Enhanced Binding of the Neural Siglecs, Myelin-associated Glycoprotein and Schwann Cell Myelin Protein, to Chol-1 (α-Series) Gangliosides and Novel Sulfated Chol-1 Analogs*

(Received for publication, July 13, 1999, and in revised form, September 8, 1999)

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Extended glycoconjugate binding specificities of three sialic acid-dependent immunoglobulin-like family member lectins (siglecs), myelin-associated glycoprotein (MAG), Schwann cell myelin protein (SMP), and siaioadhesin, were compared by measuring siglec-mediated cell adhesion to immobilized gangliosides. Synthetic gangliosides bearing the α-series determinant (NeuAc α2,6-linked to Galβ1-4GlcNAc on a gangliotetraose core) were tested, including GD1α (IV β NeuAc, III NeuAc-βGlc-βGlcNAc), GD1α with modified sialic acid residues at the III°-position, and the “Chol-1” gangliosides GT1aa (IV β NeuAc, III NeuAc, IV β NeuAc-Galβ1-3GlcNAc) and GQ1bα (IV β NeuAc, III NeuAc, II β NeuAc-Galβ1-3GlcNAc). The α-series gangliosides displayed enhanced potency for MAG- and SMP-mediated cell adhesion (GQ1bα > GT1aa, GD1α > GT1b, GD1α > G1M (nonbinding)), whereas siaioadhesin-mediated adhesion was comparable with α-series and non-α-series gangliosides. GD1α derivatives with modified sialic acids (7, 8, or 9-deoxy) or sulfate (instead of sialic acid) at the III°-position supported adhesion comparable with that of GD1α. Notably, a novel GT1aa analog with sulfates at two internal sites of sialylation (NeuAcα2,3Galβ1-4Galβ1-3GalNAc-6-sulfateβ1, 4Galβ3-sulfateβ1,4Glcβ1,1-ceramide) was the most potent siglec-binding structure tested to date (10-fold more potent than GT1aa in supporting MAG and SMP binding). Together with prior studies, these data indicate that MAG and SMP display are extended structural specificities with a requirement for a terminal α2,3-linked NeuAc and great enhancement by nearby precisely spaced anionic charges.

Siglecs (sialic acid-dependent immunoglobulin-like family member lectins) contain an N-terminal V-type Ig domain, a varying number of C-type Ig domains, a single transmembrane domain, and a short cytoplasmic tail (1, 2). The six reported siglecs (CD22, CD33, siaioadhesin, MAG, § SMP, and siglec 5) share a significant degree of sequence similarity among their V-type domains, which are required for sialic acid binding. Siaioadhesin (siglec 1), which is the largest siglec (17 Ig-like domains), is found on bone marrow macrophages and may play a role in hematopoiesis. The nervous system siglecs, MAG and SMP (siglecs 4a and 4b), each of which has five Ig-like domains, are expressed on myelinating oligodendrocytes and Schwann cells. MAG is involved in myelin maintenance and in myelin-axon interactions that mediate neuronal cytoarchitecture and the inhibition of axon regeneration after injury (3–6). SMP, which is found only in avian species, may be the avian homologue of MAG (7). Presumably, the biological functions of MAG and other siglecs require their binding to sialo-glycoconjugates on apposing cell surfaces (8). Defining the carbohydrate determinants responsible for siglec binding may help reveal their biological target specificities and provide opportunities for the design of carbohydrate mimetics to modulate siglec function.

Although each siglec recognizes a terminal sialic acid residue, the siglecs display significant differences in sialic acid linkage specificity (9). CD22 recognizes solely α2,6-linked sialic acids, MAG and SMP require α2,3-linked sialic acids, and siaioadhesin binds terminal α2,3- or α2,8-linked sialic acids (10). All siglecs demonstrate considerable sialic acid substructural specificity, with differing requirements for the sialic acid N-acetyl moiety as well as particular sialic acid hydroxyl groups (11–17). In addition, siglecs demonstrate “extended” oligosaccharide specificity, defined as preferential binding based on the neutral oligosaccharide core to which the sialic acid is attached, or preferential binding to oligosaccharides bearing multiple sialic acid residues (10, 14, 16–18).

MAG is expressed on the periaxonal membrane of myelin, directly apposed to the neural membrane, where its ligand is thought to be expressed (19). Since gangliosides carry 75–80% of the sialic acid in the brain (20), and the major brain gangliosides GD1α and GT1b express the preferred NeuAcα2,3Galβ1,3GalNAc terminal target determinant for MAG, we hypothesize that gangliosides are functional MAG ligands. A subset of neurons, those that use acetylcholine as their neurotransmitter, express a unique quantitatively minor family of gangliosides termed “Chol-1” gangliosides, initially defined by their reactivity with a polyclonal antiserum raised against cholinergic neurons (21). Chol-1 gangliosides carry an α2,6-linked sialic acid on the Galβ1-4GlcNAc of a gangliotetraose core

* This work was supported in part by National Institutes of Health Grant NS37096; a grant from the National Multiple Sclerosis Society, National Science Foundation Grant IBN–9631745, and a grant from the Paralyzed Veterans of America Spinal Cord Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported in part by National Institutes of Health Grant GM07626.

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1 The abbreviations used are: MAG, myelin-associated glycoprotein; SMP, Schwann cell myelin protein; PBS, phosphate-buffered saline.

2 Ganglioside nomenclature is that of Svennerholm (46) or as indicated in Fig. 1. cis-GM1 (NeuAcα2,3Galβ1,3GalNAcβ1,4Galβ1,3Glcβ1,1-Cer) is also referred to in the literature as “GM1b.”
Extended Carbohydrate Binding Specificities of Siglecs

**Materials**—Gangliosides GM1, GD1a, and GT1b were purchased from Matreya, Inc. (Pleasantville, PA). GQ1ba, GT1ao, GD1a, and GD1a derivatives bearing deoxysialic acids, cis-GM1, cis-GM1 isomers, and sulfated cis-GM1 analogs were synthesized de novo as described (26–29). Synthetic gangliosides were quantified by resorcinol staining as compared with ganglioside standards on TLC. Structures of synthetic gangliosides were confirmed by TLC and negative ion fast atom bombardment mass spectrometry.

**Siglec Expression—**cDNAs encoding full-length rat MAG, quail SMP, and mouse sialoadhesin were cloned into the expression vectors pCDMB (MAG and SMP) or pCDNA1/Amp (sialoadhesin) E. coli were purified by polyethylene glycol precipitation. COS-1 cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37 °C and in a humidified environment of 90% air, 10% CO2. Cells were plated at a density of 9 × 10^5 cells/100-mm diameter dish, were cultured overnight, and then were transiently transfected by exposure to 4 ml of Dulbecco's modified Eagle's medium supplemented with 2.5% fetal calf serum, 40 μg/ml DEAE-dextran, 0.1 mM chloroquine, and 3 μg of the plasmid of interest. After 3.5 h of transfection, the medium was removed, and cells were treated with 10% Me2SO (v/v) in PBS for 5 min and then were returned to growth medium. The cells were cultured for 40–50 h to allow ample siglec gene expression prior to detachment for use in adhesion assays.

**Siglec-mediated Cell Adhesion—**Adhesion assays were performed as described previously (16, 31). Gangliosides were adsorbed to the bottom of 96-well dishes in an artificial membrane monolayer with phosphatidylcholine and cholesterol. Aliquots (50 μl) of ethanol/water (1:1) containing 0.5 μM phosphatidylcholine, 2.0 μM cholesterol, and the indicated amounts of ganglioside were added to wells of a 96-well flat-bottom microwell plate. The plate was left uncovered for 90 min at ambient temperature to allow partial evaporation and lipid immobilization to occur. After adsorption, the wells were washed three times with water and then were preblocked by the addition of 100 μl/well Hepes-buffered Dulbecco's modified Eagle's medium containing 2 mg/ml bovine serum albumin. Plates were incubated for 10 min at 37 °C prior to the addition of cells.

Transfected cells were harvested by exposure to hypertonic Ca2+/Mg2+-free PBS containing 1 mM EDTA as described (18), collected by centrifugation, and resuspended at 105 cells/ml in PBS containing 2 mg/ml bovine serum albumin. Plates were incubated for 10 min at 37 °C prior to the addition of cells.

Subsequent to the adhesion incubation, nonadherent cells were removed using carefully controlled centrifugal detachment force. To avoid fluid shear, the plate was carefully immersed upright in a vat of PBS (at ambient temperature), inverted (while immersed), and placed (inverted) in an immersed custom-designed Plexiglas box, which was sealed with a gasket to exclude air (31). The inverted plate in its fluid-filled chamber was placed in a centrifuge carrier and centrifuged at 110 × g for 10 min to remove nonadherent cells, and the chamber was returned to the vat of PBS. While immersed, the plate was gently removed from the chamber but kept immersed (in the inverted orientation) for 5 min to allow any floating cells to settle away. The plate was then righted (while immersed) and removed from the vat, and excess surface PBS

**EXPERIMENTAL PROCEDURES**

**Gangliosides**—Gangliosides and ganglioside analogs used in this study. Shorthand ganglioside nomenclature is based on that of Svennerholm (46) (cis-GM1 is also referred to as “GM1α” in the literature). The “a” designation indicates a NeuAc residue linked α2,6 to the GalNAc residue in the gangliotetraose core. Chol-1 gangliosides, antigens of the polyconal cholinergic-specific Chol-1 antisem (21), include GT1ao and GQ1bo (22, 23).

![Fig. 1. Gangliosides and ganglioside analogs used in this study.](Image)

3 Synthetic details for the novel sulfated α-series ganglioside analogs will be reported elsewhere.
was removed by aspiration. At this point, all wells were full of PBS (320 μl/well), and only adherent cells remained on the well bottoms.

Adherent cells were quantified by lysis and measurement of released lactate dehydrogenase. After completion of the adhesion assay, 10 μl of 10% Triton X-100 in PBS were added to each PBS-filled well, adherent cells allowed to lyse for 10 min and then triturated thoroughly with a multichannel micropipettor. An aliquot of lysate (80 μl) from each well was transferred to a fresh plate, and 120 μl of PBS containing 0.5 mg/ml each of NADH and sodium pyruvate were added. The decrease in UV absorbance at 340 nm was measured simultaneously in 96 wells using a kinetic plate reader (Benchmark Microplate Reader, Bio-Rad). Kinetic rates were compared with those from wells containing aliquots of standard cell suspensions to calculate the percentage of cells added that remained adherent at the end of the experiment. To account for modest variations in transfection efficiency between experiments and between the different transfection vectors, values were normalized to the maximum percentage of cells adhering to positively adherent gangliosides for that lectin in that experiment, adjusted for background adhesion.

Over the course of the 14 experiments (39 separate siglec transfections) reported here, the average maximum and background adhesion values were as follows (mean ± S.E.): 75.7 ± 5.2 (maximum) and 12.2 ± 2.4 (background) for MAG; 67.2 ± 4.7 (maximum) and 13.1 ± 1.7 (background) for SMP; and 72.8 ± 5.1 (maximum) and 9.2 ± 0.9 (background) for sialoadhesin. Each value reported in the figures is the average of 3–4 replicate determinations and is expressed as mean ± S.E. Gangliosides used in these studies adsorbed comparably to the wells and remained adsorbed equally during incubation with cells (data not shown).

RESULTS

Siglec Adhesion to α-Series Gangliosides—In agreement with our previous report (17), MAG and sialoadhesin adhered with moderate potency to the two major brain gangliosides bearing terminal “NeuAcα2,3Galβ1,3GalNAc” determinants, GD1α and GT1b (Fig. 2, A and C, and Table I), whereas SMP-mediated adhesion to these gangliosides was less avid (Fig. 2B; SMP-mediated adhesion to weakly supportive gangliosides added at concentrations above 50 pmol/well was often less than maximum levels, perhaps due to charge repulsion (16)). The Chol-1 ganglioside GQ1bα was ~10-fold more potent in supporting MAG- and SMP-mediated adhesion than was the closely related major brain ganglioside lacking the α2,6 NeuAc residue, GT1b (Table I). Gangliosides without the NeuAcα2,3Gal terminus, such as GM1 (Fig. 2) and GM1α (IIIα NeuAc-Gg4OseCer, data not shown) did not support adhesion of any of the siglecs.

MAG and SMP displayed enhanced avidity for the other α-series gangliosides tested, GD1α and GT1α (Fig. 2, A and B; Table I), compared with GD1α and GT1b. In contrast to the neural siglecs, α-series gangliosides and GD1α were equally potent in supporting sialoadhesin-mediated cell adhesion (Fig. 2C). A comparison of adhesion to GD1α and cis-GM1 (Table I; structures in Fig. 1), indicates that the α2,6 NeuAc residue per se enhanced binding of siglecs 3–6-fold.

The Role of Exocyclic Glycerol Chain Hydroxyls of the α2,6-Linked NeuAc on Siglec Recognition of GD1α—The increased potency of Chol-1 gangliosides to support MAG- and SMP-mediated adhesion suggests a potential direct interaction between the α2,6 NeuAc residue and the neural siglecs. Previously, we reported that MAG required an intact sialic acid exocyclic glycerol chain on the terminal α2,3 NeuAc residue for recognition (16). In the current study, we tested the role of the exocyclic glycerol chain hydroxyls on the α2,6 NeuAc residue of GD1α using synthetic derivatives (32). GD1α, bearing a 7-, 8-, or 9-deoxylic acid linked α2,6 to GalNAc, supported comparably enhanced siglec-mediated adhesion (Fig. 3 and Table I), indicating that the hydroxyls on this exocyclic glycerol chain are not strictly required for enhanced siglec recognition. This led us to test the role of the anionic charge at the III6-position using sulfate analogs of GD1α and GT1α.

Replacing the III6 NeuAc residue of GD1α with a sulfate group (cis-GM1 III6-sulfate, Fig. 1) had no effect on MAG or sialoadhesin binding (Fig. 4, Table I) and only a modest negative effect on SMP binding, indicating that anionic charge at that position is key to enhanced binding affinity. Notably, an analog of GT1a bearing two sulfate groups (cis-GM1 variant II3, III6-bissulfate, Fig. 1) demonstrated 10-fold increased binding affinity for MAG and SMP and 2-fold for sialoadhesin, making it the most highly potent target for siglec-mediated cell adhesion tested to date (Fig. 4, Table I).

Siglec-mediated Adhesion to cis-GM1 with Altered Neutral Cores—To investigate extended recognition by siglecs based on
the gangliotetraose core, we employed synthetic derivatives of cis-GM1 (29), the minimal gangliotetraose structure bearing the NeuAc2,3Galβ1,3GalNAc terminus preferred by MAG (Fig. 1). MAG bound 3-fold better to cis-GM1 than to a matched structure with a GlcNAc replacing the core GalNAc (Fig. 5). Sialoadhesin, however, recognized both structures equally well. Surprisingly, a novel synthetic structure having the IV-Gal residue in β1,6 rather than β1,3 linkage (cis-GM1, “Gal β1,6-GalNAc” variant) supported siglec adhesion at levels similar to the parent cis-GM1 (Fig. 5). In contrast to cis-GM1, monosialogangliotetraoses GM1 (Fig. 2) and GM1α (data not shown) did not support any siglec adhesion.

**DISCUSSION**

The current study supports the hypothesis that there is extended recognition of multisialylated gangliosides by the closely related neural siglecs, MAG and SMP. For these siglecs, relative placement of the sialic acids (or sulfates) on the neutral sugar core appears to be an important factor in determining binding affinity. This and prior published data (9, 16–18) indicate that MAG and SMP require a terminal “NeuAc2,3Gal” determinant as the primary structural requirement for binding but that additional (secondary) sialic acids on the same core greatly enhance binding. Relative placement of the secondary sialic acids appears to be key, with the NeuAc2,6GalNAc determinant preferred. Thus, GD1a has higher affinity for MAG and SMP than cis-GM1, GD1a has yet higher affinity, and GT1aa is more potent than GT1b (Table I). Two alternative hypotheses fit these data: (i) MAG and SMP have primary and secondary sialic acid binding sites; or (ii) MAG and SMP are very sensitive to the precise tertiary configuration of the primary determinant (NeuAc2,3Galβ1,3GalNAc), which is in turn impacted by nearby sialic acids. NMR studies support the latter hypothesis, in that the core GalNAc and II3NeuAc of gangliosides directly interact to restrict the conformation of what would be the primary determinant (33). However, the III6NeuAc, which is yet more potent as a secondary sialic acid, may not interact in the same manner to restrict ganglioside conformation, and replacement of the III6NeuAc and II3NeuAc with sulfates greatly enhanced MAG and SMP binding. In either case, the extended structure of multisialylated gangliosides impacts the affinity of MAG and SMP binding. Importantly, the physiological significance of gangliosides bearing extended determinants has recently been established (8). Mice engineered to lack the ganglioside neutral core GalNAc transferase (UDF-GalNAc:GM3/GD3 N-acetylgalactosaminyltransferase) had progressive axon and myelin degeneration similar to the neural deficits found in MAG-deficient mice. Therefore, the extended specificity of MAG for its carbohydrate ligand may have important physiological consequences.

The extended specificity reported here conflicts with data reported recently by Strenge et al. (34), in that the oligosaccharide of GT1aa was reported to be no better than that of GM3 (sialyllactose, NeuAc2,3Galβ1,4Glc) in inhibiting MAGd1–3-Fc binding to human erythrocytes. Other structure-function differences between the Strenge et al. study and our studies were also noted, including inhibition of MAG binding
by soluble saccharides bearing 7-deoxy-NeuAc or 2-keto-3-deoxy-α-glycer-o-galactononic acid (KDN) in place of NeuAc and by certain oligosaccharides bearing only terminal α2,6-linked sialic acids. We agree with Strenge et al. that the differences are probably due to the different assays used. The current study measures binding of full-length cell surface MAG to saccharides oriented on an apposing membrane monolayer, whereas the Strenge et al. study measured the site affinity of soluble saccharides for a soluble MAG-Fc chimera engineered to have only the N-terminal three (of five) Ig-like domains. Presentation, valency, and the different forms of MAG may all contribute to the observed differences. Furthermore, it is difficult to compare the half-maximal inhibitory potency of free oligosaccharides for the MAG-Fc chimera (in the submillimolar range) with the half-maximal potencies reported here for supporting MAG adhesion (in the pmol/well range). Notably, genetically engineered mice expressing only truncated gangliosides display neuropathies similar to those in mice lacking MAG, supporting the notion that extended ganglioside specificity is of physiological importance (8).

The two neural siglecs, MAG and SMP, demonstrate the same relative trends in ganglioside binding specificity, although SMP displays characteristically lower binding avidity. These data reflect the close relationship between these two lectins, which share ~70% sequence similarity in the first two N-terminal Ig-like domains (1). Sialoadhesin, which shares ~40% sequence similarity with the neural siglecs in its first two N-terminal Ig-like domains, does not demonstrate the same extended specificity (GQ1bα, GT1bα, and GD1α demonstrated sialoadhesin binding affinities comparable with GD1α). Although extended specificity is suggested by the relatively low

**Fig. 4. Siglec-mediated cell adhesion to cis-GM1, α-gangliosides, and sulfated α-ganglioside analogs.** Adhesion of COS cells transiently transfected to express MAG (A), SMP (B), or sialoadhesin (C) to the indicated gangliosides was determined as described under “Experimental Procedures” and the legend to Fig. 2. Gangliosides used were as follows: cis-GM1 (▲), GD1α (●), cis-GM1 III-sulfate (▼), GT1αα (○), and cis-GM1 variant II,III-sulfate (◇).

**Fig. 5. Siglec-mediated cell adhesion to cis-GM1 and cis-GM1 analogs.** Adhesion of COS cells transiently transfected to express MAG (A), SMP (B), or sialoadhesin (C) to the indicated gangliosides was determined as described under “Experimental Procedures” and the legend to Fig. 2. Gangliosides used were as follows: cis-GM1 (▲); cis-GM1, “III-GlcNAc” variant (●); and cis-GM1, “Gal β1,6-GalNAc” variant (○).
affinity of sigloashein for cis-GM1 (compared with GD1a or GD1b), prior results demonstrated moderately high binding affinity of sigloashein for GM3 and even GM4, the simplest “NeuAcα2,3Gal”-bearing ganglioside (17). Together, these results suggest that sigloashein is less responsive to multiple sialic acids on the same neutral sugar core.

Sialic acid substructural specificity appears to be relatively stringent for all sigles, which require an intact exocyclic glycero side chain and are differentially responsive to the N-acyl moity (35). This contrasts with selectins, which bind to target glycosides bearing highly modified sialic acids, or the same saccharides with sulfate or carboxylate moieties in place of sialic acid (36–40). In prior studies, we demonstrated that MAG requires every hydroxyl on its primary sialic acid deter

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