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New Aspects of Siglec Binding Specificities, Including the Significance of Fucosylation and of the Sialyl-Tn Epitope*

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The siglecs (sialic acid-binding immunoglobulin superfamily lectins) are immunoglobulin superfamily members recognizing sialylated ligands. Most prior studies of siglec specificities focused on α2–3- and α2–6-sialyllactos(amin)es and on one or two of the siglecs at a time. Here, we explore several new aspects of specificities of the first six reported siglecs, using sialylated glycans presented in multivalent form, on synthetic polyacrylamide backbones, or on mucin polypeptides. First, we report that binding of siglec-1 (sialoadhesin), siglec-3 (CD33), siglec-4a (myelin-associated glycoprotein), and siglec-5 to α2–3 sialyllactosamine is affected markedly by the presence of an α1–3-linked fucose. Thus, while siglecs may not interfere with selectin-mediated recognition, fucosylation could negatively regulate siglec binding. Second, in contrast to earlier studies, we find that siglec-3 prefers α2–6-sialyllactose. Third, siglec-5 binds α2–8-linked sialic acid, making it the siglec least specific for linkage recognition. Fourth, siglecs-2 (CD22), -3, -5, and -6 (obesity-binding protein 1) showed significant binding to sialyl-Tn (Neu5Acα2–6-GalNAc), a tumor marker associated with poor prognosis. Fifth, siglec-6 is an exception among siglecs in not requiring the glycerol side chain of sialic acid for recognition. Sixth, all siglecs require the carboxyl group of sialic acid for binding. Finally, the presentation of the sialyl-Tn epitope and/or more extended structures that include this motif may be important for optimal recognition by the siglecs. This was concluded from studies using ovine, bovine, and porcine submaxillary mucins and Chinese hamster ovary cells transfected with ST6GalNAc-I and/or the mucin polypeptide MUC1.

The siglecs (**s**ialic acid-binding **i**mmuno**g**lobulin superfamily **lec**tin**s**)[1] are a class of Ig superfamily proteins (1) which show binding activity to specific glycan structures containing sialic acid (1–5). Sialic acids are acidic monosaccharides frequently found at the outer end of secreted and cell surface glycoconjugates (5–8), a good location for recognition by lectins such as the siglecs. To date, six different members of this family of lectins have been characterized (1, 2, 9–14), and a recent paper describes a potential seventh member (15). The siglecs share overall structural characteristics, with an NH_2-terminal V-set Ig domain followed by varying numbers of C2-set Ig domains. Siglec-1 (sialoadhesin, Sn) is the largest member, with 17 extracellular Ig domains, whereas siglec-3 (CD33) is the smallest, with only 2 of these domains. Each siglec has its own unique tissue distribution and specific phosphorylation sites on the cytosolic tail, indicating that each has specific functions mediated by the lectin activity. The latter suggestion is substantiated by the fact that each siglec seems to display distinctive specificities for recognition of sialic acid linkages. However, for most siglecs only a limited sampling of the wide array of sialylated glycans found in nature have been examined, primarily α2–3- and α2–6-sialyllactos(amin)es. Also, most prior specificity studies focused on one or two of the siglecs at a time. Siglec-1 is reported to prefer α2–3-linked sialyllactos(amin)e over α2–6-linked sialyllactos(amin)e and showed some binding to α2–8-linked sialic acids on glycolipids (16, 17). The exact function of siglec-1, found on macrophages in certain tissues, is unknown, although roles in hematopoiesis (18) or in cellular trafficking (19, 20) have been suggested. Siglec-2 (CD22) is only known to bind to α2–6-linked sialyllactosamines on *N*-linked glycans and is expressed exclusively on B cells, functioning as a modulator of B cell signaling (3, 9, 21–30). For siglec-3 a binding preference similar to siglec-1 has been described, with α2–3-linked sialyllactosamine being preferred over α2–6-linked sialyllactosamine. However, this was concluded only from experiments using resialylated red blood cells (11). Siglec-3 is found on myelomonocytic progenitor cells, monocytes, and macrophages and is a marker for acute myeloid leukemias (31–33), but no specific function has yet been ascribed to it. Siglec-4a (myelin-associated glycoprotein) is found only in the nervous system and is involved in maintenance of myelin sheath integrity (34, 35). Siglec-4a requires terminal α2–3-linked sialic acids for binding. However, in this case, extensive specificity studies using various gangliosides indicate that besides the terminal linkage, the underlying sugars and the presence of additional sialic acid residues may also play a role in recognition (17, 36–38). The recent discoveries of siglec-5 (13) and siglec-6 (OB-BP1) (14) revealed a subfamily of molecules with a closer homology to siglec-3. Siglec-5, expressed on neutrophils and monocytes, is reported to bind equally well to α2–3- and α2–6-linked sialyllactosamine (13). Siglec-6 is expressed on placental trophoblasts and B cells and is the only

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[1] The abbreviations used are: siglec(s), sialic acid-binding immunoglobulin superfamily lectin(s); Sn, sialoadhesin; OB-BP1, obesity-binding protein 1; G_{D3}, $II^3(NeuAc)_2$-LacCer; AUS, *Arthrobacter ureafaciens* sialidase; PAA, polyacrylamide; siglec-Fc, fusion protein of extracellular domains of a siglec with the Fc part of human IgG; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; BSM, bovine submaxillary mucin; OSM, ovine submaxillary mucin; PSM, porcine submaxillary mucin; DMB, 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; SLeX, Sialyl Lewis^x. For definitions of PAA conjugates used in this study, see Table I.

one shown so far to recognize the sialyl-Tn epitope (Neu5Acα2–6GalNAc). This is also the first siglec for which a protein ligand has been found as well, *i.e.* leptin (14). No defined biological functions have yet been attributed to siglecs-5 and -6.

As described above, several studies have examined the recognition specificity of the siglecs. However, many aspects have still not been addressed. For example, since sialyl-Tn was noted as a ligand for siglec-6, the direct recognition of the sialyl-Tn epitope has never been examined for other siglecs. Sialyl-Tn is a disaccharide found frequently on a variety of cancers and is known to be a useful diagnostic marker (39, 40). High expression of this antigen is associated with a poor prognosis in most cancers studied (39, 41–43). With regard to α2–3-linked sialic acids, fucose (Fuc) residues are frequently found attached to the underlying GlcNAc. It is not known if this fucosylation will interfere with the recognition of the nearby sialic acid residue by siglecs. Here, we have used various sialylated glycans presented in multivalent form on synthetic polyacrylamide backbones to study these issues, as well as other aspects of specificity such as linkage preference and involvement of glycerol side chain of sialic acid and of the carboxyl group of sialic acid. Additionally, mucins (either in solution or expressed on cell surfaces) were used to study further the involvement of the glycerol side chain and to examine effects of ligand presentation on siglec recognition.

EXPERIMENTAL PROCEDURES

Materials—Most of the general materials used were from Sigma Chemical Co. or Fisher Scientific. The following materials were purchased from other sources: EZ-Link™ Sulfo-NHS-biotin, Pierce; microtiter plates, Nunc; ovine and porcine (A^-) submaxillary mucins, Accurate; ganglioside G_{D3}, Matreya; protein A-Sepharose, Amersham Pharmacia Biotech; phycoerythrin-conjugated goat $F(ab')_2$ anti-human IgG (Fc-specific), CalTag Laboratories; and *Arthrobacter ureafaciens* sialidase (AUS), Calbiochem. Molecular biology reagents were from Life Technologies and Qiagen. Products for cell culture and alkaline phosphatase-conjugated streptavidin were from Life Technologies. Biotin-conjugated polyacrylamide (PAA) probes substituted with various sialylated glycans were obtained from Glycotech (44).

Cell Lines, Plasmids, Siglec-Fcs, and Transfections—COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 7.5% fetal calf serum and CHO-TAg cells in α-minimal essential medium with 10% fetal calf serum, and 1 mg/ml G418. Siglec-Fcs (extracellular domains of siglecs fused with the Fc portion of human IgG) were obtained as described in the accompanying paper (45). Plasmids encoding siglec-1-Fc and siglec-5-Fc as well as CHO cells stably expressing siglec-3-Fc were a kind gift from Dr. Paul Crocker (University of Dundee) and the plasmid encoding rat (domain 1–3) siglec-4a-Fc from Dr. Ivan Stamenkovic (Massachusetts General Hospital). CHO-TAg cells were transiently transfected with a plasmid encoding MUC1 (kindly provided by Dr. Sandra Gendler, Mayo Clinic Scottsdale) and/or a plasmid encoding mouse ST6GalNAc-I by Dr. Shuichi Tsuji (RIKEN, Japan). Transfections were performed as described in the accompanying paper (45). CHO-TAg cells (gift of Dr. John Lowe, University of Michigan) were transiently transfected using α-minimal essential medium instead of Opti-MEM.

Sialidase Treatment of Siglec-Fcs—Siglec-Fcs from 300 ml of culture supernatant were captured on 1 ml of protein A-Sepharose. Half of this was treated with 12.5 μl (50 milliunits) of AUS in 0.5 ml of 0.05 M HEPES, pH 6.9, for 3 h at room temperature. Only 12.5 μl of 0.1 M NaOAc, pH 5.5 (AUS storage buffer), was added to the other half (sham treatment). After extensive washing of the protein A-Sepharose, the AUS- and sham-treated Fcs were eluted as described (46, 47). Because AUS treatment appeared to be essential to achieve optimal binding activity only in the case of siglec-3-Fc, this molecule was routinely treated with AUS in this manner.

Analysis of Binding Properties of the Siglecs—Microtiter wells were coated overnight at 4 °C with protein A (500 ng/well) in 50 mM carbonate/bicarbonate buffer, pH 9.5. Wells were blocked with ELISA-buffer (20 mM HEPES, 1% bovine serum albumin, 125 mM NaCl, 1 mM EDTA, pH 7.45) for 1 h and incubated with siglec-Fcs (500 ng/well) for 2 h. Between incubations (all at room temperature) wells were washed three times with ELISA buffer. Biotin-conjugated PAA substituted with various sialylated glycans (1 μg/well or a range between 0.25 and 3 μg/well) or bovine, ovine, or porcine submaxillary mucins (BSM, OSM, or PSM, respectively; biotinylated with NHS-biotin, see below) (5 μg/well) were added for 2 h, followed by incubation with alkaline phosphatase-conjugated streptavidin (1:1,000) for 1 h and development with 100 μl/well *p*-nitrophenyl phosphate liquid substrate system. Absorbances at 405 nm were determined. Desialylated probes (sialyl-Tn-PAA and mucins) obtained by mild acid treatment (2 M acetic acid for 1 h for sialyl-Tn-PAA and for 3 h for the mucins at 80 °C) were used as negative controls. The acid-treated mixture was neutralized with NaOH. As a control an already neutralized mixture of NaOH/acetic acid was added to the untreated probes. The levels of biotinylation of the probes were not affected by these treatments as checked by ELISA (see below).

A lipid ELISA (48) was performed to assay binding to ganglioside G_{D3}, which was coated in MeOH onto microtiter plates (1 μg/well). After evaporation and lipid absorption the plates were blocked with ELISA buffer. Siglec-Fcs (10 μg/ml) that had been precomplexed with horseradish peroxidase-conjugated goat anti-human IgG (1:500) (for 1 h at 4 °C) were incubated for 2 h. Plates were developed with *O*-phenylenediamine and absorbances at 492 determined. Desialylated G_{D3} was obtained by mild acid treatment (2 M acetic acid for 3 h at 80 °C) or AUS treatment (12 μg of G_{D3} was treated with 40 milliunits of AUS in 100 μl of 0.1 M NaOAc, pH 5.5, for 3 h at 37 °C). After these treatments the glycolipids were lyophilized, resuspended in MeOH, and coated on microtiter plates.

Mild Periodate Treatment of Probes—Potential binding probes were treated mildly with $NaIO_4$ to specifically truncate the glycerol side chain of sialic acid (49–51). The probes (PAAs and mucins) were first treated with 2 mM $NaIO_4$ in phosphate-buffered saline for 30 min on ice in the dark. Subsequently, the aldhehydes as formed by the $NaIO_4$ treatment were reduced with 10 mM $NaBH_4$ in phosphate-buffered saline for 1 h in the dark on ice. The reaction mixtures were then diluted 5 × with ELISA buffer and used directly in the assay. For sham treatment 2 mM IO_4 and 10 mM $NaBH_4$ were incubated for 1 h on ice, then diluted with ELISA buffer, and the probes were added to this mixture just before use in the assay. For G_{D3} the $NaIO_4$ treatment (followed by $NaBH_4$) was performed on the microtiter plate before the blocking step. Analysis of DMB-sialic acid adducts was performed as described in the accompanying paper (45) on some of the periodate-treated probes (sialyl-Tn and the mucins). A shift of 1 min in HPLC elution of DMB-sialic acid adducts from the treated probes compared with the sham-treated probes confirmed truncation of the side chain of all sialic acid (data not shown). The HPLC runs of sham- and periodate-treated probes also showed that there was no loss of total sialic acid due to the treatment (data not shown).

Iodoethane Treatment of PAA Probes—PAA probes were treated with CH_3CH_2I followed by $NaBH_4$ to convert the carboxyl group of sialic acid to an alcohol (36). Typically, 10 μg of probe was lyophilized in a glass conical vial, redissolved in 35 μl of dry dimethyl sulfoxide, and incubated with 7 μl of CH_3CH_2I for 1 h at room temperature. 465 μl of ELISA buffer (without bovine serum albumin) was added, and this mixture was incubated with 10 mM $NaBH_4$ for 1 h at room temperature. For sham treatment the same procedure was performed without adding CH_3CH_2I. After these incubations the reaction mixtures were diluted 2 × with ELISA buffer and directly used in the assay. The levels of biotinylation of the probes were not affected by these treatments as checked by the ELISA described below. DMB analysis on treated probes confirmed that ~80% of the carboxylates of sialic acids were converted by the treatment (data not shown).

Analysis of Relative Biotinylation Level of the PAA Probes—Probes were coated onto microtiter plates (200 ng/well) overnight at 4 °C in 50 mM carbonate/bicarbonate buffer, pH 9.5. After blocking with ELISA buffer (1 h) the wells were incubated with alkaline phosphatase-conjugated streptavidin (1:1,000) (1 h) and developed with 100 μl/well *p*-nitrophenyl phosphate liquid substrate system, and absorbances were read out at 405 nm.

Biotinylation of the Mucins—115 μl of an EZ-Link™ Sulfo-NHS-biotin solution (1 mg/ml in H_2O) was added to 1.3 ml of a mucin solution (1 mg/ml in 0.1 M $NaHCO_3$, pH 8.3) and incubated for 2 h at room temperature. This mixture was dialyzed extensively against phosphate-buffered saline at 4 °C. The levels of biotinylation of the mucins were checked by ELISA in a similar manner as the PAAs (described above).

Base Treatment of the Mucins—Mucins were incubated with 0.1 M NaOH for 30 min at room temperature. This removes base-labile *O*-acetyl esters but leaves the rest of the glycan intact (52). This mixture was subsequently neutralized with HCl. As controls, previously neutralized mixtures of NaOH/HCl were added to the untreated mucins.

Flow Cytometry—Cells ($0.3–1 \times 10^6$) were incubated for 1 h at 4 °C with fluorescein isothiocyanate-conjugated *Sambucus nigra* agglutinin

TABLE I
Structures examined for recognition by human siglecs

Structure	Abbreviation	Presentation (backbone)	Group of sialic acid studied	
			Side chain	Carboxylate
Neu5Acα2–6Galβ1–4Glc	6′-SLL	PAA	Mild periodate	
Neu5Acα2–3Galβ1–4Glc	3′-SLL	PAA	Mild periodate	Iodoethane
Neu5Acα2–3Galβ1–4GlcNAc	3′-SLacNAc	PAA	Mild periodate	
Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc	SLeX	PAA	Mild periodate	
Neu5Acα2–6GalNAc	Sialyl-Tn	PAA, OSM, BSM[a]	Mild periodate	Iodoethane
Neu5Gcα2–6GalNAc	Sialyl-Tn (Gc)	BSM, PSM[a]	Mild periodate	
9-*O*-Ac-Neu5Ac/Gcα2–6GalNAc	*O*-Ac-sialyl-Tn	BSM	Base treatment	
Neu5Ac/Gcα2–6(Galβ1–3)GalNAc	Core 1-sialyl-Tn	PSM>>BSM	Mild periodate	
Neu5Ac/Gcα2–6(GlcNAcβ1–3)GalNAc	Core 3-sialyl-Tn	BSM>>PSM	Mild periodate	
Neu5Acα2–8Neu5Acα2–3Galβ1–4Glc-Cer	G_{D3}	Ceramide	Mild periodate	

[a] Some additional structures are present, for details, see "Results and Discussion."

(5 μg/ml) or with the various Fcs (10 μg/ml) that had been preincubated (at least 15 min at 4 °C) with 100 × diluted phycoerythrin-conjugated goat $F(ab')_2$ anti-human IgG. Binding was analyzed by flow cytometry using a Becton Dickinson FACscan machine. Mild periodate or AUS treatment of cells was performed before staining as described previously (53). Hybridoma culture supernatants containing TKH2 (anti-sialyl-Tn) antibody (kindly provided by Dr. Sen-itiroh Hakomori, University of Washington) or HFM6.2 (anti-MUC1; kindly provided by Dr. Sandra Gendler, Mayo Clinic Scottsdale) were used to stain transiently transfected CHO-TAg cells to confirm expression of sialyl-Tn and MUC1, respectively.

RESULTS AND DISCUSSION

We have examined various aspects of binding specificity of the first six reported siglecs that have not been addressed previously. Most of the experiments were ELISAs using recombinant soluble siglecs and various sialylated glycans presented in multivalent form, either on synthetic PAA backbones (PAAs) or on mucins (BSM, OSM, or PSM). In the case of the PAA probes the only sialic acid that could be tested was *N*-acetylneuraminic acid (Neu5Ac). On BSM ~70% of the sialic acid is Neu5Ac and ~30% is *N*-glycolylneuraminic acid (Neu5Gc), whereas on OSM 100% is Neu5Ac, and on PSM > 95% is Neu5Gc (data not shown). Table I provides an overview of the various sialylated ligands and presentations studied here.

Fucosylation Markedly Reduces Binding by the Siglecs—Various sialylated PAAs were used to examine importance for siglec binding of the sialic acid linkage type and of fucosylation: 6′-SLL (Neu5Acα2–6Galβ1–4Glc), 3′-SLL (Neu5Acα2–3Galβ-1–4Glc), 3′-SLacNAc (Neu5Acα2–3Galβ1–4GlcNAc) and SLeX (Neu5Acα2–3Galβ1–4(Fucα1–3)-GlcNAc). From the results (Fig. 1) it is clear that the interactions of the siglecs known previously to recognize α2–3-linked sialyllactos(amin)e (siglecs-1, -3, -4a, and -5) are reduced considerably by the presence of an α1–3-linked Fuc on the underlying GlcNAc (forming the SLeX epitope). This extends earlier findings that siglec-4a-expressing cells did not bind to SLeX-containing glycolipids (36) and that siglecs-1 and -4a binding to red blood cells could not be inhibited by SLeX-PAAs (although in the same assay monovalent haptens containing SLeX showed some inhibition capacity) (54). When fucose is present some residual binding remains. This binding is sialic acid-dependent, as shown by abrogation after mild periodate treatment (Fig. 1). Because the percentage of this residual binding varied from 1 to 10% between separate experiments, no firm conclusions can be drawn from the minor differences between the various siglecs with regard to this residual binding.

The expression of SLeX on myeloid cells is important for selectin-mediated processes in inflammatory responses and in lymphocyte homing (55). Our finding that siglec binding is diminished markedly by the presence of an α1–3-linked Fuc residue implies that this family of adhesion molecules will not compete with any selectin-mediated recognition processes. On the other hand, it is possible that fucosylation can negatively regulate siglec-binding to some cells. Interestingly, in the bone marrow the expression of SLeX is regulated developmentally: it is found on a portion of the $CD34^+$ progenitor cells (56), expressed strongly on promyelocytes, followed by a transient down-regulation during the promyelocyte/myelocyte transition stage. Expression then increases progressively during the later stages of myeloid maturation (57). This suggests that siglec-1, which is thought to interact with myeloid cells in the bone marrow (18), could only function optimally at a particular stage in myeloid development, *i.e.* the promyelocyte/myelocyte tran-

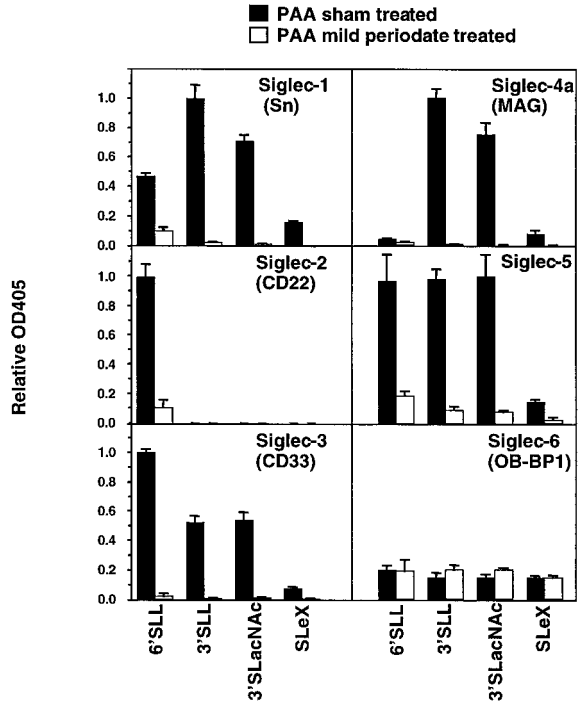

FIG. 1. **Binding of the siglecs to various sialylated PAAs: effect of fucosylation and sialic acid linkage preference.** Siglec-Fcs were immobilized via protein A on a microtiter plate at 500 ng/well as described under "Experimental Procedures" (siglec-3-Fc (CD33-Fc) was pretreated with AUS). Biotinylated polyacrylamide probes conjugated to various sialylated glycans were added, and binding was determined as described under "Experimental Procedures." For each siglec-Fc maximum binding reflected by the highest OD_{405} value was set at 1.00. (For siglec-6 the highest OD_{405} value was reached by sialyl-Tn-PAA, but this is not quantitatively compared here for any of the other siglecs because of the lower level of biotinylation of the sialyl-Tn probe). Abrogation of binding after mild periodate treatment (see "Experimental Procedures") of the PAAs shows the involvement of the glycerol side chain of sialic acid in the interaction. Data show the mean ± S.D. of triplicates. *MAG*, myelin-associated glycoprotein.

sition stage. Fucosylation could affect siglec-3 biology as well because its expression on myeloid cells is also highly regulated, being expressed on myelomonocytic precursors (where it coincides with a high level of SLeX expression) (57), monocytes, and tissue macrophages but absent from hematopoietic stem cells (58). The sialic acid binding sites of siglecs are often masked by endogenous ligands (11, 59–63), which can be unmasked either by sialidase treatment or cellular activation (53, 64). Our finding indicates that high levels of fucosylation on cell surfaces could also result in such an unmasking effect. Functional activity of siglecs through sialic acid binding could thus be regulated not only by activation-induced unmasking effects, but also by developmentally dependent fucosylation of the potential siglec ligands.

Siglec-3 Prefers α2–6-linked Sialic Acid—Earlier studies using red blood cell resialylation concluded that siglec-3 prefers to interact with α2–3-linked sialyllactosamine (11). However, we found that siglec-3 actually prefers α2–6-linked sialyllactosamine above α2–3-linked sialyllactosamine (Fig. 1). We made use of defined synthetic PAA probes, but the earlier study was done with resialylated red blood cells, where a mixture of different linkages could still confound the results if de- and/or resialylation did not proceed efficiently and/or equally well with the different sialyltransferases used. Furthermore, as expected from previous work (62), we found that AUS treatment of recombinant siglec-3 was essential for optimal binding activity. This problem was unknown at the time the initial red blood cell-based study was done (11). However, it was known that COS cells transiently transfected with siglec-3 needed to be treated with sialidase, for binding activity toward HL-60 cells, to unmask the sialic acid binding site (11). For the other recombinant soluble siglecs AUS pretreatment either made no difference (siglecs-4a and -6) or gave only a slight increase in binding activity (siglecs-1, -2, and -5) (not shown). A more dramatic effect of sialidase treatment has been described previously by others for siglec-1-Fc (65). This may reflect the intrinsic sialylation capabilities of the cells used to express the recombinant molecules.

Genetic elimination of the ST6Gal-I sialyltransferase involved in the synthesis of Neu5Acα2–6Galβ1–4GlcNAc-containing glycans results in B cell dysfunction (66). This phenotype was more severe than the phenotype caused by genetic disruption of siglec-2 (26–29). One explanation for this difference is the potential existence of other lectins besides siglec-2 which can recognize the α2–6-linked ST6Gal-I product. Our data make it clear that among these lectins we should now include siglec-3.

Siglec-5 Binds α2–8-linked Sialic Acid—To examine binding of the siglecs to α2–8-linked sialic acids, we used ganglioside G_{D3} as a probe in a lipid ELISA with all six siglecs. Only siglec-5 displayed clear binding to this glycolipid (data not shown). An earlier study also indicated that siglec-4a does not bind to G_{D3} (17). However, that same study showed that there is some binding of siglec-1 to G_{D3}. A different presentation of siglec-1, either as full-length on a cell surface (as in the earlier study) or as a recombinant chimeric molecule (as in this study) may explain this difference. The binding of siglec-5 to G_{D3} was decreased by ~85% after mild acid or AUS treatment, showing the sialic acid-dependent nature of the interaction. The interaction was decreased by 65% after mild periodate treatment of G_{D3}, indicating that it is the terminal α2–8-linked sialic acid that is being recognized. The binding to α2–8-linked sialic acid cannot be compared quantitatively with that for the other sialic acid linkages tested on the PAA probes because different assays were used. However, this finding shows that siglec-5 is even more promiscuous in its recognition for sialic acid linkages than recognized previously (13). The ability to recognize any type of sialic acid could be useful for the neutrophils and monocytes on which siglec-5 is expressed. If siglec-5 is a negative regulator of activation, as suggested by its cytosolic immunoreceptor tyrosine-based inhibitory motif (13, 67), binding to sialic acid may thus suppress activation of the neutrophils upon contact with endogenous cells bearing sialic acids in any linkage.

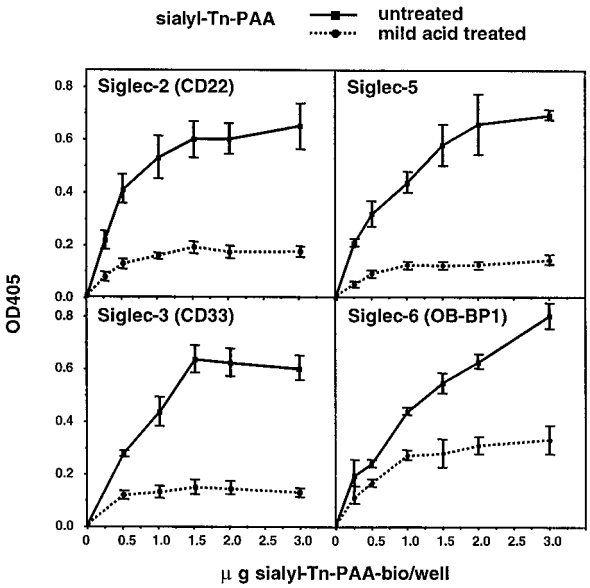

FIG. 2. **Binding of siglecs to sialyl-Tn-PAA.** Siglecs bind to sialyl-Tn-PAA (*continuous lines*), and this binding is decreased after mild acid treatment (*broken lines*). This was assayed by ELISA as described under "Experimental Procedures" and the legend to Fig. 1. Data show the mean ± S.D. of triplicates. No binding was seen with siglecs-1 (Sn) or -4a (myelin-associated glycoprotein) (data not shown).

Binding of Siglecs to Sialyl-Tn—During initial studies of siglec-6 we found for the first time that the sialyl-Tn epitope (Neu5Acα2–6GalNAc) can be a ligand for a siglec (14). Here we report that siglecs-2, -3, and -5 can also bind to sialyl-Tn (Fig. 2). No binding by siglecs-1 and -4a was noted (data not shown). This sialyl-Tn PAA probe contained a lower level of biotinylation (as examined by ELISA) compared with the other sialylated probes (6′-SLL, 3′-SLL, 3′-SLacNAc, and SLeX) all of which displayed a similar level of biotinylation (not shown). Thus, siglec binding to sialyl-Tn-PAA cannot be compared quantitatively with the other PAA probes. Regardless, reduction of binding after desialylation by mild acid treatment of the sialyl-Tn probe showed that the interaction of the various siglec-Fcs with this probe is sialic acid-dependent (Fig. 2). Mild acid treatment was used for desialylation because sialidase does not efficiently remove sialic acid from the sialyl-Tn-PAA probe (data not shown).

Although low levels of sialyl-Tn are found in some healthy tissues, such as colon (68), erythroid cells, and a subset of lymphocytes (69), it is generally considered a tumor-associated antigen. Indeed, sialyl-Tn can be used as a diagnostic marker in many cancers (39, 40), where high expression of sialyl-Tn is associated with poor prognosis (39, 41–43). The mechanism underlying the expression of this antigen on cancer cells may be a lack of expression of the glycosyltransferases that normally extend the *O*-glycan chains or the premature hypersialylation of the *O*-linked GalNAc residue, preventing the action of these extension glycosyltransferases (39, 70). De-*O*-acetylation may be another means to generate sialyl-Tn in cancers: in normal healthy colon sialyl-Tn is found in the *O*-acetylated form, whereas in colonic tumors the *O*-acetylated form is ab-

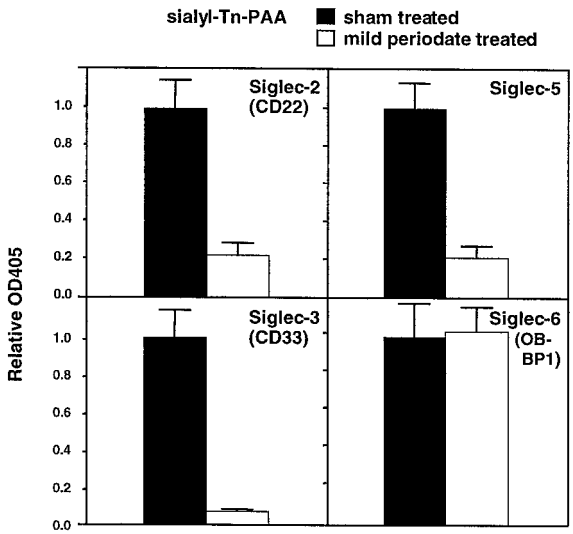

FIG. 3. **Involvement of the glycerol side chain of sialic acid in the binding of siglecs to sialyl-Tn-PAA.** Sialyl-Tn-PAA was treated with periodate and borohydride to convert the glycerol side chain of sialic acid to a mixture of C7 and C8 alcohols or sham treated as described under "Experimental Procedures." The binding of various siglecs to these sham- and periodate-treated probes was tested by ELISA (as described under "Experimental Procedures" and legend to Fig. 1). Data show the mean ± S.D. of triplicates.

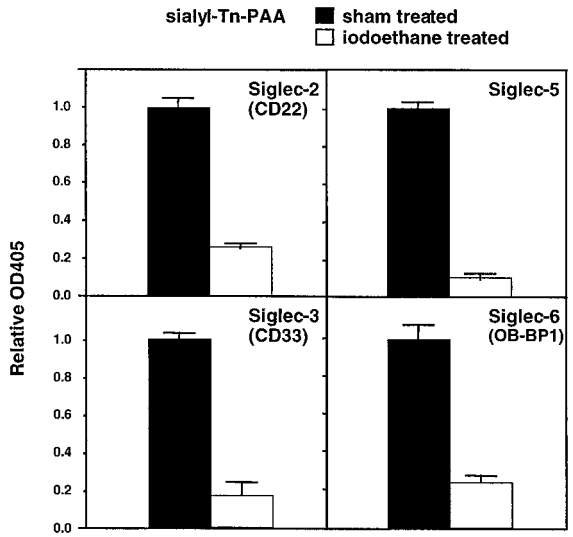

FIG. 4. **Involvement of the carboxyl group of sialic acid in the binding of siglecs to sialyl-Tn-PAA.** Sialyl-Tn-PAA was treated with iodoethane and borohydride to convert the carboxylate of sialic acid to its C1 alcohol or sham treated as described under "Experimental Procedures." The binding of various siglecs to these probes was tested by ELISA (as described under "Experimental Procedures" and the legend to Fig. 1). Data show the mean ± S.D. of triplicates.

sent (71–73). The potential selective advantage for sialyl-Tn expression on tumor cells is at present unknown. The ability of four of the siglecs to recognize a tumor-antigen like sialyl-Tn potentially links this lectin family to the progression of cancer. Of note, the four siglecs that can recognize sialyl-Tn all contain cytoplasmic motifs, such as immunoreceptor tyrosine-based inhibitory motifs and/or signaling lymphocyte activation molecule motif, which are likely to be involved in signaling events (14, 67, 74, 75). Thus, by up-regulating the expression of sialyl-Tn or by altering the spacing of this epitope on its surface (see below), a tumor cell could potentially regulate the activity of immune cells expressing siglecs and thereby influence the course of the disease.

Involvement of the Glycerol Side Chain and the Carboxyl Group of Sialic Acid in Siglec Binding—Truncation of the glycerol side chain by mild periodate oxidation is known to abrogate binding by siglecs-1, -2, and -4a (9, 17, 21, 36). Furthermore, for siglecs-1 and -4a the importance of the side chain has been shown by the use of synthetic analogs (54, 76). We show here that siglecs-3 and -5 also need the intact glycerol side chain of sialic acid for binding to the various sialylated PAA probes (Fig. 1). Surprisingly, binding by siglec-6 was not abrogated by mild periodate treatment (Fig. 3). This makes siglec-6 the first siglec for which the glycerol side chain is not directly involved in binding. To explore the role of the carboxyl group in recognition, we converted it to an alcohol on the sialyl-Tn probe by treatment with iodoethane followed by $NaBH_4$ reduction. The results show that for all four siglecs the binding to sialyl-Tn is dependent on the intact carboxyl group of sialic acid (Fig. 4). For siglecs-1 and -4a the involvement of the carboxyl group of sialic acid was proven separately by abrogation of binding to 3′-SLL after iodoethane treatment of this probe (data not shown). Thus, all six siglecs require the carboxylate group of sialic acid for binding.

For siglecs-1, -2, and -4a it is known that 9-*O*-acetylation of sialic acid generally prevents binding (54, 76–78) presumably by blocking the glycerol side chain of sialic acid. This issue was examined further by studying the binding of siglecs-2, -3, -5, and -6 to BSM, which contains 40% 9-*O*-acetylated sialic acids (as determined by DMB analysis, data not shown). Indeed, the binding of siglec-2 to BSM is increased markedly after removal of *O*-acetyl esters by base treatment (Fig. 5). The same is found for siglec-3. However, both siglecs-5 and -6 bind equally well to untreated and base-treated BSM (Fig. 5). Also, in contrast to siglecs-2 and -3, mild periodate treatment does not abrogate binding for either siglecs-5 or -6. Thus, the finding that siglec-6 does not require the glycerol side chain of sialic acid for binding is confirmed further. However, this result was unexpected for siglec-5 because the periodate treatment of the PAAs showed involvement of the glycerol side chain of sialic acid for this siglec (Figs. 1 and 3). One explanation is that besides sialyl-Tn, BSM contains the following major sialylated structures: Galβ1–3(Neu5Ac/Gcα2–6)-GalNAc-O and GlcNAcβ1–3-(Neu5Ac/Gcα2–6)-GalNAc-O (79–81). It is possible that low affinity ligands, such as sialyl-Tn-PAA, need an intact side chain to achieve binding. On the other hand, higher affinity ligands as present on BSM may not be dependent on an intact glycerol side chain of sialic acid to achieve binding. This concept may apply to siglec-3 as well, because binding to PSM is only partly abrogated by mild periodate treatment (Fig. 6). Of note, the degree of reduction in binding by siglec-2 caused by a truncated glycerol side chain also varies among the different probes tested: 6′-SLL, ~90%; sialyl-Tn PAA, ~80%; BSM, ~70%; OSM, ~80%; and PSM, ~60%. Like BSM, PSM (A^-) also contains more complex α2–6-sialylated structures: Galβ1–3(Neu5Gcα2–6)-GalNAc-O and Fucα1–2Galβ1–3(Neu5Gcα2–6)-GalNAc-O (81, 82). In this regard, another interesting finding is that while only siglec-2 binds to both PSM and OSM (98% of the glycans are sialyl-(Neu5Ac)-Tn) (83), siglecs-3, -5, and -6 only bind to the former (Fig. 6). One possibility is that the binding to PSM by siglecs-3, -5, and -6 is not mediated primarily by sialyl-Tn but by the Galβ1–3(Neu5Gcα2–6)-GalNAc-O structure known to be present on this molecule (82). Thus, the underlying and/or adjacent sugars may play an additional role in recognition, as has been found for siglec-4a (17, 36, 37, 54). Obviously, these four siglec-Fcs can recognize sialic acid in the *N*-glycolyl form, as PSM contains almost exclusively this form of sialic acid (the role of Neu5Gc is addressed further in the

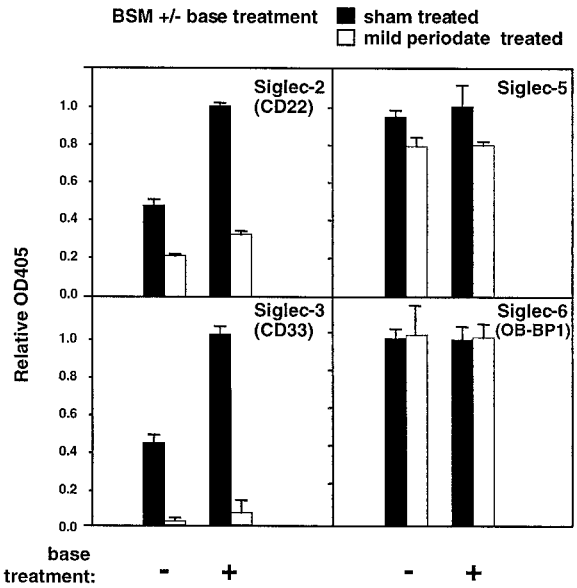

FIG. 5. **Binding of siglecs to BSM: effect of 9-*O*-acetylation of sialic acid.** BSM (biotinylated as described under "Experimental Procedures") was incubated with 0.1 M NaOH for 30 min at room temperature to remove base-labile *O*-acetyl esters and then neutralized with HCl. A control used an already neutralized mixture of NaOH/HCl. Aliquots of both untreated and base-treated BSM were also treated with mild periodate as described under "Experimental Procedures," and binding of various siglecs was tested by ELISA as described under "Experimental Procedures" and the legend to Fig. 1. Data show the mean ± S.D. of triplicates.

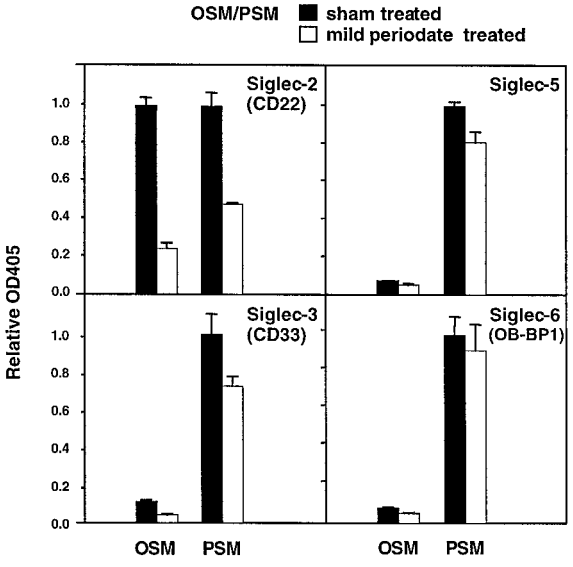

FIG. 6. **Binding of siglecs to OSM and PSM.** Binding of OSM and PSM (biotinylated as described under "Experimental Procedures") to siglecs was tested by ELISA as described under "Experimental Procedures" and the legend to Fig. 1. Aliquots of both mucins were treated with or without mild periodate as described under "Experimental Procedures." Data show the mean ± S.D. of triplicates.

accompanying paper) (45). Binding of all siglec-Fcs to mucins was abrogated after mild acid treatment, confirming the sialic acid dependence of the interactions (data not shown).

Importance of the Presentation of Sialyl-Tn for Siglec Binding—Another explanation for why only siglec-2 binds to OSM could be that the presentation of sialyl-Tn affects binding, *i.e.* the density, clustering, or spacing on the PAA backbone is more optimal than that on the polypeptide backbone of OSM. This possibility was supported by experiments done with transfected CHO-TAg cells. CHO-TAg cells transiently transfected with ST6GalNAc-I or both ST6GalNAc-I and MUC1 express easily detectable cell surface sialyl-Tn on endogenous proteins and/or on MUC1 (as shown by the IgG antibody TKH2, data not shown). Despite this expression of sialyl-Tn, no binding was found by siglecs-2, -5, and -6 (data not shown). Although the flow cytometry results with antibody TKH2 are only semiquantitative, the complete lack of binding by the recombinant siglecs (which have a similar bivalent presentation based on an Ig-Fc scaffold) indicates that presentation and/or density of sialyl-Tn can affect recognition.

The importance of presentation of sialyl-Tn for recognition by siglecs actually fits well with the findings that different antibodies specific for this epitope recognize it in two different configurations, either clustered or nonclustered (84) or need a specific cluster of sialyl-Tn groups for recognition (85). One possible mechanism for the selectivity is that the known interactions of the *O*-linked GalNAc residue with the underlying polypeptide alters the presentation of the sialic acid residue in different ways (86, 87). In another analogous situation, the recognition of terminal α2–3-linked sialic acids on *O*-glycans by polyomavirus receptors is positively regulated by a second α2–6-linked sialic acid on the GalNAc residue (88)

Conclusions and Perspectives—Here we have defined many new aspects of siglec binding specificities, including the importance of α1–3-fucosylation and of the sialyl-Tn epitope. This was achieved primarily with various sialylated glycans that are presented in multivalent form on synthetic PAA backbones. The advantage of these probes is the uniform structure of the glycans presented (44). In most instances we also confirmed earlier conclusions regarding siglec specificities, where primarily α2–3- and α2–6-sialyllactos(amin)es were used (9, 13, 14, 16, 17, 21–23, 36, 37). However, for siglec-3 we found that earlier conclusions (11) were not confirmed, most probably because of the less well defined probes used in those studies. Table II provides a summary of the recognition specificities of the siglecs based on the current work and on prior literature.

Although the PAA probes used in this study give clear indications of the selective recognition specificities of the siglecs, the experiments with mucins indicate that the natural ligands may be more complex. There may be an analogy with the history of ligand discovery in the selectin field, where the initial finding that sialylated, fucosylated glycans were recognized was followed by the discovery that these glycans were necessary but not sufficient for biologically relevant binding (89). For example, the necessity for correct presentation of the carbohydrate ligand has been clearly shown for P-selectin: tyrosine-sulfated peptides containing SLeX on a core 2-based *O*-glycan bind to P-selectin, whereas when the SLeX was presented on the same peptide using a core 1-based *O*-glycan, binding was not found (90).

This is also the first study to show that several siglecs bind to the sialyl-Tn epitope. The attachment of the *O*-linked GalNAc is a complex process under the control of an expanding family of GalNAc transferases (91–101). Together with the existence of at least four different ST6GalNAc enzymes with differing specificities which can create the Neu5Acα2–6GalNAc structure (102–106) the vertebrate Golgi can generate a wide array of different presentations of this epitope on the peptide backbone. This, together with the diversity of other possible substitutions on the GalNAc residue (core 1 or core 3, with or without extension or α2–3-sialylation) suggests avenues for further exploration of siglec ligand specificity. A search for natural macromolecular ligands also seems appropriate.

TABLE II
Recognition specificities of the human siglecs
This summary is compiled from results reported here and from data in the literature.

Siglec		Ability to recognize					Essential group of sialic acid		Based on data in this study and references
No.	Other name	Siaα2–6 Lac(NAc)	Siaα2–3 Lac(NAc)	SLeX	Siaα2–6 GalNAc[a]	Siaα2–8 Sia	Glycerol side chain	Carboxyl group	
1	Sn	+	++	−	−	−/+[b]	+	+	16, 17
2	CD22	++	−	−	+	−	+	+	9, 21–23
3	CD33	++[c]	+	−	+	−	Variable	+	11
4a	MAG[d]	−	+	−	−	−	+	+	17, 36, 37
5		+	+	−	+	+	Variable	+	13
6	OB-BP1	−	−	−	+	−	−	+	14

[a] Data concerning the possible recognition of larger *O*-glycans that include the Siaα2–6GalNAc epitope are not presented here.
[b] We did not find binding of siglec-1 to Siaα2–8Sia in immobilized G_{D3}. However, a previous study reported that cells overexpressing full-length siglec-1 (Sn) can bind to immobilized G_{D3} (17).
[c] A previous study using resialylation of erythrocytes concluded that α2–3-linked sialic acids were preferred targets for siglec-3 (CD33). However, we show here that both α2–3- and α2–6-linked sialic acids are recognized, with a preference for the latter.
[d] MAG, myelin-associated glycoprotein.

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