ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 5, pp. 496-504. © Pleiades Publishing, Inc., 2006.
Original Russian Text © E. M. Rapoport, G. V. Pazynina, M. A. Sablina, P. R. Crocker, N. V. Bovin, 2006, published in Biokhimiya, 2006, Vol. 71, No. 5, pp. 615-625.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM05-263, April 2, 2006.

Probing Sialic Acid Binding Ig-Like Lectins (Siglecs) with Sulfated Oligosaccharides

E. M. Rapoport[1], G. V. Pazynina[1], M. A. Sablina[1], P. R. Crocker[2], and N. V. Bovin[1]*

[1]*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (7-495) 330-5592; E-mail: bovin@carbohydrate.ru*
[2]*The Wellcome Trust Biocentre, School of Life Sciences, University of Dundee DD1 5EH, Scotland, UK; E-mail: p.r.crocker@dundee.ac.uk*

Received December 1, 2005
Revision received February 3, 2006

Abstract—Soluble siglecs-1, -4, -5, -6, -7, -8, -9, and -10 were probed with polyacrylamide glycoconjugates in which: 1) the Neu5Ac residue was substituted by a sulfate group (Su); 2) glycoconjugates contained both Su and Neu5Ac; 3) sialoglycoconjugates contained a tyrosine-O-sulfate residue. It was shown that sulfate derivatives of LacNAc did not bind siglecs-1, -4, -5, -6, -7, -8, -9, and -10; binding of 6′-O-Su-LacNAc to siglec-8 was stronger than binding of 3′SiaLacNAc. The relative affinity of 3′-O-Su-TF binding to siglecs-1, -4, and -8 was similar to that of 3′SiaTF. 3′-O-Su-Le^c displayed two-fold weaker binding to siglec-1 and siglec-4 than 3′SiaLe^c. The interaction of soluble siglecs with sulfated oligosaccharides containing sialic acid was also studied. It was shown that siglecs-1, -4, -5, -6, -7, -9, and -10 did not interact with these compounds; binding of 6-O-Su-3′SiaLacNAc and 6-O-Su-3′SiaTF to siglec-8 was weaker than that of the corresponding sulfate-free sialoside probes. Siglec-8 displayed affinity to 6′-O-Su-LacNAc and 6′-O-Su-SiaLe^x, and defucosylation of the latter compound led to an increase in the binding. Sialoside probes containing tyrosine-O-sulfate residue did not display increased affinity to siglecs-1 and -5 compared with glycoconjugates containing only sialoside. Cell-bound siglecs-1, -5, -7, and -9 did not interact with 6-O-Su-3′SiaLacNAc, whereas the sulfate-free probe 3′SiaLacNAc demonstrated binding. In contrast, the presence of sulfate in 6-O-Su-6′SiaLacNAc did not affect binding of the sialoside probe to siglecs. 6′-O-Su-SiaLe^x displayed affinity to cell-bound siglecs-1 and -5; its isomer 6-O-Su-SiaLe^x bound more strongly to siglecs-1, -5, and -9 than SiaLe^x.

DOI: 10.1134/S0006297906050051

Key words: sialic acid, siglecs, sulfated oligosaccharides, carbohydrate specificity

Proteoglycans and sulfated glycoproteins play an important role in intercellular interactions during inflammatory processes and malignant transformation [1]. Being receptors for integrins, growth factors, and lectins, they mediate a variety of processes such as intercellular adhesion, cell adhesion to extracellular matrix, proliferation, and apoptosis [1, 2]. For example, the initial phase of inflammation involves leukocyte adhesion to vascular endothelium and migration of leukocytes along the vascular wall as a consequence of the interaction of selectins with cognate ligands. Endothelial P-selectin binds to leukocyte glycoprotein PSGL-1; this binding mechanism includes interaction with SiaLe^x residues of a carbohydrate chain and with a cluster of tyrosine-O-sulfate residues located on the PSGL-1 polypeptide chain [3-5]. In addition, the binding of leukocyte L-selectin with 6-O-Su-SiaLe^x exposed on endothelium plays an important role in lymphocyte homing; in this case, the functionally important sulfate group is located on the carbohydrate chain [2, 6]. Lysis of virus-infected and tumor cells by natural killer (NK) cells involves binding of NK cell receptors (NKp30 and NKp46) with heparin sulfate of target cells [7]. 6-O-Su-3′SiaLacNAc is a receptor of

Abbreviations: BSA) bovine serum albumin; CHO-siglec) CHO cells transfected with siglec vector; fluo) fluorescein; Glyc–PAA) polyacrylamide glycoconjugate; PBA) PBS containing 0.2% BSA; PBS) phosphate buffered saline containing 0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.15 M NaCl, pH 7.3; siglec-Fc) chimeric protein containing full-sized extracellular region hSiglec and Fc region of human IgG; sTyr) sulfotyrosine (tyrosine-O-sulfate); TBS) 50 mM Tris-HCl buffer containing 150 mM NaCl, pH 7.5; TBA) TBS containing 0.2% BSA; Versene solution) PBS containing 0.02% EDTA.

* To whom correspondence should be addressed.

avian influenza virus H5N1, which can infect humans [8].

Recently it has been shown that the mucin fragments, sulfated carbohydrate chains 3′-O-Su-Galβ1-3GalNAcβ and 3′-O-Su-Galβ1-3GlcNAcα bind galectin-4 and galectin-1, respectively [9-11], but the biological significance of these results is unknown. Taking into consideration the increased expression of galectin-1 and galectin-4 in stomach cancer, it is possible that binding of galectin-1 and galectin-4 to sulfated glycans influences adhesion of tumor cells to extracellular matrix during metastasis.

There is convincing evidence that glycoconjugate sulfation, involving both glycan and polypeptide fragments, represents a common mechanism regulating lectin-mediated interactions. However, amongst the families of mammalian lectins, the interaction of siglecs (sialic acid binding Ig-like lectins) with sulfated glycans and the putative regulatory role (potentiation or attenuation) of sulfates in the siglec-ligand recognition has not been systemically studied. Siglecs are sialoside-binding lectins containing immunoglobulin domains that can interact with both glycoproteins and glycolipids [12-14]. There are eleven known siglecs in humans and all except siglec-4 (myelin associated protein) are found on cells of the hematopoietic and immune systems.

Siglecs are divided into two groups. Sialoadhesin (siglec-1), CD22 (siglec-2), and siglec-4 constituting the first group share 25-30% homology. The second group comprises the CD33-related siglecs, which share 50-80% homology with siglec-3 (CD33) [12, 13]. In humans, all siglecs of this group are encoded by genes located on 19q13.3-4 chromosome and mainly expressed on leukocytes of the innate immune system. The cytoplasmic region of these siglecs contains two conserved tyrosine-based motifs, including a classical immune receptor tyrosine-based inhibitory motif (ITIM), which when tyrosine phosphorylated mediates binding and activation of the protein tyrosine phosphatases SHP-1 and SHP-2. A number of studies on the structure and carbohydrate specificity of siglecs have been reported. Siglec-2 exhibits high specificity towards Neu5Acα2-6Gal, whereas siglec-1 and siglec-4 prefer Neu5Acα2-3Gal; siglecs-5, -7, -9, and -10 bind both Neu5Acα2-3Gal- and Neu5Acα2-6Gal-containing glycans. Siglec-7 and siglec-11 interact with Sia_2-containing glycans, whereas siglec-6 binds to SiaTn [14-17]. It has recently been shown that substitution of sialic acid for sulfate group in internal chains of GD1aα ganglioside results in increase in siglec-1 and siglec-4 binding [18-20]. Siglec-8 binds to 6′-O-Su-$SiaLe^x$ and siglec-9 binds to 6-O-Su-$SiaLe^x$; in both cases the binding is stronger compared with the trisaccharide 3′SiaLacNAc [21-24].

The goal of this study was to investigate systematically the interaction of siglecs with glycoconjugates containing sulfated moieties. We have studied siglec interactions with three classes of O-sulfate containing glycoconjugates. These included: 1) sulfated and non-sialylated glycoconjugates; 2) sulfated and sialylated glycoconjugates; 3) glycoconjugates containing sTyr in addition to sialylated oligosaccharides.

MATERIALS AND METHODS

The following reagents were used in this study: BSA (Serva, Germany); calf kidney α-L-fucosidase (Boehringer Mannheim, Germany); *Vibrio cholerae* neuraminidase, *p*-nitrophenyl phosphate, alkaline phosphatase labeled goat antibodies against human IgG Fc (Sigma, USA); RPMI-1640 cultivation medium, fetal calf serum, glutamine, and antibiotics-antimycotics (Invitrogen, UK).

CHO cells expressing full-length mouse siglec-1 and human siglecs-5, -7, and -9 were obtained as described [25, 26]. Supernatants containing soluble siglecs were from stably-transfected CHO cell lines. Soluble siglecs are chimeras of the extracellular regions fused to the Fc region of human IgG; siglec-1-Fc contains the first three N-terminal Ig domains [25]. Besides the wild-type form of siglec-1, we also used the Arg97Asp mutant, which inactivates the carbohydrate-binding site [26]. This mutant serves as a useful negative control in binding assays.

Neoglycoconjugates Glyc–PAA (30 kD, 20 mole % carbohydrates), Glyc–PAA–fluo (30 kD, 20 mole % carbohydrates and 1 mole % fluorescein), Glyc–PAA–sTyr (30 kD, 20 mole % carbohydrate and 10 mole % tyrosine-O-sulfate), and also tyrosine-O-sulfate polyacrylamide conjugates sTyr–PAA (30 kD; 10, 40, and 70 mole % tyrosine-O-sulfate) were synthesized as described in [27]. Table 1 shows the structures of the glycoconjugates employed in this study.

ELISA. A solution of Glyc–PAA (initial concentration 250 μM) in 0.1 M $NaHCO_3$ buffer, pH 9.6, was coated on 96-well plates Nunc Maxisorb (Nunc, Denmark) in two-fold serial dilutions. The plate was initially incubated at 37°C for 3 h and then overnight at 4°C. After incubation of the plate with TBS solution containing 2% BSA at 37°C for 1 h, a pre-complexed mixture of siglec-Fc and alkaline phosphatase-conjugated antibodies against human immunoglobulin Fc domain was added (the optimal siglec-conjugate ratio in the complex was 1 : 1 [25], and the dilution of conjugate in TBA was 1 : 320). The plate was incubated at 37°C for 3 h, then washed three times with TBA solution and after addition of *p*-nitrophenyl phosphate (1 mg/ml) in buffer containing 200 mM diethanolamine and 2 mM $MgCl_2$, pH 10.5, the absorbance was read at 405 nm using a Spectrocount 340 plate reader (Bio-Rad, USA). LacNAc–PAA was used as a negative control.

For inhibition assays, various concentrations of Glyc–PAA conjugates were preincubated with pre-com-

Table 1. List of saccharides and their designations

Designation	Structure
Neu5AcαOBn	Neu5AcαOCH$_2$C$_6$H$_4$NHCOCH$_2$–
Neu5Acα–sp	Neu5AcαOCH$_2$CH$_2$CH$_2$–
3'SiaLacNAc	Neu5Acα2-3Galβ1-4GlcNAc–sp–
3-O-Su-LacNAc	Galβ1-4(*3HSO*$_3$)GlcNAc–sp–
3'-O-Su-LacNAc	*3HSO*$_3$-Galβ1-4GlcNAc–sp–
6-O-Su-LacNAc	Galβ1-4(*6HSO*$_3$)GlcNAc–sp–
6'-O-Su-LacNAc	*6HSO*$_3$-Galβ1-4GlcNAc–sp–
4',6'-di-O-Su-LacNAc	(*4,6-HSO*$_3$)$_2$Galβ1-4GlcNAc–sp–
6'-O-Su-Lac	*6HSO*$_3$-Galβ1-4Glc–sp–
6-O-Su-Lac	Galβ1-4(*6HSO*$_3$)Glc–sp–
3',6-di-O-Su-Lac	*3HSO*$_3$Galβ1-4(*6HSO*$_3$)Glc–sp–
6'SiaLacNAc	Neu5Acα2-6Galβ1-4GlcNAc–sp–
6-O-Su-3'SiaLacNAc	Neu5Acα2-3Galβ1-4 (*6HSO*$_3$)GlcNAc–sp–
6-O-Su-6'SiaLacNAc	Neu5Acα2-6Galβ1-4 (*6HSO*$_3$)GlcNAc–sp–
3'SiaTF	Neu5Acα2-3Galβ1-3GalNAcα–sp–
6-O-Su-3'SiaTF	Neu5Acα2-3Galβ1-3 (*6HSO*$_3$)GalNAcα–sp–
SiaLe^a	Neu5Acα2-3Galβ1-3(Fucα1-4) GlcNAc–sp–
SiaLe^x	Neu5Acα2-3Galβ1-4(Fucα1-3) GlcNAc–sp–
6-O-Su-SiaLe^x	Neu5Acα2-3Galβ1-4(Fucα1-3) (*6-HSO*$_3$)GlcNAc–sp–
6'-O-Su-SiaLe^x	Neu5Acα2-3(*6HSO*$_3$)Galβ1-4 (Fucα1-3)GlcNAc–sp–
SiaTn	Neu5Acα2-6GalNAcα–sp–
Sia$_2$	Neu5Acα2-8Neu5Acα–sp–

Note: sp = OCH$_2$CH$_2$ or OCH$_2$CH$_2$CH$_2$.

plexed siglec-Fc and alkaline phophatase-conjugated antibodies against human immunoglobulin Fc at 37°C for 2 h. This mixture was added to wells containing immobilized Neu5AcαOBn–PAA (50 μg/ml for siglec-1, 10 μg/ml for siglec-4, 40 μg/ml for siglec-5) or 6′SiaLac–PAA (200 μg/ml for siglec-2, 100 μg/ml for siglec-6, 40 μg/ml for siglec-7, 10 μg/ml for siglec-10), or 3′SiaLacNAc–PAA (100 μg/ml for siglec-8, 40 μg/ml for siglec-9) in 0.1 M NaHCO$_3$ buffer, pH 9.6. The plate was incubated at 37°C for 3 h and then washed three times with TBA solution and results read after addition of *p*-nitrophenyl phosphate as described above.

Flow cytometry. The CHO cells expressing siglecs on the cell surface, CHO-siglec-1, CHO-siglec-5, CHO-siglec-7, and CHO-siglec-9, were cultivated in RPMI-1640 medium containing 10% fetal calf serum and 2 mM glutamine. Cells were detached from the plastic with Versene solution and washed by PBA centrifugation at 1200 rpm (CR3, Jouan, France). Aliquots (50 μl) of Glyc–PAA–fluo (100 μM concentration by Glyc) in PBA were added to wells of a U-shape plate (Nunc) containing $2 \cdot 10^5$ cells in 100 μl. After incubation at 4°C for 40 min, cells were washed three times in PBA followed by centrifugation at 1200 rpm. Cells were analyzed using an EPICS ELITE Coulter laser flow cytofluorimeter (Beckman, USA) at 488 nm. The cell suspension (100 μl) was mixed with 2 ml of PBS and after stirring cell fluorescence was measured at room temperature. Results were analyzed using the WiNMDI 2.8 program. In each sample, at least 5000 cells were analyzed.

Cell desialylation. Cells were washed three times in RPMI-1640 medium (without additions). An aliquot (95 μl) of cell suspension in the same medium (106 cells/ml) was mixed with 5 μl of *V. cholerae* neuraminidase (2 U/ml). The mixture was incubated at 37°C for 3 h and after this incubation the cells were washed with PBA.

The enzymatic cleavage of 6′-O-Su-SiaLe^x–PAA was carried out as follows: glycoconjugate (300 μg) was incubated overnight with calf kidney α-L-fucosidase (2 U/ml) at 37°C or with *V. cholerae* neuraminidase (2 U/ml) at 37°C for 3 h.

RESULTS

Test system for study of soluble siglecs. Siglecs as well as most monovalent lectins exhibit low affinity for their ligands. Stable binding depends on multivalent presentation and high avidity interactions. The ELISA method used in previous studies employed coating plastic wells with bivalent recombinant siglecs and measurement of their interaction with biotinylated multivalent sialosides using streptavidin. However, this system is potentially restricted by the narrow range of concentrations of the adsorbed protein due to threshold effects [25]. In the solid phase system used in the current experiments, we coated plastic wells with serial two-fold dilutions of glycoconjugate to which was added a constant concentration of soluble siglecs, precomplexed in a multivalent manner with antibodies against IgG Fc region. Figure 1a shows that the glycoconjugate Neu5AcOαBn exhibited the highest affinity to siglec-1; LacNAc (lacking sialic acid and used as the negative control) did not interact with siglec-1. The mutant siglec-1 carrying Arg97→Asp substitution in the carbohydrate-binding domain (and used

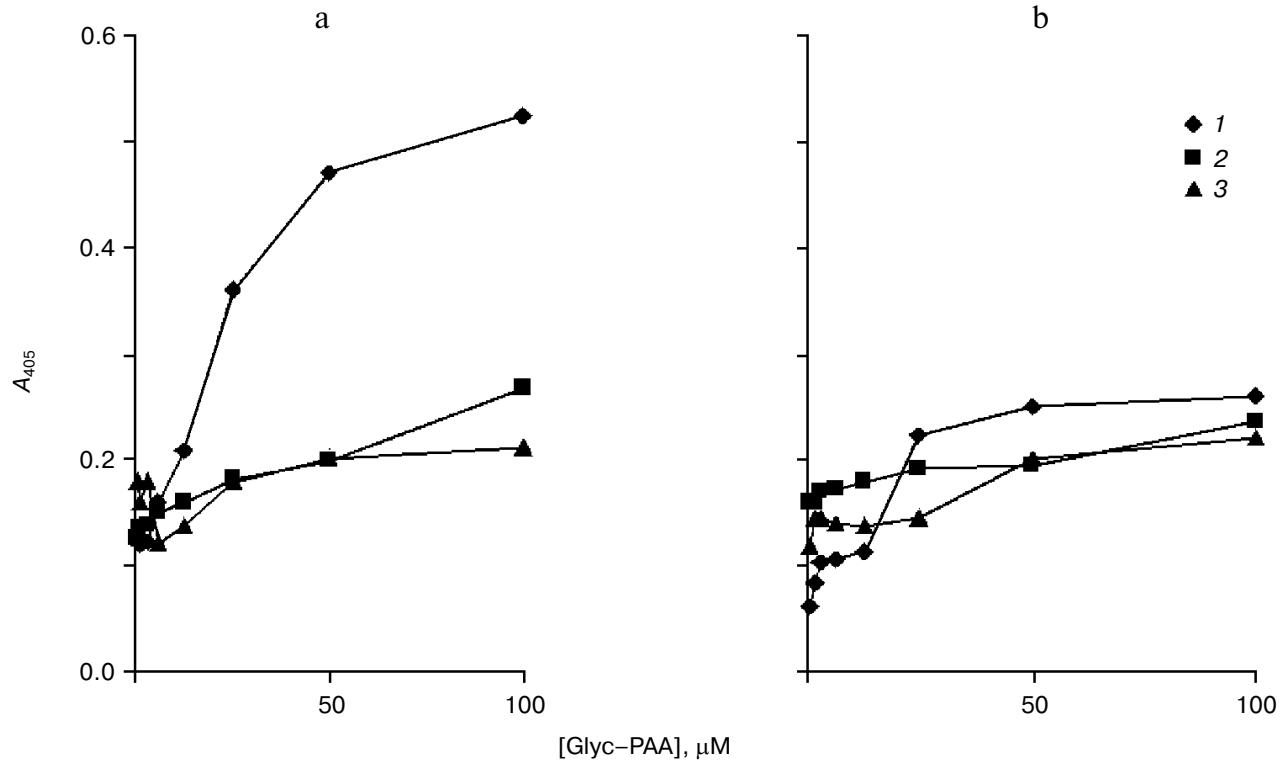

Fig. 1. Binding of siglec-1 (a) and its mutant form (b) with Neu5AcαOBn–PAA (*1*), Neu5Acα–sp–PAA (*2*), and LacNAc–PAA (*3*).

as the other negative control) did not bind Neu5AcαOBn (Fig. 1b). This is consistent with previous data [26, 28] and demonstrates the specificity of siglec–ligand binding analyzed in our system. The glycoconjugates exhibiting the highest binding with siglecs in the direct binding test were subsequently used for coating plates in the inhibitory test.

Interaction of soluble siglecs with O-sulfated Glyc–PAA lacking sialic acid. We investigated the potential inhibitory activity of sulfated derivatives of LacNAc, TF, and Lec lacking sialic acid on the interaction of soluble siglecs-1, -4, -5, and -8 with Neu5AcαOBn–PAA, siglecs-6 and -7 with 6′SiaLac–PAA, and siglecs-9 and –10 with 3′SiaLacNAc–PAA. Table 2 shows that only in one case was there evidence for interaction with a sulfated (but not sialylated) ligand, namely the interaction of siglec-8 with 6′-O-Su-LacNAc. This compound was an even more active inhibitor than 3′SiaLacNAc, whereas its isomer, 6-O-Su-LacNAc, containing sulfate at GlcNAc was inactive. In the case of other siglecs, none of the sulfated analogs of LacNAc (including disulfates 4′,6′-di-O-Su-LacNAc and 3′,6-di-O-Su-Lac) revealed high inhibitory activity. 3′-O-Su-TF exhibited inhibitory activity with respect to siglec-1, siglec-4, and siglec-8; this activity was comparable to that of 3′SiaTF. Siglec-1 and siglec-4 bound to 3′-O-Su-Le^c, but in the case of siglec-4, this binding was two times weaker than with the sialylated derivative (Table 2).

Interaction of soluble siglecs with conjugates containing sulfotyrosine. The effect of an additional sTyr residue was investigated for siglec-1 and siglec-5 using the glycoconjugate SiaLe^a–PAA–sTyr as an inhibitor. This glycoconjugate exhibited the same inhibitory activity with respect to siglec-1 and siglec-5 as SiaLe^a–PAA (without sTyr), thereby showing that incorporation of 10 mole % of sTyr (Table 3) did not influence the interaction. Polymers lacking a carbohydrate ligand but containing high levels of sTyr (up to 70 mole %) did not show any inhibitory activity.

Interaction of soluble siglecs with sulfated sialosides. We next investigated the effect of sulfate incorporation on the inhibitory activity of sialooligosaccharides. The presence of the sulfate group in 6-O-Su-3′SiaLacNAc did not influence sialoside binding to siglecs-1, -2, -4, -5, -7, -9, -10. Only in the case of siglec-8 was a significant difference observed at high concentrations of glycoconjugates: lower activity of 6-O-Su-3′SiaLacNAc compared with 3′SiaLacNAc. (Figure 2 shows the results for siglec-8 in comparison to siglecs-5 and -7.) The presence of a sulfate group in 6-O-Su-3′SiaTF did not influence inhibitory activity (data not shown).

Siglec-8 bound the fucosylated oligosaccharide 6′-O-Su-SiaLe^x and siglec-9 bound its isomer, 6-O-Su-SiaLe^x. None of the other siglecs tested interacted with the fucosylated sulfosialosides (data not shown). To evaluate the impact of the fucose residue on the interaction of siglec-8 with 6′-O-Su-SiaLe^x, the glycoconjugate was

Table 2. Inhibitory activity of sulfated glycoconjugates to siglecs-Fc- (1 and 4-10)*

Inhibitor	Relative inhibitory activity, %**							
	1	4	5	6	7	8	9	10
3'SiaLacNAc	24	0	0	n.a.	0	48	100	100
3-O-Su-LacNAc	0	0	0	n.a.	0	0	0	0
3'-O-Su-LacNAc	0	0	0	n.a.	0	0	0	0
6'-O-SuLacNAc	0	0	0	n.a.	3	70	0	0
6-O-SuLacNAc	0	0	0	n.a.	0	0	0	0
4',6'-di-O-Su-LacNAc	n.a.	0	0	n.a.	0	0	0	0
3'SiaTF	10	8	0	0	0	12	n.a.	0
3'O-SuTF	12	11	0	0	0	12	n.a.	0
3'SiaLe^c	23	15	n.a.	n.a.	n.a.	n.a.	n.a.	0
3'-O-Su-Le^c	23	8	n.a.	n.a.	n.a.	n.a.	n.a.	0
6'SiaLac	n.a.	n.a.	n.a.	100	0	n.a.	n.a.	n.a.
6-O-Su-Lac	n.a.	n.a.	n.a.	0	n.a.	n.a.	n.a.	n.a.
3',6-di-O-Su-Lac	n.a.	n.a.	n.a.	0	n.a.	n.a.	n.a.	n.a.
Neu5AcαOBn	100	100	100	n.a.	n.a.	100	n.a.	n.a.
Sia_2	n.a.	n.a.	n.a.	n.a.	100	0	0	0

* Inhibition of binding with the glycoconjugate exhibiting the highest interaction with a siglec in the direct binding assay.

** Relative inhibitory activity was calculated using the following formula: $[IC_{50}^{Glyc-PAA}/IC_{50}^{Glyc(sulfo)-PAA}] \times 100\%$, where $IC_{50}^{Glyc-PAA}$ is the 50% inhibition concentration by the glycoconjugate that exhibited the highest interaction with the siglec in the direct binding assay (see "Results" section) and $IC_{50}^{Glyc(sulfo)-PAA}$ is 50% inhibition by sulfated glycoconjugate. 0, no inhibition at 250 μM; n.a., not analyzed.

treated with the fucosidase (see "Materials and Methods" section). Defucosylation (possibly incomplete) increased binding, whereas desialylation of glycoconjugate caused total inactivation (Fig. 3).

Table 3. Inhibitory activity of conjugates containing sulfotyrosine residue*

Conjugate	Relative inhibitory activity, %**	
	siglec-1	siglec-5
Neu5AcαOBn–PAA	100	100
sTyr(10%)–PAA	0	0
sTyr(40%)–PAA	0	0
sTyr(70%)–PAA	0	0
SiaLe^a–PAA	24	0
SiaLe^a(20%)–PAA–sTyr(10%)	24	0

* Inhibition of siglec binding to Neu5AcαOBn–PAA.

** Relative inhibitory activity was calculated using the following formula: $[IC_{50}^{Neu5Ac\alpha OBn-PAA}/IC_{50}^{Glyc-PAA}] \times 100\%$, where $IC_{50}^{Neu5Ac\alpha OBn-PAA}$ is 50% inhibition by the glycoconjugate Neu5AcαOBn-PAA and $IC_{50}^{Glyc-PAA}$ is 50% inhibition by sTyr containing conjugate.

Cell-associated siglecs. The interaction between transfected CHO cells expressing cell-surface siglecs and fluorescein-labeled glycoconjugates Glyc–PAA–fluo was analyzed by flow cytometry. This approach was used previously for studying the specificity of cells expressing E-selectin [29]. Weak binding of 3′-O-Su-LacNAc was observed with cell siglecs-1 and -9 (Fig. 4). The interaction between 3′-O-Su-LacNAc and siglec-1 was stronger than that of 3′SiaLacNAc (Fig. 4). However, it should be noted that there was low binding of 3′SiaLacNAc with CHO cells expressing siglec-1 even after treatment of cells with neuraminidase [12, 13]. Binding of 3′-O-Su-LacNAc with siglec-9 expressed on CHO cells was one order of magnitude lower than that of 3′SiaLacNAc (Fig. 4).

We also investigated the interaction of CHO cell-expressed siglecs-1, -5, -7, and -9 with sulfated sialoglycoconjugates (6-O-Su-3′SiaLacNAc, 6-O-Su-6′SiaLacNAc, 6-O-Su-SiaLe^x, and 6′-O-Su-SiaLe^x) and their analogs lacking a sulfate group (3′SiaLacNAc, 6′SiaLacNAc, SiaLe^x, and SiaLe^a). Cell-surface-expressed siglecs-1 and -5 exhibited weak binding to 3′SiaLacNAc and did not interact with 6-O-Su-3′SiaLacNAc (Fig. 5a). Binding of siglec-7 and siglec-9 with 6-O-Su-3′SiaLacNAc was one order of magnitude lower than in the case of 3′SiaLacNAc. The presence of the sulfate group in the 6′-sialylated isomer (6-O-Su-

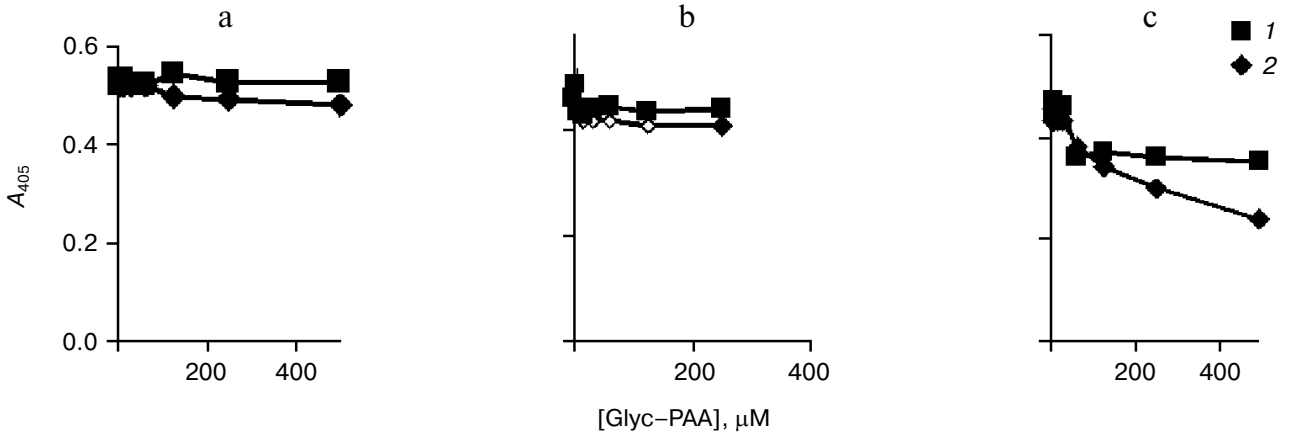

Fig. 2. Inhibition by 3′SiaLacNAc–PAA (*1*) and 6-O-Su-3′SiaLacNAc–PAA (*2*) of binding of Neu5AcαOBn–PAA to siglec-5 (a), 6′SiaLac–PAA to siglec-7 (b), and 3′SiaLacNAc–PAA to siglec-8 (c).

6′SiaLacNAc) did not influence its interaction with cell-surface-expressed siglecs-7 and -9 (i.e., it was similar to 6′SiaLacNAc) (Fig. 5b).

Cell-expressed siglecs-1, -5, -7, and -9 exhibited differences in the way they interacted with fucosylated sialoside sulfates. The interaction of siglecs-1 and -5 with $SiaLe^x$ derivatives sulfated at Gal (or GlcNAc) residues was stronger than that of $SiaLe^x$ (Fig. 6). Binding of cell-expressed siglec-7 with 6-O-Su-$SiaLe^x$ was comparable with that of $SiaLe^x$ and was lower with the isomer 6′-O-Su-$SiaLe^x$ (Fig. 6). As in the case of siglec-7, binding of CHO cells expressing siglec-9 with 6′-O-Su-$SiaLe^x$ was significantly weaker than with $SiaLe^x$ and slightly higher with the isomer 6-O-Su-$SiaLe^x$ (Fig. 6).

DISCUSSION

The limited information on the role of O-sulfate in the regulation of carbohydrate binding capacity by siglecs and other lectins (see above) stimulated the present study on the role of sulfated molecules in modulating siglec-dependent recognition. In this study we investigated the following issues: 1) whether sialic acid substitution for sulfate would retain any siglec-binding activity of oligosaccharides; 2) how introduction of sulfate into "classic" sialic acid containing oligosaccharide ligands influences their siglec-binding capacity; 3) whether sulfotyrosine residues distal to the carbohydrate chain (by analogy with P-selectin) could influence sialooligosaccharide binding with siglecs. This information is important not only for understanding the mechanisms responsible for siglec–carbohydrate recognition but might also indicate alternative binding mechanisms.

The contribution of the sialic acid component of sialooligosaccharides in determining the binding affinity of various sialic acid binding lectins differs significantly. For example, in the case of selectins, only the carboxyl group at Neu5Ac is ultimately important whereas the rest of molecule does not directly make contact with protein [30] and so substitution of sialic acid with acetate or sulfate minimally alters its affinity for selectins. Similarly, the plant lectin MAA, widely known as a reagent for Neu5Acα2-3Gal-terminated carbohydrate chains, can also bind oligosaccharides containing terminal residue HSO_3-3Gal [31]. In contrast, the influenza virus hemag-

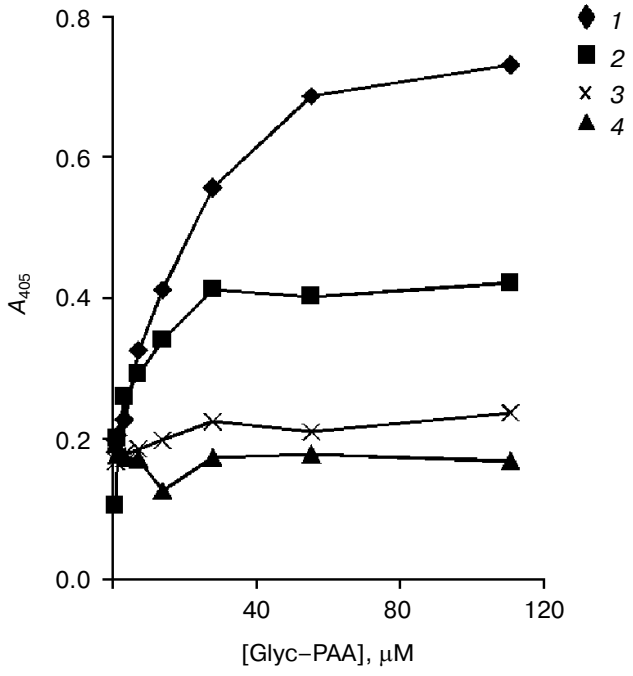

Fig. 3. Binding of 6′-O-Su-$SiaLe^x$–PAA (*2*), $SiaLe^x$–PAA (*3*), and 6′-O-Su- $SiaLe^x$–PAA to siglec-8 after treatment of the conjugate with fucosidase (*1*) or neuraminidase (*4*). Treatment with enzymes is described in the "Materials and Methods" section.

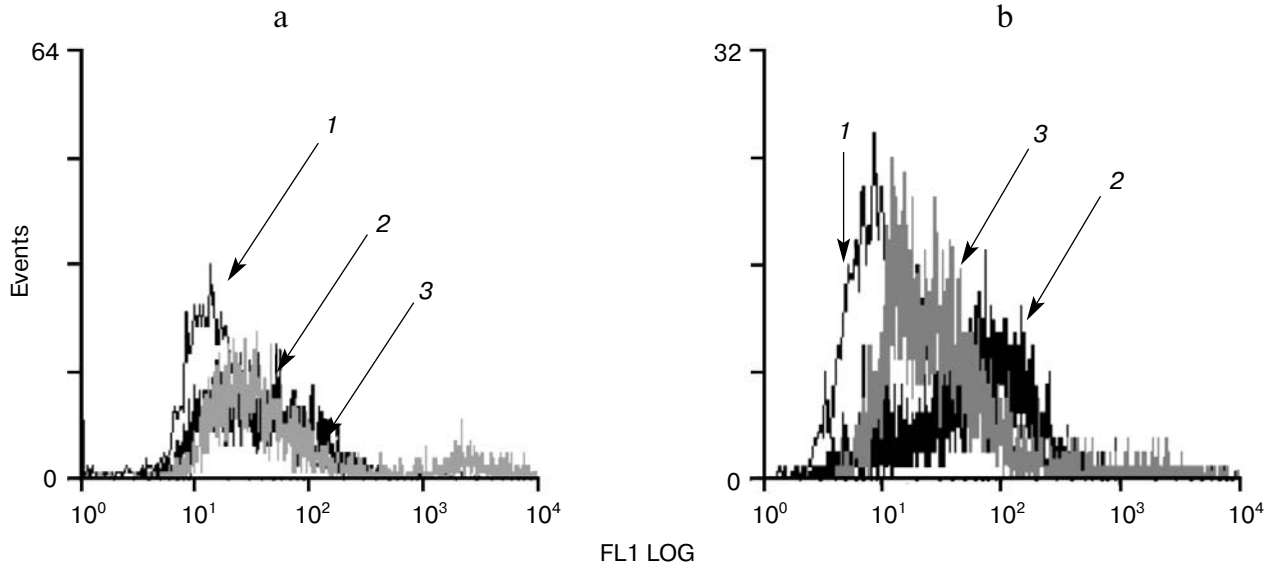

Fig. 4. Flow cytometric analysis of the interaction of 3′-O-Su-LacNAc–PAA–fluo and 3′SiaLacNAc–PAA–fluo with CHO cells expressing siglecs-1 (a) and -9 (b). Cells were pretreated with neuraminidase at 37°C for 3 h to unmask sialic acid binding sites of siglecs. *1*) Binding of 3′SiaLacNAc–PAA–fluo with CHO-WT cells (without siglecs); *2*) binding of 3′SiaLacNAc–PAA–fluo with CHO-siglec cells; *3*) binding of 3′-O-Su-LacNAc–PAA–fluo with CHO-siglec cells. Abscissa shows fluorescence intensity in selected cell population (arbitrary units), ordinate shows relative frequency of cell population. CHO-WT, mock-transfected cells, were used as negative control.

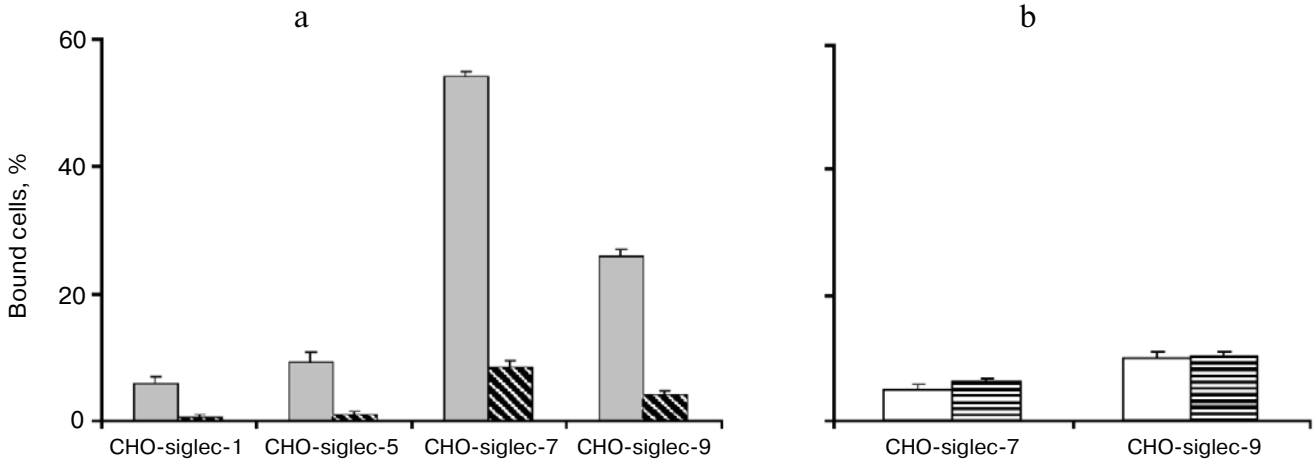

Fig. 5. a) Comparison of 6-O-Su-3′SiaLacNAc–PAA–fluo (hatched bars) and 3′SiaLacNAc–PAA–fluo (gray bars) binding to CHO-siglec cells. Cells were pretreated with neuraminidase at 37°C for 3 h to unmask siglec binding sites. Binding was analyzed by flow cytometry as described in the "Materials and Methods" section. Ordinate shows percent of CHO-siglec cells bound to Glyc–PAA–fluo glycoconjugate relative to control CHO-WT cells. b) Comparison of 6-O-Su-6′SiaLacNAc–PAA–fluo (hatched bars) and 6′SiaLacNAc–PAA–fluo (white bars) binding to CHO-siglec cells. Ordinate shows percent of CHO-siglec cells bound to Glyc–PAA–fluo glycoconjugate relative to control CHO-WT cells.

glutinin binding requires all functional groups of Neu5Ac, although a trisaccharide is a minimal requirement as a ligand for this lectin [8, 32].

The carboxyl group at C-2, the hydroxyl groups at C-8 and C-9, and the N-acyl group at N-5 of sialic acid are critical for siglec binding [33-36]. Decarboxylation or any substitution of the hydroxyl group at C-9 as well as oxidation of hydroxyls at C-9 and C-8 result in loss of ligand activity. Furthermore, the introduction of substituents at N-5 may potentiate or attenuate interaction depending on the siglec in question [33, 34]. Although the sialic acid residue is critical for binding, it should be noted that at least one neighboring monosaccharide residue is likely to contact the carbohydrate binding site of most siglecs;

however, in general, the impact of sialic versus the asialic components of ligands on binding is unknown. Thus, it is possible to speculate that the lack of Neu5Ac residue could be compensated by an interaction between sulfate and a positively charged amino acid residue of protein. In this way, a non-sialylated glycan could acquire siglec-binding activity. To test this hypothesis we investigated the interactions of several siglecs with sulfated oligosaccharides, using typical fragments of N- and O-chains of mammalian glycoproteins and glycolipids, including lactosamines with sulfate residues (Su) at O-3 and O-6 of galactose and O-6 of N-acetylglucosamine. With the notable exception of 6′-O-Su-LacNAc/siglec-8 interactions, in which binding of the sulfated ligand was higher than binding of sialylated 3′SiaLacNAc, we failed to detect significant interaction of siglecs with these sulfated oligosaccharides. Interestingly, in this case, only the 6′-O-sulfated (but not 3′-O-sulfated) analog was active.

We also investigated the impact of sulfate when present in sialooligosaccharides. Previously it was demonstrated that the presence of a sulfate group in addition to sialic acid might significantly increase ligand affinity. For example, binding of siglec-8 to 6′-O-Su-SiaLe^x (with sulfate bound to galactose) was improved when compared with its binding to non-sulfated analogs [21-23]. Also, sulfation of GD1aα ganglioside increased its affinity to siglecs-1 and -4 [18-20]. In this study, we have confirmed that sulfation of SiaLe^x (at C-6 of galactose) results in increased affinity towards siglec-8. Moreover, fucose at 6′-O-Su-SiaLe^x is not required for binding because the defucosylated analog exhibits higher affinity than the fucosylated one. It should be noted that the interaction of siglec-8 with 6′-O-Su-SiaLe^x was markedly weaker than with Neu5AcαOBn; although defucosylation of 6′-O-Su-SiaLe^x increased binding level, it nevertheless was still lower than the interaction of siglec-8 with benzylglycoside Neu5Acα (data not shown).

In earlier studies of the specificity of P-selectin, we synthesized mimetics of its natural counter-receptor, PSGL-1. This glycoprotein has a carbohydrate chain containing terminal SiaLe^x adjacent to a cluster of three sTyr residues [3, 4]. This mimetic was produced as a flexible polymer containing both sTyr and SiaLe^x. Binding of this "chimeric" polymer to P-selectin was one order of magnitude higher than that of SiaLe^x–PAA and sTyr–PAA or their mixture, i.e., there was synergistic effect of ligand binding to P-selectin [5]. We employed a similar approach in this study, but in the case of siglecs the introduction of the sTyr residue into sialooligosaccharide conjugates did not cause any enhancing effects.

When expressed at the cell surface, siglecs are oligomeric proteins and their valences are potentially higher than soluble siglecs, even after complexing [25]. In this regard, we would expect to see strong binding of cell-surface-expressed siglecs to their ligands. However, in reality this does not occur, possibly because of *cis*-interaction with cell surface glycans masking the carbohydrate-binding site [12, 13]. As in the case of soluble siglecs, cell surface siglecs did not interact with LacNAc 6-O-sulfates and disulfates.

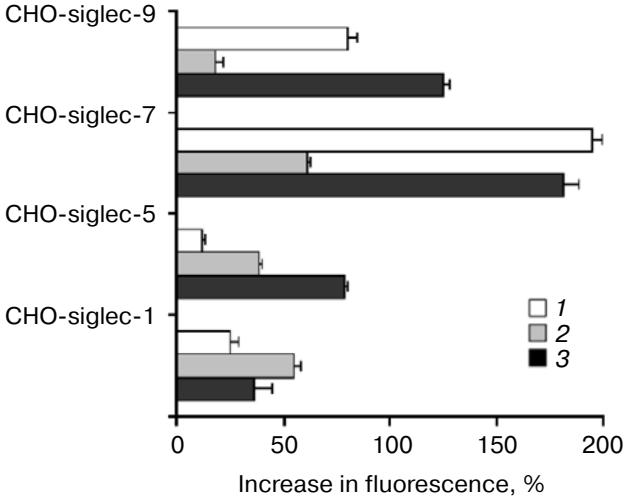

Fig. 6. Binding of SiaLe^x–PAA–fluo (*1*), 6′-O-Su-SiaLe^x–PAA–fluo (*2*), and 6-O-Su-SiaLe^x–PAA–fluo (*3*) with CHO-siglec-1, CHO-siglec-5, CHO-siglec-7, and CHO-siglec-9 cells. Abscissa shows the increase in fluorescence, calculated according to the formula $[(F_i/F_0)\cdot 100] - 100\%$, where F_i is fluorescence intensity of CHO-siglec cells after incubation with Glyc–PAA–fluo, F_0 is fluorescence intensity of CHO-WT cells after incubation with Glyc–PAA–fluo. Data represent results of one of three similar experiments.

It should be noted that there were some quantitative differences in binding of carbohydrate ligands when comparing cell-expressed and soluble forms of siglecs. First, 3′-O-Su-LacNAc–PAA exhibited a small but significant binding interaction with siglecs-1 and -9 but not to their soluble forms. Second, the presence of a sulfate group at the 6th position of GlcNAc completely abolished 3′SiaLacNAc binding to CHO-siglecs-1, -5, -7, and -9, whereas in the case of soluble siglecs this effect was observed only with siglec-8 and required high ligand concentrations.

A regulatory role of sulfation at the 6th position of the GlcNAc residue has been well documented for interaction with L-selectin and leads to a significant increase in the affinity of SiaLe^x for this lectin [3, 5]. Several studies demonstrated positive or negative contribution of O-sulfate to galectin binding with corresponding ligands [9-11]. In the case of some siglecs, we have also found bidirectional modulation of affinity, which depended on structure of oligosaccharide and nature of siglec.

This work was supported by the Russian Foundation for Basic Research (grant 01-04-49253), NIH 5 U54 GM62116-03, and also by a grant of the Russian Academy of Sciences "Physico-Chemical Biology".

REFERENCES

1. Bowman, K. G., and Bertozzi, C. R. (1999) *Chem. Biol.*, **6**, R9-R22.
2. Ley, K. (2003) *J. Exp. Med.*, **198**, 1285-1288.
3. Cummings, R. D. (1999) *Braz. J. Med. Biol. Res.*, **32**, 519-528.
4. Rodgers, S. D., Camphausen, R. T., and Hammer, D. A. (2001) *Biophys. J.*, **81**, 2001-2009.
5. Pochechueva, T. V., Galanina, O. E., Bird, M., Nifantiev, N. E., and Bovin, N. V. (2002) *Chem. Biol.*, **9**, 757-762.
6. Kanamori, A., Kojima, N., Uchimura, K., Muramatsu, T., Tamatani, T., Berndt, M. C., Kansas, G. S., and Kannagi, R. (2002) *J. Biol. Chem.*, **277**, 32578-32586.
7. Bloushtain, N., Qimron, U., Bar-Ilan, A., Hershkovitz, O., Gazit, R., Fima, E., Korc, M., Vlodavsky, I., Bovin, N. V., and Porgador, A. (2004) *J. Immunol.*, **173**, 2392-2401.
8. Gambaryan, A. S., Tuzikov, A. B., Pazynina, G. V., Webster, R. G., Matrosovich, M. N., and Bovin, N. V. (2004) *Virology*, **326**, 310-316.
9. Ideo, H., Seko, A., Ohkura, T., Matta, K. L., and Yamashita, K. (2002) *Glycobiology*, **12**, 199-208.
10. Ideo, H., Seko A., and Yamashita, K. (2005) *J. Biol. Chem.*, **280**, 4730-4737.
11. Silva, E., Teixeira, A., David, L., Carneiro, F., Reis, C. A., Sobrinho-Simoes, J., Serpa, J., Veerman, E., Bolscher, J., and Sobrinho-Simoes, M. (2002) *Virchows Arch.*, **440**, 311-317.
12. Crocker, P. R., and Varki, A. (2001) *Immunology*, **103**, 137-145.
13. Crocker, P. R. (2005) *Trends Glycosci. Glycotechnol.*, **16**, 357-370.
14. Angata, T., and Brinkman-van der Linden, E. C. M. (2002) *Biochim. Biophys. Acta*, **1572**, 294-316.
15. Yamaji, T., Teranishi, T., Alphey, M. S., Crocker, P. R., and Hashimoto, Ya. (2002) *J. Biol. Chem.*, **277**, 6324-6332.
16. Zaccai, N. R., Maenaka, K., Maenaka, T., Crocker, P. R., Brossmer, R., Kelm, S., and Jones, E. Y. (2003) *Structure*, **11**, 557-567.
17. Angata, T., Kerr, S. C., Greaves, D. R., Varki, N. M., Crocker, P. R., and Varki, A. (2002) *J. Biol. Chem.*, **277**, 24466-24474.
18. Collins, B. E., Ito, H., Sawada, N., Ishida, H., Kiso, M., and Schnaar, R. L. (1999) *J. Biol. Chem.*, **274**, 37637-37643.
19. Hara-Yokoyama, M., Ito, H., Ueno-Noto, K., Takano, K., Ishida, H., and Kiso, M. (2003) *Bioorg. Med. Chem. Lett.*, **13**, 3441-3445.
20. Ito, H., Ishida, H., Collins, B. E., Fromholt, S. E., Schnaar, R., and Kiso, M. (2003) *Carbohydr. Res.*, **338**, 1621-1639.
21. Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M. E., Alvarez, R., Bryan, M. C., Fazio, F., Calarese, D., Stevens, J., Razi, N., Stevens, D. J., Skehel, J. J., van Die, I., Burton, D. R., Wilson, I. A., Cummings, R., Bovin, N., Wong, C. H., and Paulson, J. C. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 17033-17038.
22. Bochner, B. S., Alvarez, R. A., Mehta, P., Bovin, N. V., Blixt, O., White, J. R., and Schnaar, R. L. (2005) *J. Biol. Chem.*, **280**, 4307-4312.
23. Tateno, H., Crocker, P. R., and Paulsen, J. C. (2005) *Glycobiology*, **15**, 1125-1135.
24. http://www.functionalglycomics.org.
25. Crocker, P. R., and Kelm, S. (1996) in *Weir's Handbook of Experimental Immunology* (Herzenberg, L. A., Weir, D. M., and Blackwell, C., eds.) Blackwell Sciences, Cambridge, pp. 166.1-166.11.
26. Vinson, M., van der Merwe, P. A., Kelm, S., May, A., Jones, E. I., and Crocker, P. R. (1996) *J. Biol. Chem.*, **271**, 9267-9272.
27. Pazynina, G. V., Sablina, M. A., Tuzikov, A. B., Chinarev, A. A., and Bovin, N. V. (2003) *Mendeleev Communs.*, **13**, 245-248.
28. Kelm, S., Brossmer, R., Isecke, R., Gross, H. J., Strenge, K., and Schauer, R. (1998) *Eur. J. Biochem.*, **255**, 663-672.
29. Galanina, O. E., Tuzikov, A. B., Rapoport, E. M., LePendu, J., and Bovin, N. V. (1998) *Analyt. Biochem.*, **265**, 282-289.
30. Feizi, T. (2001) in *Mammalian Carbohydrate Recognition Systems. Results and Problems in Cell Differentiation* (Crocker, P. S., ed.) Springer-Verlag, Berlin, pp. 201-223.
31. Bai, X., Brown, J. R., Varki, A., and Esko, J. D. (2001) *Glycobiology*, **11**, 621-632.
32. Matrosovich, M. N., Gambaryan, A. S., Tuzikov, A. B., Byramova, N. E., Mochalova, L. V., Golbraikh, A. A., Shenderovich, M. D., Finne, J., and Bovin, N. V. (1993) *Virology*, **196**, 111-121.
33. Kelm, S. (2001) in *Mammalian Carbohydrate Recognition Systems. Results and Problems in Cell Differentiation* (Crocker, P. S., ed.) Springer-Verlag, Berlin, pp. 153-173.
34. Strenge, K., Schauer, R., Bovin, N., Hasegawa, A., Ishida, H., Kiso, M., and Kelm, S. (1998) *Eur. J. Biochem.*, **258**, 677-685.
35. Brinkman-van der Linden, E. C., and Varki, A. (2000) *J. Biol. Chem.*, **275**, 8625-8632.
36. Varki, A. (1997) *FASEB J.*, **11**, 248-255.