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Anti-oligosaccharide antibodies as tools for studying sulfated sialoglycoconjugate ligands for siglecs and selectins

Reiji Kannagi · Katsuyuki Ohmori · Naoko Kimura

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Abstract Several sulfated sialoglycoconjugates have recently been shown to serve as ligands for selectins and siglecs. For instance, α2→3 sialylated 6-sulfo-Lewis x was found to serve as a ligand for selectins on skin-homing helper memory T cells, and α2→6 sialylated 6-sulfo-LacNAc to be a preferred ligand for CD22/siglec-2 on human naïve B cells. Monoclonal antibodies specific to sulfated sialoglycoconjugates are effective tools to dissect these ligands on minor subsets of human leukocytes.

Keywords Selectin · Siglec · CD22 · Skin-homing helper memory T-lymphocytes · Cutaneous lymphocyte antigen · Naïve B-lymphocytes

Abbreviations

CD	cluster of differentiation
CLA	cutaneous lymphocyte antigen
ELISA	enzyme-linked immunosorbent assay

Introduction

Biological functions of glycoconjugates having both sialic acid residues and sulfation in their structure have been known only recently. Perhaps the first example is an L-selectin ligand, α2→3 sialylated 6-sulfo-Lewis x, present on high endothelial venules of peripheral lymphocyte, mediating lymph node homing of naïve T cells [1–4]. A second example would be siglec-8, which has been known to recognize α2→3 sialylated 6′-sulfo-Lewis x [5, 6].

To identify specific ligands for carbohydrate-recognition molecules such as selectins and siglecs, first of all we need to show that the glycoconjugate in question binds to the carbohydrate-recognition molecules. This could be easily demonstrated by *in vitro* enzyme-linked immunosorbent assays (ELISA), in which the candidate glycoconjugates are immobilized in wells of plastic plates, and tested for the reactivity of recombinant soluble carbohydrate-recognition molecules. To date, many glycoconjugates having both sialic acid residues and sulfation in their structures are listed in the website of the consortium for functional glycomics (http://www.functionalglycomics.org/), which bind to recombinant carbohydrate-recognition molecules such as siglecs in ELISA-based assays.

The reactivity in ELISA, however, does not always warrant reactivity at the cellular level. The reactivity in ELISA also does not guarantee that the glycoconjugates serve as endogenous ligands in real physiological settings. To know the biological significance of a candidate ligand, we need to demonstrate (1) the cognate ligand is really expressed *in situ* on the cells where the carbohydrate recognition is supposed to occur; and (2) the cognate ligand is actually involved in the carbohydrate recognition occurring on the cells under physiological settings. Monoclonal anti-oligosaccharide antibodies serve as useful tools for these experiments, since they can disclose the distribution of a given cognate ligand in biological samples, and can inhibit its binding to carbohydrate-recognition molecules *in situ*. The antibodies are particularly useful when the

R. Kannagi (✉) · N. Kimura
Department of Molecular Pathology,
Research Institute, Aichi Cancer Center,
1-1 Kanokoden, Chikusaku,
Nagoya 464-8681, Japan
e-mail: rkannagi@aichi-cc.jp

K. Ohmori
Department of Clinical Pathology,
School of Medicine, Kyoto University,
Kyoto 606-8501, Japan

population of cells expressing the cognate ligands is a minor subset of cells.

The list of potentially useful monoclonal antibodies directed to glycoconjugates having both sialic acid residues and sulfate modification in their structures are listed in Table 1. In this study we will introduce several examples of the application of anti-oligosaccharide antibodies in the identification of endogenous ligands for selectins and siglecs in the defined subsets of human lymphocyte population.

Methods

Identification of selectin ligand on skin-homing human helper memory T-lymphocytes

Cutaneous lymphocyte antigen (CLA) defined by the HECA-452 antibody has been known to be specifically expressed on human skin-homing lymphocytes, and serves as a ligand for selectins [7]. Although the HECA-452 antibody reacts to the sialyl Lewis x determinant, distribution of CLA among human peripheral lymphocytes is significantly different from that of sialyl Lewis x, which is defined by classical anti-CD15s antibodies such as CSLEX-1. Thus, CLA has been thought to be a mixture of glycoconjugates highly related to, but at least partly different from, the genuine sialyl Lewis x determinant.

The HECA-452 antibody is known to have a broad specificity. It reacts to sialyl Lewis a determinant in addition to the sialyl Lewis x determinant [8]. Sometime ago we showed that the antibody reacted to α2→3 sialylated 6-sulfo-Lewis x as well as the conventional sialyl Lewis x determinant [2, 9]. This led us to test the possibility that CLA determinant expressed on skin-homing T lymphocytes would be the sialyl 6-sulfo Lewis x determinant, using a newly generated antibody G152, which was reactive to α2→3 sialylated 6-sulfo-Lewis x, but not cross-reactive to sialyl Lewis x.

The protocol for analysis of the binding of E-, P- or L-selectin to the T-lymphocyte subset is as follows [10].

1. Recombinant human E-, P- and L-selectin-IgG chimera can be obtained from various sources, such as R&D Systems Inc (Minneapolis, MN). Recombinant selectins were preincubated with affinity-purified biotinylated rabbit anti-human IgG (Dako, Glostrup, Denmark) followed by incubation with phycoerythrin–streptavidin (Dako), before application to the staining of peripheral lymphocytes. The optimum molar ratio of these molecules should be determined beforehand through preliminary experiments using the same lot of biotinylated

Table 1 Antibodies directed to sulfated and sialylated glycoconjugates

Antibody	Isotype	Determinant detected	References
Antibodies specific to sulfated and sialylated determinant			
G152	Mouse IgM	α2→3 sialylated 6-sulfo-Lewis x	[2]
G72	Mouse IgM	α2→3 sialylated 6-sulfo-Lewis x/LacNAc[a]	[2]
G2706, G27011	Mouse IgM	α2→3 sialylated 6,6′-disulfo-Lewis x	[2]
KN343	Mouse IgM	α2→6 sialylated 6-sulfo-LacNAc	[12]
Cross-reactive antibodies[b]			
HECA-452	Rat IgM	α2→3 sialylated Lewis x/a and α2→3 sialylated 6-sulfo-Lewis x	[2, 8]
2F3	Mouse IgM	α2→3 sialylated Lewis x and α2→3 sialylated 6-sulfo-Lewis x	[2, 16]
2H5	Mouse IgM	α2→3 sialylated Lewis x and α2→3 sialylated 6-sulfo-Lewis x	[2, 17]
Control antibodies			
CSLEX-1	Mouse IgM	α2→3 sialylated Lewis x[c]	[2, 18]
GL7	Rat IgM	α2→6 sialylated LacNAc[d]	[12, 19]
Antibodies to sulfated but non-sialylated determinant			
AG223	Mouse IgM	6-sulfo-Lewis x	[20]
AG107, AG105[e]	Mouse IgM	6-sulfo-Lewis x/LacNAc	[3, 21, 22]
MDC-8	Mouse IgM	6-sulfo-LacNAc[f]	[23]
PGM34	Mouse IgM	6-sulfated blood group H determinant	[24]
SU59	Mouse IgM	3′-sulfo-Lewis x[g]	[2, 25]
F2	Mouse IgM	3′-sulfo-Lewis a	[26]

[a] This antibody reacts to both α2→3 sialylated 6-sulfo-Lewis x and α2→3 sialylated 6-sulfo-LacNAc.
[b] These antibodies were shown to be directed to sialylated determinants, then later found to cross-react to some sialylated-sulfated determinants.
[c] This antibody does not cross-react to α2→3 sialylated 6-sulfo-Lewis x.
[d] This antibody does not cross-react to α2→6 sialylated 6-sulfo-LacNAc.
[e] Other antibodies having a similar specificity include AG97 and AG273.
[f] This antibody does not cross-react to 6-sulfo-Lewis x.
[g] This antibody shows a weak cross-reactivity to 3′-sulfo-Lewis a.

antibody and phycoerythrin–streptavidin reagents. Prepare a tube containing 10 μl of recombinant selectin-immune complex solution. To this, 100 μl of freshly obtained whole human blood sample (anti-coagulated using EDTA) is added, and the tube is incubated at 4°C for 30 min. After incubation, add 4 ml of PBS, and wash the cells by centrifuging at 500×*g* for 5 min. Repeat the washing procedures twice.

2. Add 50 μl of FITC-labeled anti-murine IgM antibody (100 times diluted) and incubate at 4°C for 30 min. After incubation, add 4 ml of PBS, and wash the cells by centrifuging at 500×*g* for 5 min. Repeat the washing procedure twice.
3. If necessary, add 5 μl each of APC-labeled anti-CD3 antibody SK7 (Leu4), PC5-labeled anti-CD4 antibody 13B8.2, PE-labeled anti-CD45RO antibody UCHL-1 (Leu45RO) and incubate at 4°C for 30 min.
4. Add 2 ml of BD FACS™ Lysing Solution (Becton Dickinson, diluted 10 times with distilled water) and allow to stand at room temperature for 10 min to lyse red blood cells. Centrifuge the cells at 500×*g* for 5 min and discard the supernatant. Add 4 ml of PBS, and wash the cells once by centrifugation at 500×*g* for 5 min.
5. Re-suspend the cells in 0.5 ml PBS. The stained cells are then subjected to flow-cytometric analyses.

For binding inhibition experiments, the cells are preincubated with blocking antibodies (10–40 μg/ml) for 30 min before step 2. Note that the α2→3 sialylated 6-sulfo-Lewis x determinant on peripheral T-lymphocytes is unstable, and the use of whole blood samples is necessary for maximum detection. Purification of lymphocytes using Ficoll–Hypaque solution with multiple washings is not recommended.

The experimental results indicate that the α2→3 sialylated 6-sulfo-Lewis x is expressed on a subset of helper memory T-lymphocytes, and serves as a ligand for E-, P- and L-selectins (Fig. 1). Unexpectedly, the determinant was shown to be limited to so-called central helper memory T cell subset [10]. CLA is known to be expressed eventually on other T-lymphocyte subsets, such as effector helper memory T cells and cytotoxic T cells. These results leave the possibility that

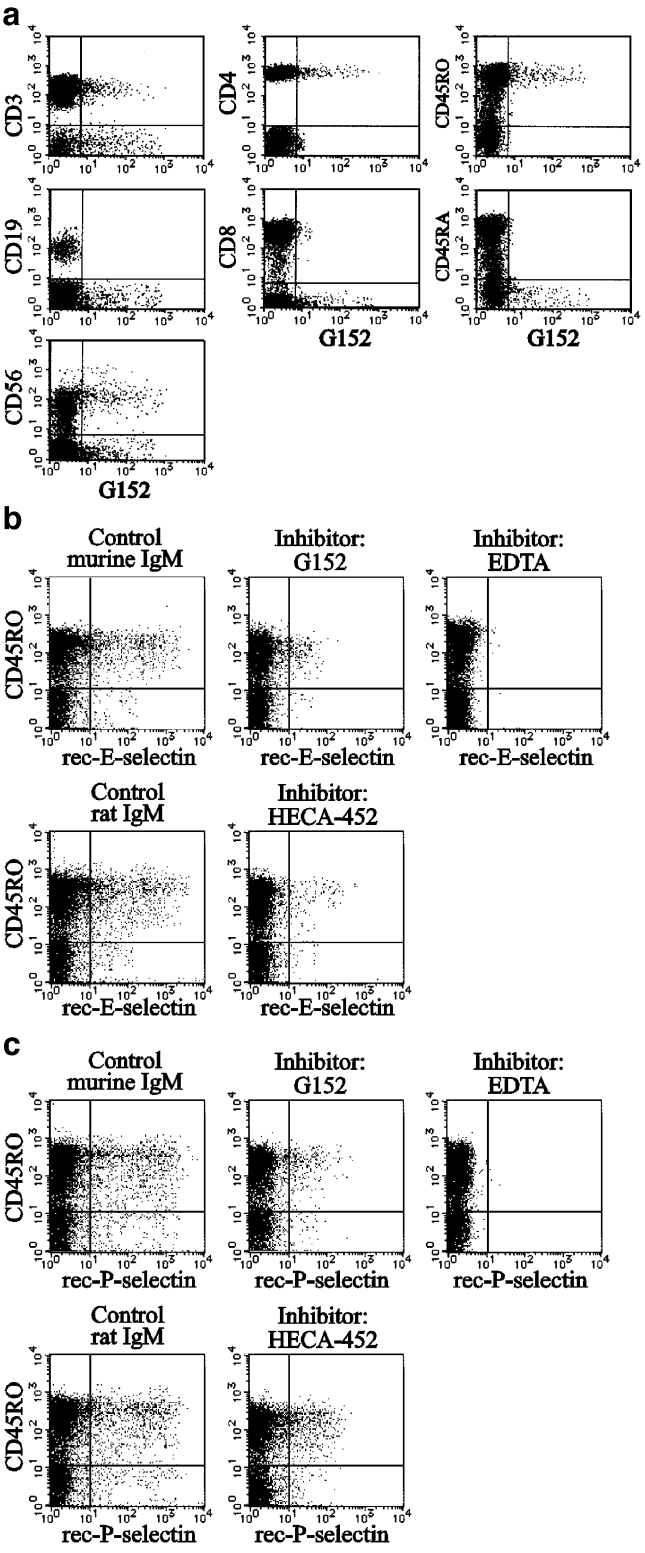

Fig. 1 Inhibition of E- and P-selectin binding to human peripheral T-lymphocytes by antibody specific to α2→3-sialylated 6-sulfo-Lewis x determinant. **a** Expression of α2→3-sialylated 6-sulfo-Lewis x determinant defined by the *G152* antibody in peripheral lymphocytes of healthy individuals. Markers for lymphocyte subset include *CD3* (T-lymphocytes), *CD4* (helper T-lymphocytes), *CD8* (killer T-lymphocytes), *CD45RA* (naive T-lymphocytes), *CD45RO* (memory T-lymphocytes), *CD19* (B-lymphocytes) and *CD56* (NK cells). Note that the α2→3-sialylated 6-sulfo-Lewis x determinant is preferentially expressed in a small subset of helper memory T-lymphocytes, which further analyses revealed to be the subset composed of skin-homing central helper memory T-lymphocytes [10]. **b** and **c** Binding of recombinant E-selectin (**b**) or P-selectin (**c**) to helper memory T-lymphocytes in the presence or absence of inhibitory antibodies (*G152*, 35 μg/ml or *HECA-452*, 20 μg/ml). Murine IgM was used as a control for G152, and rat IgM as that for HECA-452. The staining pattern with EDTA is shown as a negative control. Note that binding of E- or P-selectin-Fc to helper memory T-lymphocytes is significantly reduced by the G152 antibody as well as the HECA-452 antibody. Adopted from reference [10]

CLA detected sometimes in effector helper memory T cells and cytotoxic T cells could be the conventional sialyl Lewis x determinant.

Identification of CD22/siglec-2 ligand on human naïve B-lymphocytes

CD22/siglec-2, an important negative regulator of B-lymphocyte signalling, had long been known to recognize α2→6 sialylated glycoconjugates as specific ligands. Results of ELISA-based binding experiments suggested that sulfated α2→6 sialoglycoconjugates could be better ligands for CD22/siglec-2 [11]. Recently, we found α2→6 sialylated 6-sulfo-LacNAc to be a much preferred CD22/siglec-2 ligand on human naïve B-lymphocytes [12]. A similar method per above was applied to identify the endogenous CD22/siglec-2 ligand on human naïve B-lymphocytes. In this case, a monoclonal antibody specific to α2→6 sialylated 6-sulfo-LacNAc (KN343) was applied, with a control antibody GL7, which is specific to non-sulfated α2→6 sialylated LacNAc.

The results indicated that the α2→6 sialylated 6-sulfated LacNAc determinant is expressed on human peripheral B-lymphocytes (Fig. 2a), and that the antibody directed to α2→6 sialylated 6-sulfo-LacNAc strongly inhibited binding of CD22/siglec-2 to peripheral B-lymphocytes (Fig. 2b) [12]. In contrast, the antibody directed to non-sulfated α2→6 sialylated LacNAc showed no appreciable inhibitory activity. These findings established that α2→6 sialylated 6-sulfated glycoconjugates are much preferred ligands for CD22/siglec-2. The expression of the sulfated ligand tends to disappear when naïve B-lymphocytes differentiate into memory cells.

Remarks and conclusion

Antibodies directed to sulfated sialoglycoconjugate are useful tools for studying endogenous ligands for siglecs and selectin expressed on limited subpopulation of leukocytes. For methods for generation of anti-oligosaccharide antibodies, kindly refer to references [13, 14]. A more

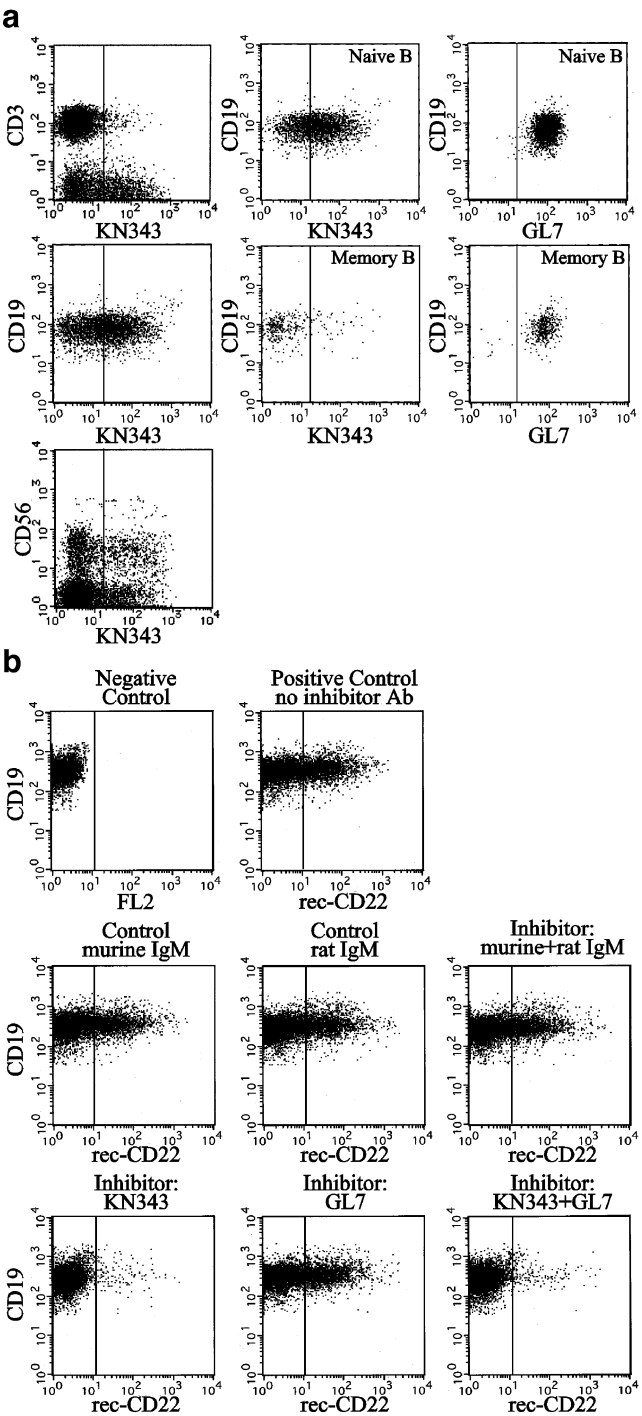

Fig. 2 Inhibition of CD22/siglec-2 binding to human peripheral B-lymphocytes by antibody specific to α2→6-sialylated 6-sulfo-LacNAc determinant. **a** Expression of α2→6-sialylated 6-sulfo-LacNAc determinant defined by the KN343 antibody in peripheral lymphocytes of healthy individuals. Markers for lymphocyte subset include *CD3* (T-lymphocytes), *CD19* (B-lymphocytes) and *CD56* (NK cells). Naïve B-lymphocytes were gated as $IgD^{+}CD27^{-}CD19^{+}$ cells, and memory B-lymphocytes as $IgD^{-}CD27^{+}CD19^{+}$ cells. Expression of non-sulfated α2–6-sialylated LacNAc determinant defined by the GL7 antibody in B-lymphocyte subsets is also shown in the *right-hand panels*. Note that the α2→6-sialylated 6-sulfo-LacNAc determinant is preferentially expressed in naïve B-lymphocytes compared to memory B-lymphocytes, while the non-sulfated α2–6-sialylated LacNAc determinant is expressed equally in both subsets. **b** Binding of recombinant CD22-Fc to $CD19^{+}$B-lymphocytes in the presence or absence of inhibitory antibodies (*KN343* and/or *GL7*, 20 μg/ml). The staining pattern without recombinant CD22-Fc is shown as a negative control, and the staining pattern by recombinant CD22-Fc without inhibitor antibody is shown as a positive control. Murine IgM was used as a control for KN343, and rat IgM as that for GL7. Note that binding of CD22-Fc to B-lymphocytes is significantly reduced by the KN343 antibody, but not by the GL7 antibody. Adopted from reference [12]

comprehensive listing of anti-oligosaccharide antibodies may be found in reference [15].

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