\(^x\) mAbs reactive with both human and mouse tissues, with the blocking ability against leukocyte trafficking mediated by all three selectins, have been established. These mAbs should be useful in determining the role of sLe\(^x\) antigen under physiological and pathological conditions.

The authors declare that they have no conflicts of interest with the contents of this article.

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Significance: sLe\(^x\) is a potential therapeutic target for CHS.

Results:

Conclusion: sLe\(^x\) is critical for both the sensitization and the elicitation phases of CHS.

Sialyl Lewis X (sLe\(^x\)) antigen functions as a common carbohydrate determinant recognized by all three members of the selectin family. The selectins are a family of three C-type lectins that mediate rapid and reversible adhesive interactions between leukocytes and vascular endothelial cells under physiological flow (1–3). L-selectin is expressed on most leukocytes, whereas E- and P-selectin are expressed on activated endothelial cells. It is also known that P-selectin is expressed on activated platelets. However, none of the conventional anti-sLe\(^x\) monoclonal antibodies (mAbs), except for one mAb, react with the high endothelial venules (HEVs) in mouse or rat peripheral lymph nodes (PLNs), where L-selectin ligands are expressed (4, 5). This situation is probably due to the fact that a large proportion of the terminal sialic acid in WT mice is in the form of N-glycolylneuraminic acid (Neu5Gc), whereas most existing mAbs react with glycans modified with N-acetylneuraminic acid (Neu5Ac) (4). The exception is the anti-sLe\(^x\) mAb 2H5 (6), which is reactive with the HEVs in rat PLNs (7), but it also binds to cells lacking α1,3-fucosyltransferase (FucT), FucT-IV, and FucT-VII and may not be very specific for sLe\(^x\) (8). Therefore, although studies using FucT-IV and FucT-VII doubly deficient (DKO) mice revealed that α1,3-fucosylation of HEV ligands and leukocytes is critical for their interaction with selectins (9, 10), the

Footnotes:

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The abbreviations used are: sLe\(^x\), sialyl Lewis X; HEV, high endothelial venules; PLN, peripheral lymph node; Neu5Gc, N-glycolylneuraminic acid; Neu5Ac, N-acetylneuraminic acid; FucT, fucosyltransferase; DKO, doubly deficient; GlcNAc6ST, N-acetylglucosaminyltransferase-6-O-sulfotransferase; LacNAc, N-acetyllactosamine; Cmah, CMP-Neu5Ac hydroxylase; CHS, contact hypersensitivity; C1β3GnT, core 1 β1,3-N-acetylgalactosaminyltransferase; C2GnT-1, core 2 β1,6-N-acetylgalactosaminyltransferase-I; PAA, polyacrylamide; MLN, mesenteric lymph node; PP, Peyer’s patch; PSGL-1, P-selectin glycoprotein ligand 1; CFSE, carboxyfluorescein diacetate succinimidyl ester.
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tissue distribution and function of sLex in mouse tissues remain unclear.

We previously reported an efficient method of generating anti-carbohydrate mAbs using mice deficient in glycan-synthesizing enzyme, in which the glycan structures formed by the missing enzyme are highly antigenic (11, 12). By immunizing N-acetylglucosamino-6-O-sulfotransferase (GlcNAc6ST)-1 and GlcNAc6ST-2 DKO mice (13) using GlcNAc6ST-2-expressing cells that had been transfected with cDNAs encoding CMP-Neu5Ac hydroxylase (Cmah), which generates CMP-Neu5Gc from CMP-Neu5Ac (14), we were able to generate mAbs reactive with mouse tissues rich in Neu5Gc (11). Because both the CDNAS of various glycosyltransferases and sulfotransferases and a number of mutant mice deficient in those enzymes are now available, we hypothesized that the method should be widely applicable for the generation of various anti-carbohydrate mAbs.

In the present study, we generated novel anti-sLex mAbs, termed F1 and F2, which react with both human and mouse sLex, by immunizing FucT-IV and FucT-VII DKO mice with 6-sulfo-sLex-expressing cells transiently transfected with an expression vector encoding Cmah. These mAbs bound both the N-acetyl and the N-glycoly forms of sLex as well as 6-sulfo-sLex, a major ligand for L-selectin expressed in the HEVs in PLNs, and efficiently blocked lymphocyte homing to PLNs and contact hypersensitivity (CHS) responses in mice. Interestingly, administration of the mAbs in the elicitation phase significantly inhibited the CHS responses and rolling of mouse leukocytes along blood vessels expressing P- and E-selectin in the inflamed ear, which could not be verified by the studies using FucT-IV and FucT-VII DKO mice that lacked selectin-mediated leukocyte trafficking in both the sensitization and the elicitation phases. These results demonstrate that our method is applicable to the generation of anti-carbohydrate mAbs to determine the biological function of glycans that have not been clarified by prior studies using gene-targeted mice.

Experimental Procedures

Mice—FucT-IV and FucT-VII DKO, GlcNAc6ST-1 and GlcNAc6ST-2 DKO, and core 1 β1,3-N-acetylglucosamyltransferase (Cβ3GnT) and core 2 β1,6-N-acetylglucosamyltransferase-I (C2GnT-I) DKO mice were back-crossed at least 5 generations to C57BL/6 WT mice and maintained as described previously (9, 13, 15). C57BL/6 WT and BALB/c Slc-nu/nu mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were treated in accordance with the guidelines of the Animal Research Committee of Hoshi University and University of Shizuoka.

Establishment of Anti-fucosylated Glycan mAbs—Anti-fucosylated glycan mAbs were generated as described previously (11) with certain modifications. In brief, CHO cells stably expressing human CD34, human Cβ3GnT, human C2GnT-I, human FucT-VII, and mouse GlcNAc6ST-2 (CHO/CD34/C1/C2/FucT-7/GlcNAc6ST-2) were transiently transfected with mouse Cmah (14) using FuGENE 6 transfection reagent (Roche Applied Science). After 48 h of transfection, the cells were suspended in PBS and mixed with Imject Alum (Pierce) at a ratio of 1:1 and injected intraperitoneally into FucT-IV and FucT-VII DKO mice three times at 2-week intervals. Four days after the final immunization, lymphocytes from the spleens of the DKO mice were fused with P3X63Ag8.653 myeloma cells (American Type Culture Collection) in the presence of PEG solution (M, 1,450; Sigma-Aldrich) and selected in the medium containing HAT (hypoxanthine, aminopterin, and thymidine) supplement (Invitrogen). The hybridoma supernatants that reacted with the HEVs of WT mice, but not with those of FucT-IV and FucT-VII DKO mice, were selected by immunofluorescence. Hybridomas secreting anti-fucosylated glycan mAbs were cloned by limiting dilution. The isotypes of the mAbs F1 and F2 thus obtained were determined to be mouse IgG1 (κ) using an isotyping kit (GE Healthcare). F1 and F2 were purified from the ascitic fluid using a caprylic acid (6-aminohecanoic acid; Wako) precipitation method. In certain cases, purified antibodies and control mouse IgG (Sigma-Aldrich) were labeled with EZ-Link Sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer’s protocols.

Immunofluorescence—Acetone-fixed, frozen sections (7-μm) from mice that had been treated with or without 300 milliunits/ml sialidase (from Streptococcus 6646K, Seikagaku) for 6 h at 37 °C were incubated with PBS containing 3% BSA (Sigma-Aldrich) to block nonspecific binding sites and then with biotinylated F1, F2, MECA-79 (BioLegend), CSLEX1 (BD Biosciences), or HECA-452 (BioLegend). After washing, the sections were incubated with streptavidin-Alexa Fluor 594 (Invitrogen) and DAPI (Roche Applied Science) and mounted using Fluoromount (Diagnostic BioSystems). Paraffin-embedded human tonsil tissue sections (kindly provided by Drs. Yoko Ishihara and Toshio Nishikawa) were deparaffinized, boiled in 10 mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA for 20 min to retrieve antigens, and stained as described above. All images were obtained using a microscope (BZ-9000; KEYENCE).

Glycan Array Analysis—Glycan array analysis was performed at the Consortium for Functional Glycomics using microarray slides (printed array version 5.0) containing 611 different glycans, as described previously (11).

Flow Cytometric Analysis—Cells were incubated with biotinylated F1, F2, mouse IgG, CSLEX1, or HECA-452, followed by streptavidin-Alexa Fluor 647 (Invitrogen). For the analysis of mouse leukocytes induced in the peritoneal cavity by thioglycollate medium (Thermo Fisher Scientific), as described below, cells were incubated with APC-Cy7-anti-mouse CD45 (BioLegend) and FITC-anti-Gr-1 (BioLegend) together with biotinylated F1, F2, mouse IgG, CSLEX1, or HECA-452 mAbs. The cells were analyzed by flow cytometry using a FACS Canto II cell analyzer (BD Biosciences). For mouse leukocyte staining, CD45+Gr-1high mouse granulocytes were gated and analyzed for their reactivity with various anti-sLex mAbs. The data were acquired and analyzed with FACS Diva software (BD Biosciences) and FlowJo software (Tree Star, Inc.).

Transient Transfection and Flow Cytometry—The CHO-K1 cell-derived mutant line Lec1 (16) was transiently transfected with pcDNA3.1/EGFP together with various combinations of pCDM8/human FucT-VII, pcDNA3/human Cβ3GnT, pcDNA3/human C2GnT-I, and pcDNA3.1/Zeo using a pipette-type electroporator (Neon Transfection System; Invitrogen) according to the protocol provided by the manufacturer.
A total of 72 h after transfection, the cells were washed with PBS, dispersed in 1 mM EDTA-PBS, and incubated with biotinylated F1 or F2 or with mouse IgG, followed by incubation with streptavidin-Alexa Fluor 647 (Invitrogen). The cells were analyzed by flow cytometry using a FACSCanto II system, as described above.

**ELISA**—The wells of a 96-well ELISA plate (Costar ELA/RIA Half Area Plate 3690, Corning, Inc.) were first coated overnight with 1 μg/ml sialyl N-acetyllactosamine (LacNAc)-polyacylamide (PAA), Le^a^-PAA, sLe^a^-PAA, or 6-sulfo-sLe^a^-PAA (GlycoTech Co.) in PBS at 4 °C. After blocking with blocking One (1:5 dilution; Nacalai Tesque, Inc.), 0.5 μg/ml F1, 0.5 μg/ml F2, 0.5 μg/ml control mouse IgG (MOPC-21, BioLegend), 1 μg/ml HECA-452 (rat IgM), or 5 μg/ml CSLEX1 (mouse IgM) in PBS containing 0.1% BSA, 0.05% Tween 20 (buffer A) was added to the wells and incubated for 1 h at room temperature. After washing, HRP-goat anti-mouse IgG (1:2,000 dilution; Zymed Laboratories Inc.), HRP-goat anti-rat IgM (1:2,000 dilution; Southern Biotech), or HRP-goat anti-mouse IgM (1:500 dilution; Zymed Laboratories Inc.) in buffer A was added to the samples treated with F1, F2, or control mouse IgG; HECA-452; or CSLEX1, respectively, and incubated for 1 h. After washing with buffer A, 1-Step Ultra TMB-ELISA HRP substrate (Thermo Fisher Scientific) was added. The reaction was terminated by the addition of 2 N H_2SO_4, and the optical density at 450 nm was measured using a 96-well spectrometer (Sunrise Rainbow RC-R, TECAN).

**Lymphocyte Homing Assay**—A lymphocyte homing assay was performed as described previously, with certain modifications (11, 13). In brief, mesenteric lymph node (MLN) lymphocytes and splenocytes from WT mice were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen). After washing, 2.0 × 10^7 cells in 200 μl of PBS were injected intravenously into recipient mice. One hour after the injection, the number of CFSE^- cells in cell suspensions prepared from the recipient lymphoid organs was determined by flow cytometry. For lymphocyte homing inhibition experiments, mice were preincubated intravenously with purified F1 or F2 (200 μg/mouse) 2 h before being injected with CFSE^-labeled lymphocytes.

**Modified Stamper-Woodruff Cell-binding Assay**—A modified Stamper-Woodruff cell-binding assay was performed as described previously (11).

**In Vitro Leukocyte Rolling Assay**—To analyze L-selectin-dependent leukocyte rolling, CHO/CD34/C1/C2/FucT-7/GlcNAc6ST-2 cells cultured as monolayers in 35-mm culture dishes (Corning Inc.) were incubated with or without 10 μg/ml sialyl P-selectin were selected by flow cytometry after staining the cells with Alexa Fluor 647-labeled anti-human CD62P (AbD Serotec). CHO-K1 cells stably expressing human P-selectin thus obtained and those expressing human E-selectin, established previously (18), were cultured as monolayers in 35-mm culture dishes and used in the rolling assay described above. In brief, human promyelocytic leukemia HL-60 cells (American Type Culture Collection) that had been preincubated with or without F1, F2, or control mouse IgG for 10 min at 4 °C were allowed to roll on CHO-K1 cells expressing P- or E-selectin. Images were taken with a CCD camera (model ADT-40S; Flovel Co., Ltd.) attached to an inverted microscope (×20 objective; Olympus CKX41).

**CHS**—The CHS responses were measured as described previously, with certain modifications (11, 13). In brief, 25 μl of 1% oxazolone (Sigma-Aldrich) in acetone/olive oil (4:1, v/v) was applied to the shaved forelegs of mice on day 0. On day 5, the ears were treated with 20 μl of 1% oxazolone (10 μl/side of the pinna). In Schedule 1, 200 μl of 0.5 mg/ml mAb in PBS or PBS alone was intravenously injected on days −1, 0, and 1. In Schedule 2, the same amount of mAb or PBS alone was intravenously injected on day 5, 30 min before the 1% oxazolone treatment was applied to the ear. Ear swelling was measured using a thickness gauge before and 24 h after the treatment. Paraffin-embedded tissue sections from the ears of the oxazolone-treated mice were stained with hematoxylin-eosin (Sigma-Aldrich) and observed using a microscope (BZ-9000; KEYENCE).

**In Vivo Leukocyte Rolling Assay**—To observe leukocyte rolling in the skin, hair removal cream (Veet HPS-a, Reckitt Benckiser Japan Ltd.) was applied to both sides of the ear. After 5 min, the ear was gently cleaned with gauze. CHS responses were then elicited as described above. To observe in vivo leukocyte rolling, 0.5 mg/ml rhodamine 6G in 200 μl of saline was injected intravenously to label leukocytes fluorescently in the mouse peripheral blood 0.5 or 14 h after the challenge with 1% oxazolone on day 5. The mice were then anesthetized and mounted on the microscope stage. The ear was flattened and held in place by glass slides, and leukocyte rolling along the vein in the inflamed ear was observed with a CCD camera installed on an inverted fluorescence microscope (CKX41, Olympus) and was recorded for 3 min.

**RT-PCR**—Total RNA was purified from the ears of C57BL/6 WT mice using TRIzol reagent (Invitrogen). cDNA was synthesized using a PrimeScript RT-PCR kit (TaKaRa) and subjected to RT-PCR. The primers used were as follows: P-selectin, 5’-GCTTCAGGACAATGGACATG-3’ and 5’-ACTCGGTATGTTTCTAGGTG-3’; E-selectin, 5’-CCTCGACAGAGGAAGCTCAGAAT-3’ and 5’-TCACACCTCAGAGAGCTACTACCG-3’; 5’-TGAATCCTTGGCATCATGGAAC-3’ and 5’-TAAACGCAGCTCAGTACAGTCCG-3’.
The PCR cycle (94 °C for 30 s, 61 °C for 30 s, and 72 °C for 60 s) was repeated 50 times for P-selectin. The PCR cycle (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s) was repeated 50 times for E-selectin. The PCR cycle (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s) was repeated 30 times for \( H_9252 \)-actin.

Statistical Analysis—Dunnett’s test (for Figs. 7E, 8A and 9B and C) or Student’s t test (for Fig. 7A–C) was used for the determination of statistically significant differences between experimental groups. All results are expressed as the mean ± S.D.

Results

Generation of Anti-sLex mAbs—To obtain anti-fucosylated glycan mAbs that recognize glycan epitopes on mouse HEVs, FucT-IV and FucT-VII DKO mice were immunized with CHO cells stably expressing CD34, C1β3GnT, C2GnT-I, FucT-VII, and GlcNAc6ST-2 (CHO/CD34/C1/C2/FucT-7/GlcNAc6ST-2 cells) that had been transiently transfected with an expression vector encoding Cmah (14). The splenocytes of the immunized mice were used to generate hybridomas by cell fusion with mouse myeloma cells. The culture supernatants were screened for their immunoreactivity with the HEVs of WT mice and for their lack of immunoreactivity with the HEVs of FucT-IV and FucT-VII DKO mice deficient in sLex biosynthesis in the HEVs (9). As a result, two independent hybridoma clones, secreting the anti-sLex mAbs F1 and F2 (mouse IgG1, \( H_9260 \)), were established.

To determine the carbohydrate-binding specificity of the mAbs F1 and F2, immunofluorescence staining using frozen sections of PLNs from various KO mice was performed (Fig. 1A). F1 and F2 bound well to the HEVs of GlcNAc6ST-1 and GlcNAc6ST-2 DKO (13) and C1β3GnT and C2GnT-I DKO (15) mice. These mAbs did not bind to PLN HEVs in FucT-IV and FucT-VII DKO mice (9). In contrast, the mAb MECA-79, which recognizes sulfated extended core 1 \( O \)-glycans (19), bound to the HEVs in FucT-IV and FucT-VII DKO but not to those in the other two strains of mutant mice. The staining intensity of F1 and F2, but not that of MECA-79, was significantly diminished after sialidase treatment of the tissue sections (Fig. 1B). Therefore, these results indicate that sialylation and \( \alpha,3 \)-fucosylation, but not sulfation, are required for the binding of F1 and F2 to HEVs.

The above mentioned results obtained using C1β3GnT and C2GnT-I DKO mice suggest that F1 and F2 can interact with sLe\(^a\) on N-glycans. In support of this notion, treatment of the...
PLN tissue sections of C1β3GnT and C2GnT-I DKO mice with N-glycosidase F as described previously (11) reduced the staining intensity with these mAbs. Furthermore, both of the mAbs specifically bound stable transfectants expressing 6-sulfo-sialyl sLe\(^x\) on their N-glycans (CHO/CD34/FucT-7/GlcNAc6ST-2) as well as those expressing 6-sulfo- sLe\(^x\) on both N- and O-glycans (CHO/CD34/C1/C2/FucT-7/GlcNAc6ST-2) (Fig. 2A). To examine whether these mAbs can also interact with sLe\(^x\) on O-glycans and glycolipids, transient transfection of CHO-K1 cell-derived mutant Lec1 cells (16) and flow cytometric analysis were performed. CHO-K1 cells lack C1β3GnT and C2GnT-I. In addition, Lec1 cells lack N-acetylgalactosaminyltransferase-I, which forms complex- and hybrid-type N-glycans (16). Therefore, transient transfection of Lec1 cells with a human FucT-VII expression vector together with an EGFP expression vector resulted in the expression of sLe\(^x\) only on glycolipids. As shown in Fig. 2B, Lec1 cells transiently transfected with FucT-7 reacted with both F1 and F2, indicating that both of these mAbs can interact with sLe\(^x\) on glycolipids. Further co-transfection of the Lec1 cells with C1β3GnT (C1/FucT-7) or C2GnT-I (C2/FucT-7) resulted in a significant increase in the cells reactive with the mAbs. These results indicate that F1 and F2 can interact not only with sLe\(^x\) on N-glycans but also with that on glycolipids and O-glycans.

Glycan Array Analysis of F1 and F2—To determine the fine carbohydrate-binding specificities of F1 and F2, glycan array screening was performed (Fig. 3 and supplemental Table S1). As shown in Fig. 3, both F1 and F2 interacted with sLe\(^x\) terminated with Neu5Ac (Glycan 256) as well as with that terminated with Neu5Gc (Glycan 283). Consistent with immunofluorescence studies, F1 and F2 also bound well to 6-sulfo-sLe\(^x\) (Glycan 253), which is abundantly expressed in PLN HEVs. In contrast, F1 and F2 failed to interact with the Lex structure (Glycan 153) or with α2,3-sialylated LacNAc (Glycan 261). In addition, these mAbs did not bind to other related glycans lacking sialic acid (Glycans 170, 80, 291, 130, 73, and 114). Importantly, both of the mAbs failed to interact with an isomer of sLe\(^x\), or sLe\(^x\) (Glycan 240), bearing β1,3-linked galactose and α1,4-linked fucose residues. In addition, these mAbs bound to terminal sLe\(^x\) on a di-LacNAc backbone (Glycan 258) but not to VIM-2 antigen bearing α1,3-linked fucose attached to the internal GlcNAc residue (Glycan 219). Both of the mAbs bound well to sLe\(^x\) on the GlcNAcβ1–2Man present in N-glycans (Glycan 535) but only weakly or not at all to sLe\(^x\) on the core 2-branched O-glycans (Glycan 330), suggesting that the core 1 branch of the O-glycan chain may interfere with the accessibility of the terminal sLe\(^x\) on the core 2 branch to the mAbs. A comparison between F1

3 R. Matsumura and H. Kawashima, unpublished observation.

**FIGURE 2. Binding of the mAbs F1 and F2 to sLe\(^x\)-expressing cells.** A, CHO-K1, CHO/CD34/FucT-7/GlcNAc6ST-2, and CHO/CD34/C1/C2/FucT-7/GlcNAc6ST-2 cells were stained with biotinylated F1, F2, or control mouse IgG (0.5 μg/ml), followed by Alexa Fluor 647-conjugated streptavidin (1.0 μg/ml), and were analyzed by flow cytometry on a FACSComp II, as described under “Experimental Procedures.” B, Lec1 cells were transiently transfected with an expression vector encoding EGFP together with (FucT-7) or without (Mock) a vector encoding human FucT-VII. C1/FucT-7, cells were co-transfected with expression vectors encoding EGFP, FucT-VII, and C1β3GnT. C2/FucT-7, cells were co-transfected with expression vectors encoding EGFP, FucT-VII, and C2GnT-I. Cells were stained with biotinylated F1, F2, or control mouse IgG (2.0 μg/ml), followed by streptavidin-Alexa Fluor 647 (1.0 μg/ml). Cells were analyzed by flow cytometry. The percentage of cells in the blue box relative to those in the red box is indicated.
and F2 suggests that the two mAbs have similar binding specificities, although F2 appears to bind sLex more avidly than F1 does (Glycans 256, 283, 253, and 535).

**Binding of F1 and F2 to Human and Mouse HEVs and Leukocytes—** To compare the reactivity of F1 and F2 with that of the previously reported anti-sLex mAbs CSLEX1 (20) and HECA-452 (21), immunofluorescence studies were performed using human tonsil and mouse PLN tissue sections (Fig. 4A). In human tonsils, F1, F2, and HECA-452, but not CSLEX1, bound to MECA-79+/H11001 HEVs as well as infiltrated leukocytes. In contrast, F1 and F2, but not CSLEX1 and HECA-452, bound to mouse MECA-79+/H11001 HEVs.

Flow cytometric analysis of human promyelocytic leukemia HL-60 cells and mouse leukocytes expressing human and mouse sLex, respectively, indicated that all four anti-sLex mAbs examined bound to HL-60 cells (Fig. 4B). In contrast, F1 and F2, but not CSLEX1 and HECA-452, bound to CD45+/H11001 Gr-1high mouse granulocytes.

To determine the reasons for the differential reactivity of the mAbs described above, ELISA using immobilized PAA-oligosaccharides was performed (Fig. 5A). Consistent with the results of the glycan array screening, F1 and F2 bound sLeX and 6-sulfosLeX-PAA. HECA-452 also bound to these oligosaccharides with certain cross-reactivity with LeX-PAA. CSLEX1 bound only to sLeX without sulfation, which is probably the reason why CSLEX1 did not bind HEVs in human tonsil sections. To determine the preference of the mAbs for terminal sialic acid species, flow cytometric analysis using CHO cells stably expressing P-selectin glycoprotein ligand 1 (PSGL-1), C2GnT-I, and FucT-VII (CHO/PSGL/C2/FucT-7) and CHO cells additionally expressing Cmah (CHO/PSGL/C2/FucT-7/Cmah) was performed (Fig. 5B). F1 and F2, but not HECA-452, reacted with cells expressing Cmah. This result is consistent with a previous report showing the nonreactivity of HECA-452 with the N-glycolyl form of sLeX (4). In addition, the reactivity of CSLEX1 was only slightly diminished in the presence of Cmah, as described previously (4), which is probably because the lack of reactivity of CSLEX1 with mouse leukocytes is mainly due to other sialic acid modifications, such as O-acetylation, as suggested previously (4). These results, together with the results in Fig. 3, indicate that F1 and F2 bind both Neu5Ac- and Neu5Gc-terminated sLeX, whereas the other two mAbs prefer Neu5Ac-terminated sLeX.

**Inhibition of Selectin-mediated Leukocyte Rolling by F1 and F2—** sLex is recognized by all three members of the selectin family. To examine the ability of F1 and F2 to block selectin-mediated leukocyte rolling under physiological flow conditions, a parallel plate flow chamber assay was performed. As shown in Fig. 6A, CHO/CD34/C1/C2/FucT-7/GlcNAc6ST-2 cells supported L-selectin-dependent rolling of mouse leukocytes. The rolling was markedly blocked by F1 and F2. Rolling of HL-60 cells on P- and E-selectin transfectants was also significantly blocked by F1 and F2 (Fig. 6, B and C).

**Inhibition of Lymphocyte Homing by F1 and F2—** To assess the functional effects of the newly generated mAbs in vivo, a lymphocyte homing assay was performed (Fig. 7). F1 and F2 nearly completely inhibited lymphocyte homing to PLNs and MLNs in WT mice (Fig. 7A). These mAbs also significantly inhibited lymphocyte homing to Peyer’s patches (PPs). Consistent with these findings, the mAbs specifically stained HEVs in PLNs, MLNs, and PPs in WT mice, but not those in FucT-IV and FucT-IV/-VII DKO mice, in the immunofluorescence analysis (Fig. 7D). In addition, a modified Stamper-Woodruff cell-binding assay indicated that F1 and F2 significantly inhibited the binding of fluorescently labeled leukocytes to HEVs in PLN tissue sections, similar to the MECA-79 antibody (Fig. 7E). The binding observed in this assay was dependent on L-selectin because the anti-L-selectin mAb MEL-14 (22) completely blocked the cell binding. Thus, these results indicate that both F1 and F2 inhibited the interaction between L-selectin on leukocytes and its ligands on HEVs.
Previously, it was reported that lymphocyte homing was diminished by ~75% in GlcNAc6ST-1 and GlcNAc6ST-2 DKO mice and that the residual homing was probably mediated by unsulfated sLe^a, as suggested by our group and others (13, 23). To determine whether this is the case, the ability of F1 and F2 to effect the residual homing in the sulfotransferase DKO mice was examined (Fig. 7). Both F1 and F2 nearly completely blocked lymphocyte homing to PLNs and MLNs, indicating that the previous hypothesis was correct. It was also reported previously that lymphocyte homing to PLNs in C1GnT-1/2GnT-I DKO mice was only partially diminished (15). To confirm the importance of N-glycans in the residual lymphocyte homing in these mutant animals, a short term homing assay using WT mice and C1GnT-1/2GnT-I DKO mice, which lack sulfated O-glycans, was performed. Both F1 and F2 nearly completely inhibited the residual lymphocyte homing to PLNs in the C1GnT-1/2GnT-I DKO mice (Fig. 7C). These results suggest that the sLe^a- or 6-sulfo-sLe^a-containing N-glycans expressed in the HEVs mediate the residual lymphocyte homing in C1GnT-1 and 2GnT-I DKO mice.

Inhibition of CHS Responses by F1 and F2—To further examine the effects of F1 and F2 in vivo, their effects on CHS responses were examined. In Schedule 1, in which mice were injected intravenously with F1 or F2 on three consecutive days during the sensitization phase, ear swelling and leukocyte infiltration were significantly blocked by the treatment with F1 or F2 (Fig. 8, A and B). Under the same protocol, mAb S2, which recognizes 6-sulfo-sialyl LacNAc and 6-sulfo-sLe^a, inhibited CHS responses, as described previously (11). ELISA indicated that 11.8 ± 4.07 µg/ml F1 and 0.07 ± 0.05 µg/ml F2 persisted in the sera on day 5 after separately intravenously injecting these mAbs according to Schedule 1 (Fig. 8E), suggesting that administration of the mAbs during the sensitization phase might affect not only hapten sensitization but also leukocyte infiltration in the elicitation phase. To examine the effects of the mAbs in the elicitation phase, the mAbs were injected intravenously.
into the hapten-sensitized mice 30 min before the second oxazolone challenge (Schedule 2). Interestingly, even under this protocol, both ear swelling and leukocyte infiltration were significantly blocked by the mAb treatment (Fig. 8, C and D), suggesting that sLe^a-dependent leukocyte infiltration in the elicitation phase is critical for CHS responses.

**Inhibition of in Vivo Rolling of Leukocytes by F1 and F2 during the Elicitation Phase of CHS Responses**—To determine if F1 and F2 actually inhibited leukocyte rolling in vivo, leukocyte rolling along the vein in the inflamed ear was analyzed by fluorescence intravital microscopy. As shown in Fig. 9A, two peaks of leukocyte rolling were observed, at 30 min and 14 h after elicitation. Both of these peaks of leukocyte rolling were significantly blocked by intravenously injecting F1 or F2 30 min prior to the elicitation (Fig. 9, B and C). RT-PCR analysis indicated that P-selectin was expressed at all time points examined, whereas E-selectin was expressed at 6 and 14 h after elicitation and faintly at 24 h (Fig. 9D), suggesting that P- and E-selectin differentially contribute to leukocyte rolling at different time points in CHS responses.

**Discussion**

We previously developed an efficient method for generating anti-carbohydrate mAbs by immunizing mice deficient in glycan-synthesizing enzyme with transfectants that overexpress the glycan epitopes formed by the enzyme (11). In the present study, we examined whether this method is applicable to the generation of other anti-carbohydrate mAbs. To this end, FucT-IV- and FucT-VII-deficient mice were immunized with 6-sulfo-sLe^a-expressing transfectants, and novel anti-sLe^a mAbs, termed F1 and F2, were generated. Before immunization, Cmah cDNA was transiently transfected into 6-sulfo-sLe^a-expressing cells to modify the terminal sialic acid. This strategy successfully generated mAbs that were reactive with mouse tissues rich in Neu5Gc (4).

Glycan array analysis indicated that both F1 and F2 were highly specific for the sLe^a and 6-sulfo-sLe^a structures. Both of the mAbs bound well to sLe^a on the GlcNAcβ1–2Man present in N-glycans (Glycan 535). However, F2 bound only weakly to sLe^a on core 2-branched O-glycans (Glycan 330), and F1 failed to bind to this glycan, suggesting that the core 1 branch of the O-glycan chain may interfere with the accessibility of terminal sLe^a on the core 2 branch to these mAbs. We speculate that both F1 and F2 should make contact with the sLe^a structure on the core 2 branch of an O-glycan from such an angle that the core 1 branch might sterically interfere with the access. The reason why only F2 could bind to this glycan is probably that F2 has higher affinity toward sLe^a compared with F1. Indeed, F2 bound sLe^a more avidly than F1 did in our glycan array analysis. Previously, it was reported that PSGL-1-derived N-terminal sulfoglycopeptide modified with sLe^a-containing core 2 O-glycans could support interaction with P-selectin (24, 25). Because both F1 and F2 significantly blocked P-selectin-mediated leukocyte rolling (Fig. 6), F1 and F2 probably bound to sLe^a-containing glycans on PSGL-1 near its N terminus. We hypothesize that sLe^a-containing glycans other than those on core 2-branched structures might serve as the binding site for F1.

Previous studies indicated that lymphocyte homing was partially diminished in GlcNAc6ST-1 and GlcNAc6ST-2 DKO mice (13, 23). Although those reports suggested that the unsulfated sLe^a structure was involved in the residual homing in the sulfotransferase DKO mice, a definitive conclusion could not be reached because of the lack of mAbs reactive with mouse sLe^a. Our present study now clearly shows that the residual homing to PLNs in these mice is nearly completely dependent on unsulfated sLe^a (Fig. 7). In addition, it has also been reported that lymphocyte homing to PLNs was only partially diminished, by 55%, in C1β3GnT and C2GnT-1 DKO mice in a short term homing assay (15). Further studies in that work showed the importance of N-glycans in lymphocyte homing, although it was not clear whether sLe^a or its related structure on N-glycans is involved in the residual lymphocyte homing. In the present study, by using specific mAbs, we provided evidence that the residual homing observed in C1β3GnT and C2GnT-1 DKO mice is most likely mediated by N-glycans modified with sLe^a or 6-sulfo-sLe^a structures (Fig. 7). Because the majority of lymphocyte homing to the PLNs in C1β3GnT and C2GnT-1 DKO mice was blocked by anti-sulfated glycan mAb S2 in our previous studies (11) and by mAb CL41 with similar carbohydrate binding specificity (26), it is likely that N-glycans modified with 6-sulfo-sLe^a play a major role in the residual lymphocyte homing to PLNs in these mutant mice.

Both F1 and F2 were reactive with PP HEVs and significantly inhibited lymphocyte homing to PPs (Fig. 7). These results are consistent with a previous report using FucT-IV and FucT-VII DKO mice, in which short term lymphocyte homing to PP was significantly inhibited (9). In contrast, GlcNAc6ST-1 and GlcNAc6ST-2 DKO mice did not show any reduction of lymphocyte homing to PPs (13, 23). Furthermore, mAb S2 did not inhibit lymphocyte homing to PPs, although this mAb was reactive with PP HEVs (11). Therefore, these findings collectively suggest that unsulfated sLe^a structure plays an important role in lymphocyte homing to PPs. The residual homing to PP in the presence of F1 or F2 could be due to α4β7 integrin and MadCAM-1 interaction (27) or CD22-mediated homing of B cells (28).

Interestingly, F1 and F2 blocked CHS responses not only when administered in the sensitization phase but also when administered in the elicitation phase in mice (Fig. 8). Because F1 and F2 nearly completely abrogated lymphocyte homing to the PLNs, where immune responses occur, these results suggest that F1 and F2 inhibited CHS responses at least in

**FIGURE 5. Binding of anti-sLe^a mAbs to synthetic glycans and to transfectants expressing PSGL-1 modified with the NeuSAs or NeuSgc form of sLe^a.** A, the binding of anti-sLe^a mAbs and control mouse IgG to a plate coated with PAA coupled with various synthetic glycans was analyzed by ELISA. B, CHO/K1 cells; CHO/PSGL/C2/FucT-7 cells stably expressing PSGL-1, C2GnT-1, and FucT-VII; and CHO/PSGL/C2/FucT-7/Cmah cells stably expressing PSGL-1, C2GnT-1, FucT-VII, and Cmah (4) were stained with biotinylated anti-sLe^a mAbs or control mouse IgG (2.0 μg/ml), followed by streptavidin–Alexa Fluor 647 (1.0 μg/ml) and were analyzed by flow cytometry.
part by blocking L-selectin-dependent lymphocyte homing to the draining lymph nodes during the sensitization phase. However, because a small amount of F1 (11.8 ± 4.07 μg/ml) and F2 (0.07 ± 0.05 μg/ml) persisted in the sera on day 5 after intravenous injection, we think it likely that mAbs administered during the sensitization phase might have blocked not only L-selectin-dependent hapten sensitization but also P- and E-selectin-dependent leukocyte infiltration.

**FIGURE 6. Inhibition of selectin-dependent leukocyte rolling by F1 and F2 in vitro.** A, numbers of rolling leukocytes from mouse spleens that had been pretreated with or without (None) MEL-14 (10 μg/ml) for 30 s and added to CHO/C34/C1/C2/FucT-7/GlcNAc6ST-2 cells pretreated with or without (None) F1, F2, or control mouse IgG (10 μg/ml). B and C, numbers of rolling HL-60 cells that had been pretreated with or without F1, F2, or control mouse IgG (50 μg/ml) for 30 s and added to CHO-K1 cells expressing human P-selectin (B) or E-selectin (C). Error bars, S.D. of three measurements.

**FIGURE 7. Inhibition of lymphocyte homing by F1 and F2.** A–C, CFSE-labeled lymphocytes (2.0 × 10^7 cells) were injected into the tail vein of WT (A), GlcNAc6ST-1 and GlcNAc6ST-2 DKO (B), or C1βGnT and C2GnT-I DKO mice (C). One hour after injection, CFSE-labeled lymphocytes in lymphocyte suspensions from the PLNs, MLNs, and PPs were quantified by flow cytometry. The mice were preinjected with F1 or F2 (200 μg/mouse) or with PBS 2 h before the injection of CFSE-labeled lymphocytes. Lymphocyte homing to different lymphoid organs in the mAb-injected animals is shown as a percentage of that observed in PBS-injected animals, which was set as 100%. Bars, mean ± S.D. (error bars). In A, n = 5. In B and C, n = 4. **, p < 0.01; ***, p < 0.001 versus PBS-injected control mice. D, binding of the mAbs F1 and F2 to HEVs in mouse PLNs, MLNs, and PPs. Immunofluorescence of acetone-fixed, frozen sections of PLNs, MLNs, and PPs from WT mice and FucT-IV and FucT-VII DKO mice reacted with biotinylated F1 or F2 (0.5 μg/ml), followed by streptavidin-Alexa Fluor 594 (0.5 μg/ml). Nuclear staining was performed with DAPI (blue). Dotted lines, outline of HEV. Bar, 100 μm. E, inhibition of the L-selectin-dependent adhesion of splenocytes to PLN HEVs by F1 and F2 in a modified Stamper-Woodruff cell-binding assay. The number of splenocytes that had been preincubated with or without 10 μg/ml MEL-14 that bound to a PLN HEV that had been preincubated with or without 10 μg/ml F1, F2, or MECA-79 is plotted. The number of HEVs analyzed per sample (n) is indicated at the bottom. The horizontal red lines represent the average number of leukocytes bound per HEV. ***, p < 0.001 versus treatment with mAb control.
in the elicitation phase. In accordance with this possibility, F1 and F2 more efficiently inhibited CHS responses than S2 that selectively inhibits L-selectin-dependent lymphocyte homing to PLNs (11) under Schedule 1. Administration of F1 and F2 to the hapten-sensitized mice before the second hapten challenge (Schedule 2) clearly indicated that blocking leukocyte infiltration in the ear during the elicitation phase was effective in blocking the CHS responses. We also provided evidence that F1 and F2 indeed block leukocyte rolling in the inflamed ear in vivo, as evidenced by intravital fluores-
cence microscopy (Fig. 9). These findings suggest the interesting clinical implication that blocking acute leukocyte infiltration could be an effective way to block allergic reactions, even in allergen-sensitized individuals.

Because both F1 and F2 bound not only mouse but also human tissues and leukocytes, these mAbs will be useful for assessing the expression of sLeX in pathophysiological situations in both humans and mice. HEV-like vessels are induced at various sites of chronic inflammation. For example, MECA-79-reactive HEV-like vessels are induced in human gastric mucosa infected with *Helicobacter pylori* (29), in ulcerative colitis (30), and in rheumatoid arthritis (31). Because F1 and F2 strongly block selectin-mediated leukocyte trafficking, those mAbs will be useful for examining the roles of sLeX and its related structures at sites of chronic inflammation using animal models.

In conclusion, we have developed novel anti-sLeX mAbs reactive with both human and mouse tissues. Because these mAbs block leukocyte trafficking mediated by all three selectins in vivo and can be used in immunohistochemical as well as flow cytometric studies, future studies using these mAbs will advance our understanding of the role of sLeX in health and disease.

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