Sialoside Specificity of the Siglec Family Assessed Using Novel Multivalent Probes:

Identification of Potent Inhibitors of Myelin-Associated Glycoprotein*

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RUNNING TITLE: Sialoside specificity of the Siglec family

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SUMMARY

Ten of the eleven known human siglecs or their murine orthologs have been evaluated for their specificity for over twenty-five synthetic sialosides representing most of the major sequences terminating carbohydrate groups of glycoproteins and glycolipids. Analysis has been performed using a novel multivalent platform comprising biotinylated sialosides bound to a streptavidin-alkaline phosphatase (SAAP) conjugate. Each siglec was found to have a unique specificity for binding sixteen different sialoside-SAAP probes. The relative affinities of monovalent sialosides were assessed for each siglec in competitive inhibition studies. The quantitative data obtained allows a detailed analysis of each siglec for the relative importance of sialic acid and the penultimate oligosaccharide sequence on binding affinity and specificity. Most remarkable was the finding that myelin-associated glycoprotein (Siglec-4) binds with 500-10,000 fold higher affinity to a series of mono- and di-sialylated derivatives of the O-linked T-antigen (Galβ(1-3)GalNAcαOThr) as compared to α-methyl-NeuAc.
INTRODUCTION

Siglecs (sialic acid-binding immunoglobulin superfamily lectins) are a structurally and functionally related family of cell surface receptors that bind to sialic acid containing carbohydrate groups of glycoproteins and glycolipids as ligands. Eleven members have been identified in the human genome including sialoadhesin (Siglec-1), CD22 (Siglec-2), CD33 (Siglec-3), myelin-associated glycoprotein (MAG; Siglec-4), and Siglecs-5-11 which are closely related to CD33 and believed to have arisen by gene duplication (1-4). In the murine genome, eight siglec genes have been identified comprising orthologs for sialoadhesin, CD22, CD33, MAG, and Siglec-10 (designated mSiglec-G), and three other CD33 related siglecs (Siglec-E, -F and -H) that have no clear-cut human ortholog (3). All siglecs have between 2-17 extracellular Ig domains. In each case, the distal most N-terminal Ig domain is comprised of a characteristic ‘V-set’ domain that is responsible for binding sialic acid containing ligands. Siglecs may have one or more immune receptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail which are believed to be involved in regulation of receptor mediated signal transduction (2-4).

Most siglecs are expressed on one or more haemopoietic cell types including B cells, NK cells, eosinophils, neutrophils, dendritic cells and minor subsets of CD8 T cells. The exception is MAG, which is found exclusively expressed in oligodendrocytes and Schwann cells. While the functions of the siglecs are still emerging, it is clear that they participate in a variety of cell-cell interactions and signaling pathways. For example, CD22 is well established to be an important modulator of B cell receptor signaling through recruitment of a protein-phosphatase (SHP-1) by
the phosphorylated ITIM motifs in its cytoplasmic domain (5-7). Recent evidence from several laboratories suggests that CD33 and CD33-related siglecs can also mediate recruitment of protein-tyrosine phosphatases and generate inhibitory signals in activated and differentiating leukocytes (1,8-17). Sialoadhesin and MAG have no ITIM motifs in their cytoplasmic domains. However, sialoadhesin is abundantly expressed on the surface of bone marrow macrophages and mediates the binding of immature granulocytes and has also been implicated in interactions with T cells during immune responses (18). MAG (Siglec-4) has been suggested to be important in maintenance of the myelin sheath and to inhibit neurite outgrowth in postnatal neurons (19,20).

While there is ample evidence that the functions of siglecs are modulated by the interaction with sialic acid containing ligands, establishing detailed mechanisms remains a challenge due to the ubiquitous distribution and structural diversity of sialyoligosaccharides on glycoproteins and glycolipids (3,7,11,21). More approachable has been the analysis of the specificity of siglecs to the sialoside sequence itself. The crystal structure for the sialoadhesin V-set domain with bound 3’-sialyllactose (NeuAcα(2-3)Galβ(1-4)Glc), revealed that sialoside binding is stabilized predominately by contacts with the sialic acid, including a salt bridge between the C-2 carboxyl-group and a highly conserved Arg at residue 97 (22). Yet, numerous reports have documented that the siglecs differ in their specificity towards other elements of sialoside sequences (2,3). For example, CD22 exhibits high specificity for the Siaα(2-6)Gal linkage (23-25), while sialoadhesin and MAG preferentially bind sialosides with the Siaα(2-3)Gal linkage (23,26), and Siglecs-7 and -11 exhibit preferred binding to sialosides with the Siaα(2-8)Sia linkage (1,27,28). There are also clear differences in the ability of siglecs to recognize naturally occurring sialic acids. Human sialoadhesin binds N-acetylneuraminic acid (NeuAc) but
not N-glycolylneuraminic acid (NeuGc), while human CD22 binds both and murine CD22 preferentially binds NeuGc. Addition of a 9-O-acetyl group or truncation of the sialic acid to the C-7 analog with periodate abolishes binding of several siglecs examined (27,29-33).

To date, most of the information obtained on siglec specificity has been gleaned from a variety of different assay methods involving the binding of recombinant siglecs or siglec-Fc chimeras to enzymatically modified erythrocytes, various multivalent sialoside probes, or immobilized gangliosides (23,24,27,28,31,34-38). By their very nature, such multivalent assays typically yield qualitative information on the relative binding affinities of the sialoside sequences being studied. Several reports have evaluated sialoside specificity using competitive inhibition with monomeric sialosides yielding more quantitative comparisons of sialoside specificity, but only a few (1-3) of the siglecs have been compared in any given study (25,26,28,39,40). As a result, while important and useful information has been obtained, current information on the sialoside specificity of siglecs is somewhat fragmentary and does not readily yield to direct comparisons between members of the family.

In this report we have systematically examined the specificity of siglecs representing ten of the eleven members of the human siglec family or their murine orthologs against 28 synthetic and structurally defined sialosides representing the major terminal sialoside sequences found on carbohydrate groups of mammalian glycoproteins and glycolipids. This has been accomplished through the use of a novel and versatile multivalent sialoside probe platform based on adsorption of biotinylated synthetic sialosides to a commercial streptavidin-alkaline phosphatase conjugate (SAAP). Such probes can be rapidly assessed for binding siglec-Fc chimeras adsorbed to plastic wells of a microtiter plate. Binding is reversible and can be inhibited by free monovalent
sialosides allowing assessment of their affinity as inhibitors of probe binding. The results reveal that each siglec exhibits a unique specificity against the panel of sialosides examined. Most striking is the specificity of MAG which binds to monomeric sialosides based on O-linked oligosaccharides with an affinity 500-10,000 times that observed for the preferred ligands of the other siglecs.
EXPERIMENTAL PROCEDURES

Materials: Streptavidin-alkaline phosphatase (SAAP) was from Sigma (St. Louis, MO, cat. No. S2890). All other commercial chemical reagents used in this study were of highest purity available. α1-acid glycoprotein (α1-AGP) was a gift from the late Karl Schmid.

Preparation of Sialosides: Sialyloligosaccharides for inhibition studies and for conjugation to biotin were prepared with chemoenzymatic methods. Compounds 3-21 were synthesized as previously described (41-43). Compound 22 was prepared from 9 by treatment with 0.04 M NaIO₄ at 0°C for 1 hr and further purified by size exclusion chromatography. Compound 23 was prepared from 9 by 1 hr treatment with methyl iodide (40 eqv.) in DMSO and subsequent overnight treatment with ethanolamine (75 eqv.) and further purified by size exclusion chromatography. Sialylation of synthetic T- and Tn-antigen intermediates gave compounds 26 and 28 (data not shown). Compound 27 was prepared by sialylation of commercial disaccharide glycoside Galβ(1-3)GalNAcαOBn (Calbiochem, San Diego, CA) using porcine ST3GalI. Characterization and purity of the synthesized compounds were verified by ¹H-NMR and Maldi-FTMS spectrometry (data not shown). N-acetylneuraminic acid (1) and di-sialyllactose (NeuAcα(2-8)NeuAcα(2-3)Galβ(1-4)Glc were from Sigma. N-acetylneuraminic acid α-methyl glycoside (2) and sialyl LewisX probe (P) were purchased from GlycoTech (Bethesda, MD).

Preparation of Biotinylated Sialoside probes: General procedure for biotinylation of N-linked 2-azido ethyl sialosides: Each of the sialoside derivatives 4, 5, 7, 9, 10, 12, 13, 19 and 20 (10-
50µmol) were dissolved in methanol-water (1:9 by volume, 0.5-2 mL) and hydrogenolysed over 
Pd/C (10%, 5-10 mg) in H₂-atmosphere at room temperature. Sodium hydrogen carbonate (a 
final concentration of 100 mM, pH 8-8.5) and sulfo-NHS-LC-LC-Biotin or sulfo-NHS-
Biotin (Pierce Chemicals Inc., Rockford, IL) (3 eqv.) was added to the mixture. When the 
reaction showed completion (analyzed by silica gel thin layer chromatography, eluent: 
ethylacetate-methanol-acetic acid-water; 4:3:3:2, by volume), the crude mixture was filtered 
and purified by gel filtration chromatography (Sephadex G15, 1 x 70 cm, equilibrated in 5% n-
BuOH), generating the biotinylated sialoside probes A-C, E-H, K, L and O in 75-85% yield 
(Table I).

General preparation of biotinylated O-linked-sialosides containing the amino L-
threonine aglycon: Fully protected N-(9-Fluorenylmethyloxycarbonyl)-O-[4,6-di-O-2-
azido-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-
galactopyranosyl]-L-threonine phenacylester or N-(9-Fluorenylmethyloxycarbonyl)-O-[2-
acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-
galactopyranosyl)-α-D-galactopyranosyl]-L-threonine phenacylester (44-46) (20-100 µmol) 
(Fig. 1), were completely deprotected by Zemplen conditions (1-4 mL, 0.1% NaOMe/MeOH) 
and neutralized by Dowex (50W-1x8, 200-400 mesh, H⁺-form). The mixture was filtered and 
purified by gel filtration chromatography (Sephadex G15, 1 x 70 cm, equilibrated in 10 mM 
sodium hydrogencarbonate, pH 8.0). Appropriate fractions were collected and lyophilized. The 
crude product was resuspended in water (1-2 mL) and sulfo-NHS-LC-LC-Biotin (3 eqv.) was 
added to the mixture. When the reaction was completed (analyzed by silica gel thin layer
chromatography, eluent: ethylacetate-methanol-acetic acid-water; 4:3:3:2, by volume), the mixture was filtered and purified by gel filtration chromatography (Sephadex G15, 1 x 70 cm, equilibrated in 5% n-BuOH) to give the corresponding biotinylated T- and Tn-epitopes respectively. The biotinylated T-, and Tn-epitopes were sialylated by the donor substrates CMP-NeuAc or CMP-NeuGc with porcine ST3Gal I and / or chicken ST6GalNAc I using reaction conditions as described (42) to give probes, D, I J and M respectively (Table I) in 60-80% yield. Characterization and purity (>95%) for the synthesized compound was verified by 1H-NMR and Maldi-FTMS spectrometry (data not shown). The α2-8-sialoside probe (N), was prepared from commercial disialyllactose (Sigma) and amino-POE-biotin (Pierce) by reductive amination as described previously (47,48).

**Preparation of Siglecs:** Siglec-Fc chimeras containing the amino terminal 3 (mSiglec-1, m/hSiglec-2, hSiglec-6, hSiglec-7, hSiglec-8, hSiglec-9), 4 (hSiglec-5) or 5 (mSiglec-4, hSiglec-10) Ig domains fused to the Fc region of human IgG1 were generated as described previously (23,34,35,49-53). CHO cells stably expressing the siglec-Fc chimeras for murine sialoadhesin and Siglec-2, human Siglec-3, -5, -7, -8, -9 and -10 were grown in GMEMα (First Link Ltd, Brierly Hill, UK). Plasmids encoding human Siglec-2 and –6-Fc chimeras (a generous gift of Dr. Ajit Varki) were transiently transfected into COS cells using Effectene (Qiagen, Valencia CA). All siglec-Fc proteins were purified from culture supernatant by affinity chromatography using a HiTrap Protein A column (Amersham Pharmacia Biotech, Uppsala, Sweden) and dialyzed into sodium phosphate buffer (10 mM, pH 7.5) containing sodium azide (0.05%) for storage at 4ÚC. Purity was established by SDS-PAGE.
**ELISA Assays:** Microtiter wells were coated overnight at 4°C with protein A (1 µg/well) in carbonate / hydrogen carbonate buffer (100 µL, 50 mM, pH 9.0). Wells were blocked with ELISA-buffer (200 µL, 20 mM HEPES, 0.5% BSA (bovine serum albumin), 125 mM NaCl, 1 mM EDTA, pH 7.50) for 1 hr at 37°C, and washed again (ELISA-buffer, 5 x 200 µL). Siglec-Fc chimeras (0.25-1.0 µg/well) were immobilized for 1 hr at 37°C in ELISA-buffer supplemented with 4 mM CaCl₂, and *Vibrio cholerae* neuraminidase (50 mU/mL). After washing with ELISA-buffer (5 x 200 µL), a dilution of sialoside-SAAP conjugate (50 µl containing 0.25-1 µg protein) was added to the wells and incubated for 20 min at room temperature. The sialoside-SAAP conjugate was prepared by mixing 2 volumes of a biotinylated sialoside solution in deionized water (55 µM) and 1 volume of 1 mg/mL streptavidin-alkaline phosphatase (Sigma, S2890) in 50 mM Na cacodylate buffer, pH 7.4, and left at room temperature for 30 min. Prior to use it was further diluted to the desired concentration (5-20 µg SAAP /ml) in ELISA-buffer.

For the inhibition assays, sialosides (50 µL, 20 mM-4 nM) were first added to the wells followed by the sialoside-SAAP conjugate (50 µL, 0.5-1.0 µg/well) and incubated 20 min at room temperature. The wells were washed with ELISA-buffer (5 x 200 µL) and developed with *p*-nitrophenyl phosphate liquid substrate system (50 µL/well, Sigma). Absorbance at 405 nm was determined by a Spectrocount plate reader (Packard Instruments, Meriden, CT). Wells without siglec-Fc chimeras or non-sialylated probes were used as negative controls. Assays were performed in duplicates throughout. From the competition curves the IC₅₀ could be calculated by non-linear regression analysis (PRISM, GraphPad Software, Inc. San Diego, CA). For each
set of competitive inhibition experiments, a reference compound was included as a positive control. As the IC\textsubscript{50} for reference compounds ranged from 0-25% between assays, the relative inhibitory concentration (rIC) of a given compound was calculated as the ratio of IC\textsubscript{50} values for a test compound to the reference compound.

\textit{Biacore Assays:} Flow cells of a CM5 sensor chip, equilibrated in HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) were activated with protein A (300 µg/mL, 10 mM NaOAc pH 4.8), at a flow rate of 10 µL/min for 10 min using standard EDC/NHS coupling (Biacore). Typically 12000 RU of protein A was immobilized. Sialidase (\textit{Vibrio cholerae}) treated hCD22-Fc chimera or human IgG (7 µg/mL, as control), was loaded over the protein A flow cells until response units of 5000 RU were immobilized. All analyses were performed at a flow rate of 5 µL/min using HBS as eluent. Injection times were 3 min followed by 1 min dissociation. No regeneration after each sample was necessary. For competitive inhibition studies, sialosides ranging from 5-5000 µM, were mixed with a fixed concentration of α\textsubscript{1}-AGP (10 or 50 µM). Each experiment was followed by an internal control of the specific sialoside mixed with asialo-α\textsubscript{1}-AGP and subtracted from the sample containing the α\textsubscript{1}-AGP. Subtractions were also made with the reference cell containing immobilized human IgG.
RESULTS

Synthesis of Monomeric Sialosides of Glycoprotein and Glycolipids. The goal of this study was to investigate the relative specificity of 10 of the 11 known siglec5 towards sialoside sequences that commonly occur in the glycans of potential natural ligands. For this purpose, sialosides representing terminal sequences on N-linked and O-linked carbohydrate groups of glycoproteins and on glycolipids were synthesized using chemoenzymatic methods, largely as described previously (41-43). Briefly, neutral core structures of Galβ(1-4)Glc (lactose), Galβ(1-3)GlcNAc (lacto-N-biose I) and Galβ(1-4)GlcNAc (N-acetyllactosamine or LacNAc) were synthesized with a short alkyl aglycon terminated with an azido group, and were then sialylated enzymatically in α2-3 and/or α2-6 linkage to galactose. Sialosides containing both NeuAc and NeuGc were synthesized in parallel since NeuAc is the predominant sialic acid in humans and NeuGc is a major sialic acid in mice (23)(Table I). These compounds were used as monovalent sialosides with no further modification, or were further modified by reduction of the azido group to the amine and conjugation to N-hydroxy succinimido-activated biotin as described in Experimental Procedures (Table I). The GalNAc (Tn-antigen) and Galβ(1-3)GalNAc (T-antigen) cores were prepared as the α-glycosides of threonine and sialylated in α2-3 linkage to galactose and/or α2-6 linkage to N-acetylgalactosamine. To prepare the corresponding biotinylated derivatives, it was more efficient to first biotinylate the O-linked precursors GalNAcαOThr(OMe) or Galβ(1-3)GalNAcαOThr(OMe) prior to the enzymatic sialylation (42) (Fig. 1).

Development of ELISA-type Assay Using Sialoside-SAAP Probes. Commercially available
multivalent sialoside conjugates consisting of a polyacrylamide polymer with pendent sialosides have proven to be useful probes of siglec function in both in vitro assays employing siglec-Fc chimeras and siglecs expressed on cell surfaces (49,51,52,54,55). Our initial attempts to use these conjugates in an ELISA-type assay to quantitatively measure probe binding to siglec-Fc chimeras in the presence and absence of free sialosides, however, proved to be highly variable between experiments and even within a single experiment.

As a simple alternative to the sialoside-polyacrylamide probes, we investigated the use of probes comprised of sialoside-biotin conjugates adsorbed to streptavidin-alkaline phosphatase (SAAP) based on our preliminary experiments indicating that such probes would bind to siglec-Fc chimeras bound to wells of a protein A coated microtiter plate. Taking advantage of the high affinity of biotin for streptavidin, probes are simply prepared by mixing a biotinylated sialoside with a solution of SAAP (Sigma Chemical Company, S2890). Mixing at a ratio of 4 moles of sialoside per mole of streptavidin (approximately 2 nmol sialoside / 10 µg SAAP) gives optimal binding of the probes to immobilized siglec-Fc chimeras, with no increase in probe binding observed at higher ratios of sialoside to SAAP (data not shown).

A representative example of optimization of probe binding (NeuGcα(2-6)Galβ(1-4)GlcNAc-SAAP (L)) to a siglec-Fc chimera (Siglec-10) is shown in Fig. 2A. Various concentrations of siglec-Fc were added to protein A coated wells of a microtiter plate, and the adsorbed siglec-Fc chimera was overlaid with serially diluted sialoside-SAAP probe over a range of 0.04 to 5 µg/well. After 30 minutes the plates were washed and bound probe was detected with p-nitrophenyl phosphate (pNPP). Binding of the streptavidin-alkaline phosphatase probe was stable between 5 and 10 washes with buffer (data not shown). Saturation of the wells
with siglec-Fc chimera typically occurred at a range of 0.25-0.5 µg well. For all experiments shown, each batch of purified siglec-Fc chimera was similarly analyzed prior to use to select an amount sufficient to saturate the wells of the microtiter plate.

The kinetics of the binding of sialoside-SAAP probe L to Siglec-10 is shown in Fig. 2B. Binding of the probe increased gradually up to 60 minutes and was maximal by 120 minutes. No further increase in probe binding was apparent over 24 hours (not shown). In the presence of excess (4 mM) monovalent sialoside 20, probe binding was reduced to background. Moreover, addition of 4 mM sialoside 20 at 30 min reduced probe binding to the same level as when it was added at 0-time, indicating that binding of the sialoside-SAAP probe is reversible.

Two commercial sources of streptavidin-alkaline phosphatase (SAAP) were evaluated in this assay system. The product obtained from Sigma reproducibly yielded sialoside-SAAP probes that behaved as described above, while a similar product from Pierce yielded probes that showed no binding to siglec-Fc chimeras in the ELISA assay format. Analysis of the two preparations by a Superdex 200 column revealed that the Sigma product was entirely excluded in the void volume, while the Pierce product was heterogeneous and largely included. Analysis by SDS gel electrophoresis showed that both were heterogeneous, but the Sigma preparation had a distinct band at H 230-250 kDa and gradient of larger conjugates, while the Pierce material had predominately lower molecular weight species of 60-250 kDa (data not shown).

**Siglec Specificity Towards the Sialoside-SAAP Conjugates.** A panel of sixteen sialoside-SAAP probes (A-P, Table I) was prepared from their corresponding biotinylated sialosides as described in Experimental Procedures. Each was evaluated for their ability to bind to eleven siglec-Fc
chimeras representing ten of the eleven known human siglecs (2-3 and 5-10) or their murine orthologs (1, 2 and 4). Probes were tested in duplicates for each siglec in at least two separate experiments, and results were highly reproducible when done on different days and with different batches of probe and siglec-Fc chimera. Results in Fig. 3 show representative experiments for ten of the siglecs. Each probe bound to a different set of siglecs except for probes C and G, which bound to the same set of six siglecs. Conversely, each siglec exhibited a unique specificity for binding the 16 sialoside-SAAP probes examined. At the extremes, mCD22 (Siglec-2) and hSiglec-5 bound only 2 of the probes, while hSiglecs-8, -9 and -10 exhibited binding above background for 12 of the 16. Also examined was hSiglec-6 which bound only to probe I (NeuGcα(2-6)GalNAc-R) when 2 µg Siglec-Fc chimera/well was used to coat each well (not shown).

Interestingly, probes H and O have the identical sialoside sequence, NeuAcα(2-6)Galβ(1-4)GlcNAc-R and differ only in the length of the biotin spacer (See Table I). Yet, of the seven siglecs that bound to probe H, only Siglecs-9 and -10 bound to probe O with the short spacer. This result clearly demonstrates that the length of spacer arm can influence the interaction of a sialoside sequence with a given siglec in this assay.

To determine the effect of siglec-Fc density on the microtiter plate on probe binding, several siglecs were evaluated for their ability to bind probes at sub-saturating levels. This was performed as a titration experiment examining the ability of all 16 probes to bind to Siglecs-1, -7 and -10 to microtiter wells coated with 2-fold dilutions of the respective siglec-Fc chimera. Results shown in Fig. 4 compare the sialoside-SAAP specificity of each siglec coated at saturating levels (0.5 µg/well) to the specificity seen when coated at the lowest level that
supported the binding of one or more probes. At sub-saturating levels, murine sialoadhesin bound preferentially to the NeuAcα(2-3)Galβ(1-4)Glc(NAc)-R sialosides (A and B) including the fucosylated structure Siayl-LeX (P). Similarly, at low density, Siglec-7 bound only the α2-8-sialyllactose (N) probe, and Siglec-10 bound only the NeuGcα(2-6)Galβ(1↑4)Glc(NAc)-probes K and L.

**Siglec Specificity Towards Monomeric Sialosides.** Binding of multivalent carbohydrate ligands to carbohydrate binding proteins is dependent on the structure of the carbohydrate as well as the nature of the multivalent display afforded by the carrier. In order to examine the specificity of the siglecs for sialoside sequences in the absence of the variables of multivalent display and steric interactions of the carrier, we sought to compare the relative affinity of monovalent sialosides for each siglec using a competition assay. To accomplish this, a single ‘high affinity’ sialoside-SAAP probe was selected for each siglec, and this was then used to detect siglec in the presence of increasing concentrations (4 nM – 10 mM) of each monovalent sialoside. A sub-saturating level of probe was used to increase the sensitivity of the assay so the observed IC50 value would approximate the Kd value of the inhibitor being evaluated. A comparison of the inhibition curves obtained for selected sialosides with mCD22, hCD22, hSiglec-7 and hSiglec-10 are shown in Fig. 5. As is evident, each siglec exhibits a unique specificity towards the panel of monovalent sialosides evaluated, differing in the sialoside that is the most potent inhibitor, and the rank order of the inhibitory potencies of the other sialosides.

Results obtained for inhibition of eleven siglecs against a panel of 24 sialosides is shown in Table II. Results are expressed as relative inhibitory potency compared to a reference
compound selected as the most potent inhibitor of the four sialosides comprising the sequence Siaα(2-3 or 2-6)Galβ(1-4)GlcNAc-R, where Sia is either NeuAc or NeuGc. The actual IC50 value for the reference sialoside is shown at the bottom of the Table, and the relative inhibitory potency is based on the formula [Relative Inhibitory Potency (%) = (IC50 reference sialoside / IC50 comparison sialoside) x 100]. Thus, the reference sialoside has a value of 100, higher affinity or more potent inhibitors have values >100, and less potent inhibitors have values less than 100.

Comparison of the siglecs reveals striking difference in their overall affinities for binding the four reference compounds, with IC50 values ranging from ~0.15 mM for murine and human CD22 (Siglec-2) to 3.7 mM for CD33 (Siglec-3), and no inhibition at the highest concentration tested for hSiglec-6. For each siglec, a comparison of the relative inhibitory potency of the simplest sialoside, α-methyl-NeuAc (3), with that of the reference compounds shows that the oligosaccharide sequences in the reference compounds increase binding affinity from 3 to >50 fold. Of the four reference compounds, Siglecs-1, -4 and -9 preferentially bound the Siaα(2-3)Gal linkage while human and murine CD22 (Siglec-2) are the only siglecs that exhibit a clear preference for the Siaα(2-6)Gal linkage. Siglecs-1, -4 and -9 also exhibited preference for NeuAc over NeuGc, while in contrast murine CD22 (Siglec-2) and Siglec-10 exhibit a clear preference for NeuGc over NeuAc. The other siglecs exhibit little preference or more complex preferences for sialic acid type and linkage represented in the reference compounds. The observed results are in accord with previous reports that have evaluated the specificity of sialoadhesin, CD22, MAG and Siglec-7 by competitive inhibition with sialosides in other assay systems (25,26,28,39,56).
With several notable exceptions, few sialosides exhibited higher affinities than the reference compounds. Siglec-7 exhibited highest affinity for disialyllactose (24) containing the NeuAcα(2-8)NeuAcα(2-3)Gal- sequence, with an IC₅₀ value of 0.5 mM, nearly 8 times more potent than the related 3-sialyllactose (4) without the NeuAcα(2-8)NeuAc linkage. The enhanced avidity for Siglec-7 for the NeuAcα(2-8)NeuAc linkage was previously noted by Yamaji et al. (28), and accounts for the preferential binding of the corresponding sialoside-SAAP probe (N) in Fig. 4. Substitution of the N-acetyl group on the GlcNAc moiety to the phalamido group on reference compound 5 to give compound 6 increased affinity 2.7 fold for sialoadhesin (Siglec-1).

Striking results were obtained with a series of monovalent sialosides corresponding to sialylated derivatives of Tn (GalNAcα-O-Thr) and T (Galβ(1-3)GalNAcα-O-Thr) analogs. Comparison of the simplest, α2-6 sialylated-Tn 14 (NeuAcα(2-6)GalNAc) with the related reference compound 13 (NeuAcα(2-6)Gal) reveals an increase affinity of 3-4 fold for MAG and Siglec-10, with little difference in affinity seen for the other siglecs. However, the additional galactose in the branched α2-6 sialylated T antigen (17) results in decreased affinity for Siglecs-2, 9 and 10, and increased affinity for Siglecs-1, -4, -5, -7 and -8. Most remarkable is the 100 fold increase in affinity observed for MAG (Siglec-4) for the α2-6 sialylated-T antigen (17). Equally, remarkable are the affinities of MAG for the α2-3 sialylated- (8) and α2-3/α2-6 disialylated- (18) T antigens with inhibitory potencies 300 and 1,500 higher than the reference compound (5), respectively.

Potent Inhibition of MAG (Siglec-4) by O-linked Sialosides. To better understand the structural
features of the $O$-linked sialosides that confer their high affinity for MAG, a direct comparison of their inhibitory potencies with several reference compounds was conducted. Complete titration profiles of MAG with nine sialosides are shown in Fig. 6. While the neutral disaccharide Galβ(1-3)GalNAcαOThr showed no inhibition at 10 mM (not shown), attachment of sialic acid in either the NeuAcα(2-3)Gal (8) or NeuAcα(2-6)GalNAc linkage (17) exhibited IC$_{50}$ values of 1.6 and 10 µM, respectively, reflecting >1000 fold increase in affinity by addition of either sialic acid. The disialylated compound (18) was even more potent with an IC$_{50}$ value of 0.3 µM. Also of note is the importance of the galactose (compare 14 and 17) and the αOThr aglycon (compare 8 with 25 and 27).

*Competitive Inhibition Assays Using Surface Plasmon Resonance (Biacore).* To determine how the IC$_{50}$ values obtained in the competitive ELISA assay compared with another equilibrium based assay, human CD22 affinity for monovalent sialosides was evaluated in a plasmon resonance ‘BIACORE’ based assay. In this assay, siglec-Fc chimera was adsorbed to a protein A coated sensor chip (Experimental Procedures). Human α1-acid-glycoprotein (α1-AGP), which contains N-linked oligosaccharides terminated with the NeuAcα2-6/3Gal linkage, was used as the high molecular weight analyte in the mobile phase. Increasing concentrations of α1-AGP caused a change in surface density that could be measured in response units (RU), and binding was rapidly and quantitatively reversed when α1-AGP was omitted from the solvent. A Hill plot of the binding isotherm gave an apparent K$_d$ of 42 µM and a slope of ≈ 0.8, suggesting no cooperative binding of sialic acids on the α1-AGP (not shown). Inhibition of a fixed
concentration of α1-AGP (50 µM) by increasing concentrations of the monovalent sialoside 13 (NeuAcα(2-6)Galβ(1-4)GlcNAc) produced a normal binding isotherm for a competitive inhibitor (Fig. 7). IC50 values obtained at α1-acid-glycoprotein concentrations of 10 and 50 µM were 351 and 629 µM, respectively. Using the formula $K_i(app) = \frac{IC_{50}}{(1+[\alpha_1-AGP]/K_d)}$, nearly identical $K_i(app)$ values of 283 and 284 µM were obtained. Those $K_i$ (apparent $K_d$) values are comparable (within a 2-fold) to the IC50 values obtained for the same sialoside (13) in the ELISA-based competition assay (IC50 = 150 µM)(Table II).
DISCUSSION

Coincident with the discovery that siglecs were members of a homologous gene family, Kelm et al. demonstrated that three siglecs, sialoadhesin, MAG and CD22, exhibited differential specificity towards sialoside sequences containing the NeuAcα(2-3)Gal and NeuAcα(2-6)Gal linkage using a multivalent assay system employing resialylated erythrocytes (23). Subsequently, many of the siglecs have been evaluated to various extents for their linkage specificity, α(2-3), α(2-6) and α(2-8), and/or ability to recognize the NeuAc and NeuGc forms of sialic acid using a variety of different assays (3,4). Several reports have evaluated the specificity of one or several siglecs using competitive inhibition of multivalent binding assays by monovalent sialosides analogous to the studies reported here (25,26,28,39,40). However, most have used multivalent assays based on binding of siglecs to sialosides attached to a polyacrylamide or other polymeric carrier (1,34,37,49,52-55,57-59), to gangliosides adsorbed to microtiter plates (30,36,38) or to neo-glycoprotein conjugates (27,28).

In this report seven human and four murine siglecs have been compared for their relative affinity for 28 monovalent sialosides comprising most of the terminal sequences found in carbohydrate groups of glycoproteins and glycolipids. Relative affinities were obtained as IC50 values by competitive inhibition of the binding of a multivalent sialoside-SAAP probe, which was used at sub-saturating levels (See Fig. 5) to maximize the sensitivity of the inhibition such that the IC50 value obtained would approximate its Kd. In this regard, it is notable that the IC50 value observed for sialoadhesin and its reference compound 5 (NeuAcα(2-3)Galβ(1-4)GlcNAc) of 1.3 mM is comparable to the Kd of 0.8 mM obtained for the same sialoside by NMR
experiments (60). Similarly, the IC$_{50}$ values of 0.14 mM and 0.15 mM obtained for murine and human CD22 (Siglec-2) with their reference compounds 20 ($\text{NeuGc}\alpha(2-6)\text{Gal}\beta(1-4)\text{GlcNAc}$) and 13 ($\text{NeuAc}\alpha(2-6)\text{Gal}\beta(1-4)\text{GlcNAc}$) respectively are comparable to the K$_d$ values obtained by Bakker et al. for the binding of soluble mCD22 to immobilized sialosides (0.12 - 0.26 mM at 25°C) (56), and to the apparent K$_i$ (K$_d$) of 0.28 mM obtained for hCD22 in the competitive plasmon resonance experiment reported here (Fig. 7). Although Powell et al. (25) obtained a lower K$_d$ of 0.032 mM for hCD22 at 4°C, this likely reflects a strong temperature dependence on K$_d$ values as demonstrated for mCD22 by Bakker et al. (0.05 mM at 5°C to 0.27 mM at 37°C) (56). Providing that other siglecs exhibit a similar temperature dependence on affinity for their ligands, the true K$_d$ values at physiologic temperature (37°C) may be somewhat higher than the IC$_{50}$ values obtained at room temperature in this report.

The results shown in Table II allow detailed quantitative comparison of the specificity of the siglec family towards naturally occurring sialosides, and evaluation of the importance of oligosaccharide sequence on binding affinity. Striking is the fact that sialic acid (NeuAc$\alpha$OMe, 2) is a very low affinity ligand for all siglecs examined with IC$_{50}$ values ranging from 2.4 mM to >10 mM. All siglecs exhibited higher affinities towards one or more of the various natural sialoside sequences evaluated. While each siglec exhibited a unique specificity, the increase in affinity afforded by the penultimate oligosaccharide sequence for the highest affinity sialoside ranged from as little as 3 fold for sialoadhesin (mSiglec-1) to greater than 12,000 fold for MAG (mSiglec-4). With the exception of MAG, the affinity exhibited for each siglec towards its
preferred sialoside ligand is still low, with IC$_{50}$ values ranging from 0.14 - 3.8 mM.

Several well documented elements of siglec specificity detected in multivalent assays are reflected in the sialoside affinities by the quantitative data summarized in Table II. The high specificity of human and murine CD22 (Siglec-2) for sialosides with the Sia$\alpha$(2-6)Gal$\beta$(1-4)Glc(NAc) sequence (23,25), reflects a difference in binding affinity for $\alpha$2-6 and $\alpha$2-3 sialosides of at least 60 fold. No other siglec exhibited a preference for these $\alpha$2-6 sialosides. The preference of sialoadhesin (mSiglec-1) and MAG (mSiglec-4) for the NeuAc$\alpha$(2-3)Gal sequence over the NeuAc$\alpha$(2-6)Gal sequence (26,27,30,34,36) is consistent with the 2-3 and 5-7 fold increased affinity, respectively, for sialosides with the $\alpha$23 linkage (4 and 5) over those with the $\alpha$26 linkage (12 and 13), in good agreement with results obtained by Strenge et al. (26). A preference of Siglecs-9 and 10 for sialosides with the $\alpha$23 linkages seen here (1.5-2 fold) was not recognized previously using multivalent probes (51,59,61,62). Yamaji et al. demonstrated a unique preference of Siglec-7 for the NeuAc$\alpha$(2-8)NeuAc sequence found in the ganglioside GD3 based on competitive inhibition of binding to a multivalent GD3-BSA probe using ganglioside micelles (28). As shown in Fig. 5 and Table II, disialyllactose (24) corresponding to the free oligosaccharide sequence of GD3, exhibited 5-10 fold higher affinity than any other monosialylated oligosaccharide. Binding of NeuAc$\alpha$(2-8)NeuAc linkages has also been demonstrated for Siglec-11, which was not studied in this report (1).

Previous reports have also demonstrated that siglecs exhibit differential specificity for sialic acids commonly found in mammalian species including NeuAc, NeuGc and 9-O-Acetyl-NeuAc (3,4,29,32). Indeed, while human CD22 binds equally well to $\alpha$26 sialosides containing either NeuAc or NeuGc, murine CD22 exhibits a strong preference for NeuGc (32,39,63).
shown here, the preferential affinity of mCD22 for NeuGc reflects a 10-20 fold higher affinity for sialosides containing NeuGc over the same sequences containing NeuAc (12 & 13 compared to 19 & 20). Conversely, murine sialoadhesin and murine MAG have been demonstrated to exhibit negligible binding to NeuGc containing oligosaccharides in various multivalent assay systems (27,30,32,39). This preference for NeuAc is reflected in the increased affinity of these siglecs for NeuAc over NeuGc by 2 and 3-8 fold, respectively (Table II). No previous report has directly compared the sialic acid preferences of the human CD33 related siglecs (3, 5-10) using defined sialoside sequences. As shown in Table II, Siglecs-3, -5 and -8 show no clear preference for NeuGc and NeuAc. In contrast Siglec-9 shows a 2-fold preference for NeuAc over NeuGc. Conversely, Siglec-7 and Siglec-10 show a consistent preference for NeuGc of 1.5 and 2-4 fold, respectively. Siglec-10 was unique among all siglecs in binding the NeuGcα(2-3)Galβ(1-3)GlcNAc sequence as its highest affinity ligand. Because these are human siglecs, and humans are unique among mammalian species in being incapable of making NeuGc, these differences are of significant interest from an evolutionary perspective (32), and for the potential to create specific sialoside probes unique to each siglec.

The series of O-linked sialosides studied in this report reveal several unrecognized elements of specificity among murine and human siglecs. Most siglecs recognized the sialyl-Tn antigen (NeuAcα(2-6)GalNAcα-OThr, 14), with similar affinity to sialosides with the NeuAcα(2-6)Gal sequence (12, 13). The notable exceptions were MAG (Siglec-4) and Siglec-10, which bound to the sialyl-Tn sequence with 3-4 fold higher affinity. Addition of a branched galactose, as represented in the α2-6-sialylated-T antigen Galβ(1-3)[NeuAcα(2-6)]GalNAcα-OThr (17), had little effect on the affinity for CD33 and Siglec-8, but significantly decreased affinity for
CD22 (40 fold), Siglec-9 (4 fold), and Siglec-10 (6 fold). In contrast, the same substitution dramatically enhanced affinity 100 fold for MAG (Siglec-4) and Siglec-6 and to a lesser extent for sialoadhesin, Siglecs-5 and Siglec-7 (1.5-2 fold). This oligosaccharide was the most potent inhibitor of the binding of the multivalent sialoside probe I (Table II, compound 14). The additional sialic acid in the α2-3, α2-6-disialyl-T antigen (18) further enhanced affinity towards MAG (33-fold) and sialoadhesin (2-fold), resulting in the highest affinity observed for either of these two siglecs towards the naturally occurring sialoside sequences evaluated in this report.

Comparison of the affinity of MAG for disialyl-T antigen (18) and its biosynthetically related O-linked oligosaccharides is summarized in Fig. 8. Striking is the observation that the non-sialylated Core 1 structure, Galβ(1-3)GalNAcα-OThr, shows no inhibition at 10 mM, while addition of sialic acid to either the 6 position of GalNAc or the 3 position of Gal increases affinity 1000 fold or more, and both sialic acids increase affinity over 30,000 fold. These results suggest that there may be two independent sites for binding sialic acid on MAG as suggested previously by others from analysis of MAG binding to ganglioside ligands (26,64,65). This conclusion was also reached by Vinson et al. who mutated the conserved Arg118 essential for sialic acid binding in sialoadhesin (22,60,66) and found that MAG still mediated sialic acid dependent effects on neurite outgrowth (67).

The ganglioside GD1α (and GQ1bα) contains the same terminal sequence as di-sialyl-T antigen (18), and has been reported to bind to MAG as a high affinity ligand (30). Yet, the free oligosaccharide of GD1α was found to be only 3 fold more potent an inhibitor of MAG than α2-3sialyllactose (4) (26), while the disialyl-T antigen was a 1500 fold more potent inhibitor than
α2-3sialyllactose in this study. The primary difference between the structure of the GD1α oligosaccharide and disialyl-T antigen (18) is that the terminal oligosaccharide, NeuAcα(2-3)Galβ(1-3)[NeuAcα(2-6)]GalNAc, is linked β(1-4) to lactosylceramide in the former case, and is α linked to Thr in the latter. These differences appear to be critically important. Indeed, comparison of the linear sequence NeuAcα(2-3)Galβ(1-3)GalNAc linked in either β linkage (26), or α linkage (27) to an aromatic aglycon shows that the α linkage is favored, and comparison of the aromatic aglycon (27) to the threonine aglycon (8) favors threonine by another 30 fold (Table II). Thus, both the α anomic linkage, and the threonine aglycon contribute to the unique high affinity of the sialylated-T antigens (8, 17, and 18).

The relevance of the high affinity of MAG for these monomeric O-linked sialosides to natural ligands of MAG in situ is not clear at the present time. Several reports provide compelling evidence for gangliosides GT1b (and GD1a) as the major neuronal sialoside ligands for MAG (65,67-70). MAG has been shown to interact with glycoprotein carbohydrates in a sialic acid dependent manner (71). However, while MAG has been shown to bind to the neuronal Nogo receptor with high affinity, and that this interaction comprises a major mode of MAG induced inhibition of neurite outgrowth, binding to the Nogo receptor appears to be sialic acid independent (72,73). Regardless of the ultimate biological significance of the high affinity interactions with the analogs of O-linked sialosides reported here, we anticipate that such compounds and their derivatives will prove to be important tools in the analysis of in vitro and in vivo sialic acid dependent functions of MAG.

Although analysis of siglec specificity using multivalent sialosides has revealed many important elements of specificity seen with monovalent sialosides, multivalent presentation of
ligands can differentially influence the observed specificity in several distinct ways. Multivalent constructs can amplify small differences in binding affinity, or differentially alter binding affinity through steric interactions between the carrier and the ligand-binding site. Amplification of small differences in affinity is well documented for differential binding of influenza virus hemagglutinin to NeuAcα(2-6)Gal and NeuAcα(2-3)Gal linkages on cell surface glycoprotein ligands. While the intrinsic binding affinity of the human H3 hemagglutinin to these ligands is 2.5 mM and 3.7 mM, respectively (74,75), this difference is amplified over 1000 fold in multivalent binding assays (76,77), and is sufficient to exert pressure for selection of receptor variants in natural and model host species (78,79). Such amplification of small differences in affinity can readily explain the observed preference of sialoadhesin for multivalent displays of α(2-3) sialosides over α(2-6) sialosides, and for NeuAc over NeuGc despite only 2-3 fold differences in affinity for the corresponding monovalent sialosides.

How two ligands might be differentially impacted by physical properties imposed by the carrier used in the multivalent constructs is more difficult to assess. Such factors are clearly evident from the data reported here and elsewhere. While both Siglec-5 and Siglec-7 exhibit similar affinity for sialosides with the NeuAcα(2-3)Gal and NeuAcα(2-6)Gal linkages (Table II), only sialoside-SAAP probes with NeuAcα(2-3)Gal linkage bound to Siglec-5, and only probes with the NeuAcα(2-6)Gal linkage bound to Siglec-7 (Fig. 3). Yet binding of the same sialosides attached to polyacrylamide showed equivalent binding of both linkages to Siglec-5 and Siglec-7 (34,49,55,58,59). Conversely, high affinity binding of SLeX-SAAP (P) to sialoadhesin (Figs. 3 and 4) was not seen with the corresponding SLeX-polyacrylamide probe by Brinkman et al. (49). There is also little correlation between the intrinsic affinity of the sialosides
(e.g. IC50 values in Table II) and the ability of the corresponding SAAP probe to bind to immobilized siglec chimeras (Fig. 3). Indeed, the SAAP probe D (NeuAcα(2-3)Galβ(1-3)GalNAcαOThr) failed to bind to MAG (Siglec-4) even though the corresponding monovalent sialoside 8 was one of the highest affinity ligands of any of the siglecs (IC50 = 1.6 µM). It should be recognized that such considerations may be equally relevant to siglec recognition of sialosides displayed on glycoproteins or glycolipids, and can determine which glycoproteins are effective ligands in vivo.

The systematic analysis of siglec specificity towards an extensive panel of sialosides was made possible by the use of a novel and flexible multivalent sialoside probe design consisting of biotinylated sialosides adsorbed to commercial streptavidin-alkaline phosphatase. Of paramount importance was the ability of the probes to bind to siglec chimeras adsorbed to a microtiter plate, and yield well behaved binding isotherms with low backgrounds and high reproducibility. In addition to their utility in microtiter plate-based binding assays, we have also found that the probes labeled with FITC are also well suited for binding to cell surface siglecs in flow cytometry experiments (unpublished results). These probes can be simply and inexpensively prepared with synthetic biotinylated sialosides as described here. Many of the biotinylated sialosides used in this report are also available with a shorter spacer from a commercial source (Glycotech) and with the long spacer from the Consortium for Functional Glycomics (http://web.mit.edu/glycomics/consortium/).
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FOOTNOTES

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The abbreviation used are: $\alpha_1$-AGP, $\alpha_1$-acid glycoprotein; Alloc, allyloxycarbonyl; BSA, bovine serum albumin; Da, Dalton; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GD3, $\text{II}^3(\text{NeuAc})_2$-LacCer; GD1a, $\text{IV}^3\text{NeuAc,II}^3\text{NeuAc-Gg}_4\text{Cer}$; GD1a,$\text{IV}^3\text{NeuAcIII}^6\text{NeuAcGg}_4\text{Cer}$; GT1b, $\text{IV}^3\text{NeuAcII}^3(\text{NeuAc})_2$-Gg$_4$Cer; GQ1ba, $\text{IV}^3\text{NeuAc,III}^6\text{NeuAc,II}^3(\text{NeuAc})_2$-Gg$_4$Cer; GMEM$\alpha$, Glasgow’s modified Eagle’s medium $\alpha$; ITIM, immunoreceptor tyrosine-based inhibition motif; Ig, immunoglobulin; LacNAc, $N$-acetyllactosamine (Gal$\beta$(1-4)GlcNAc); MAG, myelin-associated glycoprotein; Maldi-FTMS, matrix-assisted laser-desorption ionization fourier transformed mass spectrometry; NeuAc, n-acetyleneuraminic acid; NeuGc, n-glycolyneuraminic acid; NMR, nuclear magnetic resonance; Pht, phtalamide; rIP, relative inhibitory potency; SAAP, streptavidin-alkaline phosphatase; Sia, sialic acid; Siglec, sialic acid-binding lectin of the immunoglobulin superfamily; SLe$^X$, sialyl lewiss$^X$ tetrasaccharide (III$^3$Fuc,$\text{IV}^3\text{NeuAc-nLc}_4$); SPR, surface plasmon resonance; ST6Gal I,
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FIGURE LEGENDS

**Fig. 1. Preparation of biotinylated \( O \)-linked \( \alpha-2-6 \)-sialosides.** Acetylated T- and Tn-epitopes (Ia and Ib), were deprotected by Zemplen deacetylation and purified by size exclusion chromatography. Free amino groups were biotinylated by \( N \)-hydroxy-succinimide activated LC-LC-Biotin (Pierce) followed by size exclusion chromatography. The biotinylated galactosides were sialylated by chST6GalNAcI using CMP-NeuAc or CMP-NeuGc as sugar nucleotide donors (as described in Experimental Procedures).

**Fig. 2. Optimization of sialoside-SAAP probe binding to siglec-Fc chimeras. (Panel A.)** Sialoside-SAAP probe L was evaluated for its binding to hSiglec-10 coated at various concentrations on a 96 well microtiter plate. Wells were coated as described in Experimental Procedures with \( n(1.0\mu g), \Delta(0.5\mu g), \Omega(0.25\mu g), u(0.125\mu g), l(0.06\mu g), q(0.03\mu g) \) of the Fc-chimera of Siglec-10. Two-fold serial dilutions of sialoside-SAAP probe were applied and incubated for 20 min at room temperature. (Panel B.) Kinetics of SAAP-probe binding to Siglec-10. Sialoside-SAAP probe L (0.25 \( \mu g/50 \mu L \)) was added to wells of a microtiter plate previously coated with Siglec-10 (0.5 \( \mu g/well \)) and incubated for up to 120 min. Binding was evaluated in the absence (q), or (O,l) presence of 4 mM monovalent sialoside (20) added before (O) or 30 min after (l) addition of the probe. Bound probe was detected with pNPP as described in Experimental Procedures.

**Fig. 3. Sialoside-SAAP probe binding to Siglecs-1-10.** Each siglec was evaluated for its ability to bind to 16 sialoside-SAAP probes (Table I) as described in Experimental Procedures. A saturating level of each siglec was established in separate experiments as illustrated in Fig. 2.
Values are the mean ±S.D. of duplicate determinations. The asterik (*) indicates the probe chosen for each siglec for use in subsequent competitive inhibition experiments (See Fig. 5 and Table II).

**Fig. 4. Preferred sialoside-SAAP specificity revealed at lower siglec density.** The effect of siglec density on probe binding specificity was analyzed for sialoadhesin (mSiglec-1), hSiglec-7 and hSiglec-10 immobilized at 0.5 or d 0.12 µg siglec-Fc/well, using the panel of 16 sialoside-SAAP probes (Table I) as described in Experimental Procedures.

**Fig. 5. Comparison of the inhibition of sialoside-SAAP probe binding to selected siglec by monovalent sialosides.** Two fold dilutions of selected sialosides (8 µM – 10 mM) were compared for their ability to inhibit binding of sialoside-SAAP to human and murine CD22 (Siglec-2), hSiglec-7 and hSiglec-10. Sialosides were added to microtiter wells coated with siglec as described in Experimental Procedures. For each siglec, a selected sialoside SAAP probe (see ‘*’ in Fig. 3) was added and incubated for 20 min at room temperature as described in Experimental Procedures. After washing, the bound probe was detected with the pNPP. Values are the mean ±S.D. of duplicate determinations.

**Fig. 6. Competitive inhibition of sialoside-SAAP probe (A) to myelin-associated glycoprotein (MAG) using monovalent O-linked sialosides.** A concentration gradient (8 µM – 10 mM) of the indicated sialosides were added to microtiter wells coated with the Fc-chimera of MAG (mSiglec-4; 0.5 µg/well). Sialoside-SAAP probe A (0.5 µg/well) was added and incubated for 20 min. After washing, bound probe was detected with pNPP as described in Experimental Procedures. Values are the mean ±S.D. of duplicate determinations. Relative IC50 values for each
sialoside were calculated using the GraphPad software program.

**Fig. 7. A competitive binding assay with α1-acid glycoprotein and an α2-6-sialoside to hCD22 using Surface Plasmon Resonance (SPR).** hCD22-Fc chimera was captured onto a protein A immobilized CM5 sensor chip. Various concentrations of the inhibitor compound 13 (0-5000 µM) was mixed with a fixed concentration of α1-AGP (10 µM) and binding to the chip was measured. The reference cell contained IgG immobilized onto protein A. Shown are the response curves for each concentration of compound 13, corrected for the response generated by a matched sample containing compound 13 and no α1-AGP run immediately after the corresponding test sample. The inset shows a plot of the plateau RU vs concentration of compound 13, from which the Kᵢ values were calculated.

**Fig. 8. Inhibitory potencies of related O-linked sialosides for MAG (Siglec-4).** Illustrated is the structural relationship of O-linked sialosides and the IC₅₀ values obtained for inhibition of probe binding to murine MAG as described in Fig. 6. Arrows connect structures that differ by a single sugar residue. The reference number for each sialoside is underlined.
Table 1. Synthetic sialosides and biotinylated sialoside conjugates. Monovalent sialosides (3-28) were prepared as described in Experimental Procedures. 2-azidoethyl-glycosides (A-C, E-H, K, L and O) were hydrogenolyzed over Pd/C followed by Biotinylation with N-hydroxy-succinimide activated-Biotins. O-linked sialosides (D, I, J and M) were biotinylated as described in Figure 1 and Experimental Procedures. The α-2,8-sialoside-probe (N) was prepared from commercial disialyllactose (Sigma, St. Louis, MO) and biotinylated with amino-biotin (II) by reductive amination using sodium cyanoborohydride followed by acetic anhydride. Compound P was purchased from Glycotech (Bethesda, MD).

| Cpd. # | Sialoside Structure | Aglycon (R) | Biotin Spacer | Probe Name |
|--------|-------------------|-------------|--------------|------------|
| 1      | NeuAc             | R = CH₃     |              |            |
| 2      | NeuAcαOR         | R = CH₃     |              |            |
| 3      | NeuAcα(2-3)GalβOR | R = CH₃     |              |            |
| 4      | NeuAcα(2-3)Galβ(1-4)GlcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | A          |
| 5      | NeuAcα(2-3)Galβ(1-4)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | B          |
| 6      | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | C          |
| 7      | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | D          |
| 8      | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | E          |
| 9      | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | F          |
| 10     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | G          |
| 11     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | H/O        |
| 12     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | I          |
| 13     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | J          |
| 14     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | K          |
| 15     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | L          |
| 16     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | M          |
| 17     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | N          |
| 18     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | P          |
| 19     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | Q          |
| 20     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | R          |
| 21     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | S          |
| 22     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | T          |
| 23     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | U          |
| 24     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | V          |
| 25     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | W          |
| 26     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | X          |
| 27     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | Y          |
| 28     | NeuAcα(2-3)Galβ(1-3)GlcNAcβOR | R = CH₃, CH₂N, | LC-LC-Biotin | Z          |

* Alloc = allyloxycarbonyl; Pht = phtalamide

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![Diagram](https://via.placeholder.com/150)
Table 2. Relative inhibitory potency of monovalent sialosides towards Siglecs 1-10. Monovalent sialosides were evaluated for their inhibitory potency toward each Siglec as described in Figure 5, using a matched sialoside-SAAP probe indicated by the * symbol in Figure 3 (for hSiglec-6, probe J was used). The value for each sialoside was calculated using the software program GraphPad. A reference compound for each Siglec was selected as the most potent sialoside inhibitor of the four N-acyl-sialyllactosamine compounds (5, 9, 13, 20). Relative inhibitory potency for each sialoside is calculated as a percentage of the reference compound (100%) according to the formula: (Value) = (IC50 reference compound / IC50 sialoside) x 100.

| Cpd. # | Sialoside† | Siglecs (Relative Inhibitory Potency %)‡ |
|--------|------------|-----------------------------------------|
|        |            | m1 | h2 | m2 | h3 | m4 | h5 | h6 | h7 | h8 | h9 | h10 |
| 1      | NeuAc      | 30 | 0  | 0  | 62 | 12 | 26 | 42 | 32 | 0  | 12 | 0   |
| 2      | NeuAcO R1  | 73 | 0  | 0  | 76 | 192| 47 | 39 | 32 | 62 | 48 |     |
| 3      | NeuAc(2-3)GalβSR1 | 89 | 0  | 84 | 94 | 50 | 57 | 49 | 63 | 45 |     |
| 4      | NeuAc(2-3)Galβ(1-4)GlcβOR2(A) | 100 | 0  | 100| 100| 73 | 66 | 38 | 100| 42 |     |
| 5      | NeuAc(2-3)Galβ(1-4)GlcNAcβOR2(B) | 100 | 0  | 100| 100| 73 | 66 | 38 | 100| 42 |     |
| 6      | NeuAc(2-3)Galβ(1-4)GlcNPhβSR3 | 270 |     |     |     |     |     |     |     |     |     |     |
| 7      | NeuAc(2-3)Galβ(1-3)GlcNAcβOR2(C) | 65 | 0  | 0  | 85 | 94 | 56 | 45 | 49 | 45 | 43 |     |
| 8      | NeuAc(2-3)Galβ(1-3)GalNAcτOR2(D) | 65 | 0  | 85 | 29600| 28 | 41 | 43 | 16 | 20 |     |
| 9      | NeuGcort(2-3)Galβ(1-4)GlcNAcβOR2(E) | 32 | 0  | 61 | 46 | 100| 95 | 66 | 62 | 100|     |
| 10     | NeuGcort(2-3)Galβ(1-3)GlcNAcβOR2(F) | 27 | 0  | 61 | 48 | 64 | 74 | 70 | 33 | 166|     |
| 11     | NeuAc(2-6)GalβSR1 | 40 | 51 | 3  | 43 | 14 | 32 | 0  | 75 | 91 | 28 | 22 |
| 12     | NeuAc(2-6)Galβ(1-4)GlcβOR2(G) | 46 | 88 | 3  | 43 | 21 | 60 | 68 | 60 | 37 | 35 |     |
| 13     | NeuAc(2-6)Galβ(1-4)GlcNAcβOR2(H/O) | 38 | 100| 5  | 58 | 14 | 60 | 0  | 82 | 100| 45 | 23 |
| 14     | NeuAc(2-6)GalNAcτOR4(I) | 33 | 79 | 2  | 75 | 40 | 44 | 0  | 75 | 63 | 55 | 83 |
| 15     | NeuAc(2-6)Galβ(1-4)GlcNAcτSR5 | 79 | 8  | 63 |     |     |     |     |     |     |     |     |
| 16     | NeuAc(2-6)Galβ(1-4)GlcNPhβSR1 | 78 | 125| 14 | 75 | 60 |     |     |     |     |     |     |
| 17     | Galβ(1-3)[NeuAc(2-6)]GalNAcτOR4(J) | 70 | 2  | 0  | 89 | 4640| 69 | 100| 142| 63 | 12 | 13 |
| 18     | NeuAc(2-3)Galβ(1-3)[NeuAc(2-6)]GalNAcτR4 | 138 | 38 | 2  | 96 | 154000 | 118 | 50 | 117| 65 | 34 | 22 |
| 19     | NeuGcort(2-6)Galβ(1-4)GlcβOR2(K) | 68 | 33 | 59 | 6 | 54 | 87 | 69 | 20 | 60 |     |     |
| 20     | NeuGcort(2-6)Galβ(1-4)GlcNAcβOR2(L) | 15 | 100| 100| 4  | 61 | 100| 35 | 14 | 68 |     |     |
| 21     | NeuGcort(2-6)GalNAcτOR4(M) | 11 | 41 | 17 | 0  | 60 | 53 | 0  | 86 | 91 | 20 | 38 |
| 22     | NeuAc(7-Aldehyde)α(2-3)Galβ(1-4)GlcNAcβOR2 | 0  | 34 | 4  | 0  |     |     |     |     |     |     |     |
| 23     | NeuAc(1-ethanolamide)α(2-3)Galβ(1-4)GlcNAcβOR2 | 0  | 34 | 4  | 0  |     |     |     |     |     |     |     |
| 24     | NeuAc(2-8)NeuAcO(2-3)Galβ(1-4)Glc(N) |     |     |     |     |     |     |     |     |     |     |     |
| 25     | NeuAc(2-5)Galβ(1-3)GalNAcβSR7 | 178 |     |     |     |     |     |     |     |     |     |     |
| 26     | NeuAc(2-3)Galβ(1-3)GalNAcτOR6 | 943 |     |     |     |     |     |     |     |     |     |     |
| 27     | NeuAc(2-6)Gal(2N2)SR7 | 36 |     |     |     |     |     |     |     |     |     |     |

IC50 Reference compound (mM) 1.29 0.15 0.14 4.72 0.49 0.62 3.80 2.33 3.07 0.94 0.60

† Monovalent sialoside sequences used in competitive inhibition experiments (See Table 1 for R group). Letters in parentheses indicate corresponding biotinylated sequences used for preparation of sialoside-SAAP probes used in Figure 3.
‡ Blank = Not tested; (0) = No inhibition at 10 mM.
**Ia**  
R₁ = β-2,3,4,6-tetra-O-acetyl galactopyranoside  
or  
R₁ = Ac  

**Ib**  
chST6GalNAc-I  
CMP-NeuAc or CMP-NeuGe  

1) NaOMe/MeOH  
2) Gel filtration  
3) NHS-LC-LC-Biotin  
4) Gel filtration  

**Probe**  

I: R₁ = H, R₂ = H  
M: R₁ = H, R₂ = OH  
J: R₁ = β-galactopyranoside, R₂ = H
**Structure** | **IC$_{50}$ (μM)**
---|---
[ ] NeuAcoα2-6GalβSR$_1$ (11) | 3900.0
[ ] NeuAcoα2-6Gal(2N$_3$)βSR$_7$ (28) | 1300.0
[ ] NeuAcoα2-6GalNAcαOR$_4$ (14) | 1100.0
[ ] NeuAcoα2-3Galβ1-3GalNAcβSR$_7$ (25) | 260.0
[ ] NeuAcoα2-3GalβSR$_1$ (3) | 250.0
[ ] NeuAcoα2-3Galβ1-3GalNAcαOR$_8$ (27) | 49.0
[ ] Galβ1-3(NeuAcoα2-6)GalNAcαOR$_4$ (17) | 10.0
[ ] NeuAcoα2-3Galβ1-3GalNAcαOR$_4$ (8) | 1.6
[ ] NeuAcoα2-3Galβ1-3(NeuAcoα2-6)GalNAcαOR$_4$ (18) | 0.3
NeuAcα2,6GalNAcαThr
14 (1100 μM)

Galβ1,3GalNAcαThr
(>10,000 μM)

NeuAcα2,3Galβ1,3GalNAcαThr
8 (1.6 μM)

NeuAcα2,6

NeuAcα2,3Galβ1,3
18 (0.3 μM)
Sialoside specificity of the Siglec family assessed using novel multivalent probes: Identification of potent inhibitors of myelin associated glycoprotein
Ola Blixt, Brian E. Collins, Ingrid M. van den Nieuwenhof, Paul R. Crocker and James C. Paulson

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