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Gangliosides are neuronal ligands for myelin-associated glycoprotein

(lectins/sialoadhesins/Chol-1 gangliosides/nerve regeneration)

LYNDA J.-S. YANG*, CYNTHIA B. ZELLER*, NANCY L. SHAPER†, MAKOTO KISO‡, AKIRA HASEGAWA‡, ROBERT E. SHAPIRO§, AND RONALD L. SCHNAAR*¶

*Department of Pharmacology and Molecular Sciences, †The Oncology Center, and §Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ‡Department of Applied Bioorganic Chemistry, Gifu University, Gifu 501-11, Japan

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ABSTRACT **Nerve cells depend on specific interactions with glial cells for proper function. Myelinating glial cells are thought to associate with neuronal axons, in part, via the cell-surface adhesion protein, myelin-associated glycoprotein (MAG). MAG is also thought to be a major inhibitor of neurite outgrowth (axon regeneration) in the adult central nervous system. Primary structure and *in vitro* function place MAG in an immunoglobulin-related family of sialic acid-binding lectins. We report that a limited set of structurally related gangliosides, known to be expressed on myelinated neurons *in vivo*, are ligands for MAG. When major brain gangliosides were adsorbed as artificial membranes on plastic microwells, only GT1b and GD1a supported cell adhesion of MAG-transfected COS-1 cells. Furthermore, a quantitatively minor ganglioside expressed on cholinergic neurons, GQ1bα (also known as Chol-1α-b), was much more potent than GT1b or GD1a in supporting MAG-mediated cell adhesion. Adhesion to either GT1b or GQ1bα was abolished by pretreatment of the adsorbed gangliosides with neuraminidase. On the basis of structure–function studies of 19 test glycosphingolipids, an α2,3-*N*-acetylneuraminic acid residue on the terminal galactose of a gangliotetraose core is necessary for MAG binding, and additional sialic acid residues linked to the other neutral core saccharides [Gal(II) and GalNAc(III)] contribute significantly to binding affinity. MAG-mediated adhesion to gangliosides was blocked by pretreatment of the MAG-transfected COS-1 cells with anti-MAG monoclonal antibody 513, which is known to inhibit oligodendrocyte–neuron binding. These data are consistent with the conclusion that MAG-mediated cell–cell interactions involve MAG–ganglioside recognition and binding.**

Nerve cell function depends upon appropriate contacts between the neuron and other cells in its immediate environment (1). These include specialized glial cells, oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system, which ensheathe the neuronal axon with myelin, an insulating structure of multilayered membranes (2). Myelin is required for efficient nerve impulse conduction but has other profound biological effects. The inability of nerves to regenerate after CNS injury in adults may be due largely to the axon's inability to grow when in contact with CNS myelin (3, 4). Identification of the cell-surface constituents on neuronal axons and myelin membranes that interact with each other to control cell behavior may facilitate efforts to enhance nerve regeneration as well as modulate myelination.

Myelin-associated glycoprotein (MAG), a quantitatively minor protein constituent of CNS (1%) and peripheral nervous system (0.1%) myelin, is implicated in myelin–axon interactions based on its *in vivo* location and *in vitro* binding properties. In the CNS, MAG is located exclusively on myelin membranes juxtaposed to axons, where it contributes to maintenance of the periaxonal cytoplasmic collar (5). Purified MAG incorporated into liposomes binds specifically to neuronal processes in cell culture (6–8). This binding is blocked by an anti-MAG monoclonal antibody (mAb 513) that also inhibits neuron–oligodendrocyte adhesion *in vitro* (7).

One consequence of MAG–axon binding is inhibition of neurite outgrowth from CNS neurons. Cultured primary CNS neurons fail to extend neurites on a substratum of MAG-expressing CHO cells, whereas neurite extension proceeds readily on cells transfected with the reverse (control) construct (9). Recombinant MAG adsorbed to a culture surface inhibits neurite outgrowth from neuroblastoma cells in culture, as does a mixture of detergent-solubilized myelin proteins (10). Immunodepletion of MAG from the solubilized myelin proteins reduces their neurite outgrowth inhibitory activity by more than half. These data implicate MAG as a major neurite outgrowth inhibitory factor in myelin. If true, axonal ligands for MAG may be key elements controlling nerve regeneration in the CNS.

MAG is a member of the immunoglobulin superfamily with structural features related to those of other adhesion molecules (11–14). It is a transmembrane protein with a large extracellular domain consisting of five immunoglobulin-like repeats bearing broad similarity to adhesion molecules such as NCAM and close similarity to a subclass of immunoglobulin-like adhesion molecules termed the "sialoadhesin family" or "I-type lectins" (15, 16). Sialoadhesin family members (MAG, sialoadhesin, CD22β, and CD33) are lectins that hemagglutinate red blood cells only if they bear sialic acids in particular glycosidic linkages. MAG binds best to 2,3-linked sialic acid on a Gal(β1→3)GalNAc core structure (15). In the nervous system, this determinant is often carried on gangliosides, a large and varied family of sialic acid-containing cell-surface glycosphingolipids (17, 18). Accordingly, we found that immobilized Fc–MAG chimera binds to a ganglioside-containing radioligand [GT1b conjugated to radioiodinated bovine serum albumin (BSA)] (15). This observation suggested that gangliosides may be physiological ligands for MAG. However, MAG has different neuronal binding properties as an integral membrane protein than as a fragment containing the extracellular domains of the molecule (6). Therefore, binding studies using full-length MAG in a membrane milieu may more closely approximate the physiologic binding properties of MAG. We show here that full-length MAG expressed on the surface of COS cells mediates highly specific cell adhesion to immobilized gangliosides. The specificity of carbohydrate binding defines a

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Abbreviations: MAG, myelin-associated glycoprotein; CNS, central nervous system; mAb, monoclonal antibody; BSA, bovine serum albumin; LDH, lactate dehydrogenase.

¶To whom reprint requests should be addressed.

limited set of structurally related gangliosides, known to be expressed on myelinated neurons *in vivo*, as ligands for MAG.

MATERIALS AND METHODS

Gangliosides. The ganglioside nomenclature of Svennerholm (19) is used where applicable. Structures for other glycosphingolipid abbreviations are indicated below (20). Gangliosides used in these studies were from the following sources: GA1 (Gg_4Cer, where Gg_4 is gangliotetraose), GM1, GD1a, and GD1b were from EY Laboratories; GM3 was from Sigma; GQ1b was from Accurate; GD3 and sulfatide (3-sulfate-GalCer) were from Matreya; GM2 was purified from human Tay–Sachs brain; GQ1bα (Chol-1α-b, $IV^3NeuAc,III^6NeuAc,II^3(NeuAc)_2$-$Gg_4Cer$), GT1$\beta$ [$IV^3NeuAc,III^6(NeuAc)_2$-Gg_4Cer], GQ1β [$IV^3(NeuAc)_2,III^6(NeuAc)_2$-$Gg_4Cer$], and GM1$\alpha$ [III^6NeuAc-Gg_4Cer) were chemically synthesized *de novo* (ref. 39 and A.H., unpublished work); GM1b (*cis*-GM1, IV^3NeuAc-Gg_4Cer) was from Robert Yu, Medical College of Virginia, Richmond; 2,3SnLc (IV^3NeuAc-nLcCer) and 2,6SnLc (IV^6NeuAc-nLcCer) were from Paul James, Glycomed, Alameda, CA; SLex (IV^3NeuAc,III^3Fuc-nLcCer) was from Brian Brandley, Glycomed; and SGGL [IV^3GlcU(3-sulfate)-nLcCer] was prepared from bovine cauda equina (21).

MAG-Transfected COS Cells. Plasmids containing cDNA encoding the long form of MAG were constructed by excising the full-length cDNA from pGEM-L-MAG (from B. D. Trapp, Cleveland Clinic, Cleveland, OH) with *Apa* I and inserting the fragment in either the forward (pCDM8-MAG) or reverse orientation (control) into the expression vector pCDM8 (22) using *Bst*XI/*Eco*RI adapters (Invitrogen). The size of the MAG expression product was confirmed by *in vitro* translation (TNT system; Promega), resulting in a protein of ≈70 kDa produced by the forward construct only. Sequencing of 600 bp from the 3′ end of the insert confirmed the presence of rat MAG (long form) cDNA. COS-1 cells were transiently transfected with pCDM8-MAG or the reverse construct, using a high-efficiency DEAE-dextran procedure (23). After 60 hr, cells were detached from plates in a phosphate-buffered saline (PBS) solution (308 mM NaCl/16 mM Na_2HPO_4/3 mM KH_2PO_4/5.4 mM KCl/1 mM EDTA), collected by centrifugation, and resuspended in Dulbecco's phosphate-buffered saline. Expression of MAG was confirmed immunocytochemically by using a Coulter Epics flow cytometer. Cells (4×10^5) were incubated for 30 min at 4°C with anti-MAG antibody at 30 μg/ml (mAb 513; Boehringer Mannheim) in 30 μl of PBS containing BSA at 20 mg/ml. Cells were washed by centrifugation and stained for 30 min at 4°C with a 1:100 dilution of phycoerythrin-conjugated polyclonal goat anti-mouse IgG $F(ab')_2$ (Tago). At least 5000 cells were evaluated for each flow cytometric profile. Background fluorescence was defined using an irrelevant mouse IgG1 isotype control antibody (Coulter).

Ganglioside Adsorption to Microwells. Gangliosides and other glycosphingolipids were evaporated from storage solvent (chloroform/methanol/water, 4:8:3) and resuspended at the desired concentrations in ethanol containing 1 μM phosphatidylcholine and 4 μM cholesterol. An equal volume of water was added, 50 μl was placed into microwells of a 96-well Serocluster plate (Costar), and plates were incubated uncovered for 75–120 min to allow efficient lipid adsorption (24). Plates were washed with water and preblocked by adding 100 μl per well of Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) containing BSA (1 mg/ml) and incubating for 10 min at 37°C. To quantitate ganglioside immobilization selected wells were further incubated in 300 μl of the above buffer for 10 min at 4°C followed by 50 min at 37°C to mimic the cell-adhesion experiment (see below). Wells were then washed with water and emptied. Gangliosides were recovered by extraction with 100 μl per well of 1-butanol for 30 min (25). Recovered gangliosides were analyzed by quantitative thin-layer chromatography (26).

MAG-Mediated Cell Adhesion. MAG- or control-transfected COS cells were collected from plates 36–48 hr after transfection and suspended at 2×10^5 cells per ml in Hepes-buffered DMEM containing BSA at 1 mg/ml. An aliquot of cell suspension (200 μl) was added to each lipid-adsorbed and preblocked microwell. Plates were incubated for 10 min at 4°C to allow cells to settle and then were transferred to 37°C for 50 min. To remove nonadherent cells after incubation, the plate was immersed, upright, in a vat of PBS, inverted, and placed in an immersed custom-designed Plexiglas box that was sealed with a gasket to exclude air. The inverted plate in its fluid-filled chamber was placed in a centrifuge carrier and centrifuged at $24 \times g$ to gently remove nonadherent cells. The box was again immersed in a vat of PBS and the plate was removed and righted (while immersed), surface fluid was removed by aspiration, 20 μl of 10% Triton X-100 was added and mixed, and 80 μl per well was removed to a fresh 96-well plate. Cell adhesion was quantitated by measuring lactate dehydrogenase (LDH) activity in the cell lysate after addition of 120 μl of 0.1 M potassium phosphate buffer, pH 7.0 containing 0.7 mM NADH and 4.7 mM pyruvate. The decrease in absorbance at 340 nm as a function of time was measured simultaneously in each well with a Molecular Devices UV multiwell kinetic plate reader.

RESULTS AND DISCUSSION

cDNA encoding MAG was cloned into the mammalian expression vector pCDM8 in the correct orientation or in the reverse orientation (control). Plasmids were transiently transfected into COS-1 cells using a highly efficient DEAE-dextran procedure. MAG expression was confirmed by flow cytometric analysis of intact cells stained with mAb 513, which binds to a conformational epitope in the third extracellular immunoglobulin-like domain of MAG (27). Most MAG-transfected COS cells bound mAb 513; relative fluorescence of ≈80% of the MAG-transfected cells ranged from 5- to 200-fold above average background fluorescence (Fig. 1). In contrast, COS cells transfected with control plasmid were indistinguishable from background, as were MAG- or control-transfected COS cells stained with an irrelevant isotype-matched control antibody (data not shown).

MAG-transfected COS cells adhered in a concentration-dependent manner to microwell surfaces on which the ganglioside GT1b was adsorbed as an artificial membrane monolayer with phosphatidylcholine and cholesterol (Fig. 2). MAG-transfected COS cells did not bind to the artificial membrane lacking GT1b, and control-transfected COS cells did not bind to GT1b-adsorbed or control surfaces. Consistently, 30–40% of the MAG-transfected cells adhered to wells adsorbed with ≥50 pmol of GT1b, whereas <8% bound to control lipid-adsorbed surfaces. These data demonstrated the ability of a purified endogenous neuronal glycoconjugate, ganglioside

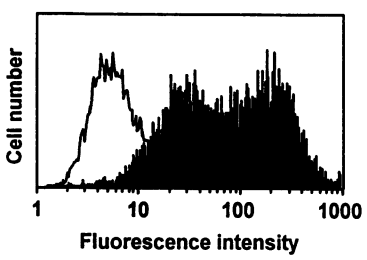

FIG. 1. MAG expression by transfected COS cells. COS cells transfected with pCDM8-MAG (filled) or control plasmid (open) were stained with anti-MAG mAb 513 and analyzed by flow cytometry.

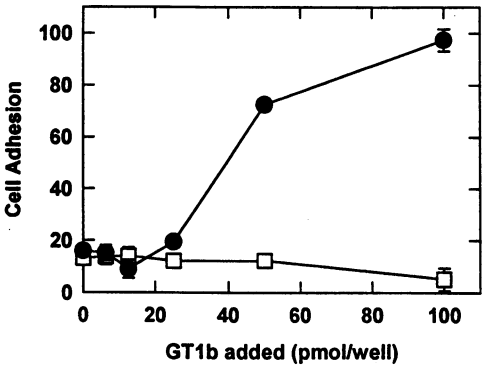

FIG. 2. MAG-mediated adhesion of transfected COS cells to GT1b. Adhesion of MAG-transfected (●) and control-transfected (□) COS cells to microwells adsorbed with the indicated amounts of GT1b was measured. All wells (including those with no GT1b) were coadsorbed with phosphatidylcholine at 25 pmol per well and cholesterol at 100 pmol per well. Cell adhesion is reported as LDH activity (ΔA_{340} per min $\times 10^3$). Values are the mean ± SE of quadruplicate determinations.

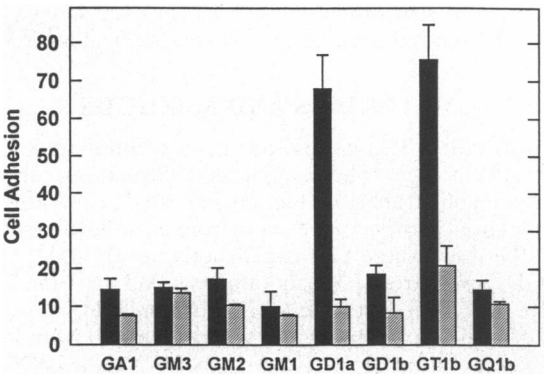

FIG. 3. MAG-mediated adhesion of transfected COS cells to major brain gangliosides. The indicated gangliosides were adsorbed at 50 pmol per well as in Fig. 2. The efficiency and stability of adsorption of the different gangliosides is comparable (24). Adhesion of MAG-transfected (solid bars) and control-transfected (hatched bars) COS cells to each ganglioside was measured. Cell adhesion is reported as LDH activity (ΔA_{340} per min $\times 10^3$). Values are expressed as the mean ± SE of quadruplicate determinations.

GT1b, to support MAG-mediated cell adhesion and defined conditions for subsequent carbohydrate specificity studies.

Among the major brain gangliosides, only GT1b and GD1a supported MAG-mediated cell adhesion (Figs. 3 and 4). Adhesion required a terminal NeuAc(α2→3)Gal determinant (compare GD1a to GM1) but was abrogated by a terminal α2,8-linked sialic acid (compare GT1b to GQ1b). Notably, NeuAc(α2→3)Gal presented on a different saccharide core (GM3 or 2,3SnLc) did not support adhesion. This observation, along with the enhanced binding of GD1a or GT1b compared with GM1b, indicates that the gangliotetraose core is recognized along with the sialic acid on the internal galactose residue.

The interpretation of these results depends on the assumption that gangliosides that supported adhesion were similarly immobilized on the microwells compared to those that did not support adhesion. This assumption was tested by recovering and quantitating the adsorbed gangliosides from selected wells. The results are consistent with the above interpretations of carbohydrate specificity. For example, GD1a (which supported cell adhesion) adsorbed at 31 pmol per well (of 100 pmol per well added), whereas GD1b (which did not support adhesion) adsorbed at 38 pmol per well. Glycolipids with larger head groups adsorbed at somewhat lower densities as reported (25). Thus GT1b (which supported cell adhesion) adsorbed at 18 pmol per well (of 100 pmol per well added), whereas GQ1b (which did not support adhesion) adsorbed at 20 pmol per well.

The above structure–function data have a notable correlate in development. Ganglioside patterns change dramatically during early brain development in all vertebrates, including humans (28–31). In general, GD3 predominates during the period of neuroblast proliferation and initiation of neurite-genesis but is supplanted by GD1a when myelination begins. It is possible that the coincident developmental expression of

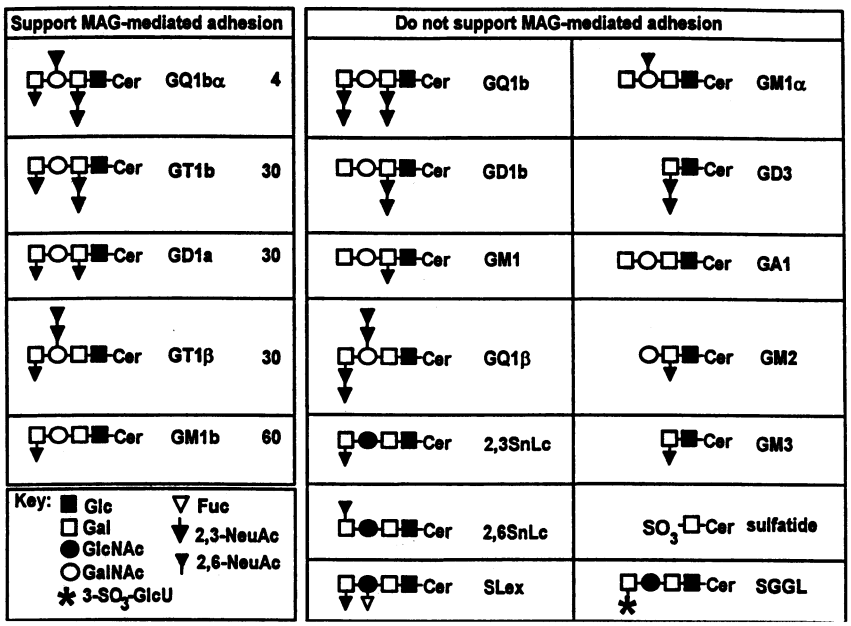

FIG. 4. Structure–function studies of MAG-mediated cell adhesion to gangliosides and related glycosphingolipids. Each of 19 glycolipids was tested at a variety of concentrations for its ability to support adhesion of MAG-transfected COS cells as in Fig. 2. The five structures that supported cell adhesion are shown at the left, with the amount required to support half-maximal cell adhesion (pmol/well) indicated. Structures that did not support any cell adhesion are shown at the right. Each was tested at a maximum of 100–200 pmol per well. Note that NeuAc–NeuAc bonds are all α2,8; other NeuAc bonds are as noted in the key.

MAG and gangliosides that bind to MAG are part of a mechanism to stabilize neurite outgrowth late in brain development.

To confirm that MAG-mediated ganglioside adhesion depended on terminal sialic acids, microwells adsorbed with GT1b were incubated with *Vibrio cholerae* neuraminidase. This enzyme cleaves all α2,3- α2,6-, and α2,8-linked NeuAc residues, except the α2,3-NeuAc linked to the internal galactose on gangliosides (thereby converting GT1b to GM1, see Fig. 4). Neuraminidase-treated microwells did not support adhesion of MAG-transfected cells, whereas control-treated microwells supported adhesion (Fig. 5*A*). Recovery and analysis of the gangliosides from selected neuraminidase-treated microwells confirmed that enzyme pretreatment did not desorb gangliosides from the wells and that the enzyme converted most of the GT1b to GM1 (with no GD1a or GD1b detected, data not shown). Very little (<0.02%) of the neuraminidase activity remained associated with the wells after pretreatment and washing before cell addition (measured on selected wells using the colorimetric substrate *p*-nitrophenyl-α-*N*-acetylneuraminic acid). Furthermore, pretreatment of the MAG-transfected COS cells with neuraminidase did not reduce their subsequent adhesion to ganglioside-adsorbed microwells. Therefore, the terminal sialic acids on the adsorbed gangliosides are required to support adhesion of MAG-transfected COS cells.

Pretreatment of MAG-transfected COS cells with mAb 513, which blocks MAG–liposome binding to neurons in culture and inhibits oligodendrocyte–neuron binding (6–8), blocked MAG-mediated cell adhesion to GT1b-adsorbed surfaces (Fig. 5*B*). Treatment of the same cells with an isotype-matched control antibody had no inhibitory effect. Monoclonal antibody 513 recognizes a protein epitope on the extracellular domain of MAG (M. Schachner, personal communication). These data are consistent with the interpretation that the same

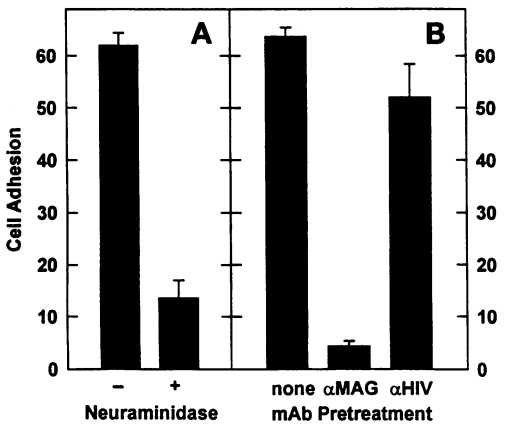

FIG. 5. Inhibition of MAG-transfected COS cell adhesion to GT1b by neuraminidase and anti-MAG mAb 513. (*A*) For neuraminidase treatment, 70 pmol of GT1b was adsorbed to microwells as in Fig. 2. The wells were washed with water and incubated for 10 min at 37°C in neuraminidase buffer (154 mM NaCl/4 mM $CaCl_2$/50 mM sodium acetate, pH 5.5) with or without *V. cholerae* neuraminidase (Calbiochem) at 0.3 unit/ml. Wells were washed with water, and adhesion of MAG-transfected COS cells was measured. (*B*) For mAb inhibition studies, MAG-transfected COS cells (130,000 cells per ml) were preincubated for 1 hr at 0°C in Hepes-buffered DMEM containing BSA at 1 mg/ml, mAb 513 at 100 μg/ml (Boehringer Mannheim), and commercial mAb additives (0.5 mM potassium phosphate, raffinose at 5 mg/ml). Control preincubations were in the same medium without antibody or in medium containing irrelevant isotype-matched mAb (anti-human immunodeficiency virus) at 100 μg/ml. After the wells were preblocked, medium was removed and replaced with 300 μl of pretreated cell suspension. Cell adhesion is reported as LDH activity (ΔA_{340} per min × 10^3). Values are the mean ± SE of triplicate determinations.

polypeptide domain on MAG is responsible both for neuronal cell adhesion and ganglioside recognition.

A surprising additional finding arose from ganglioside structure–function studies. Quantitatively minor gangliosides, termed "Chol-1" gangliosides, are the targets of antibodies highly specific for cholinergic neurons and their processes throughout the nervous system (32–34). Chol-1 gangliosides are related to the major brain gangliosides but have an additional sialic acid linked α2,6 to the GalNAc in the gangliotetraose core. Chol-1 antibodies do not cross react with any proteins on Western blots, yet bind to gangliosides on cholinergic neurons in organisms ranging from *Torpedo* to humans. The role of Chol-1 gangliosides in cholinergic neuronal function is unknown. Upon testing synthetic Chol-1 gangliosides, one of them, GQ1bα, was nearly 10-fold more potent than GT1b at supporting MAG-mediated cell adhesion (Figs. 4 and 6). GQ1bα also supported adhesion of a greater maximum percentage of MAG-transfected COS cells (70–80%) compared to other gangliosides tested, suggesting that a lower cell-surface concentration of MAG was sufficient to support adhesion to GQ1bα compared to GT1b or other gangliosides (Fig. 6). Binding of MAG-transfected COS cells to GQ1bα was completely blocked by pretreatment of the adsorbed ganglioside with *V. cholerae* neuraminidase (data not shown).

The enhanced ability of GQ1bα to support MAG-mediated cell adhesion was unanticipated for two reasons. (*i*) We had demonstrated that α2,6-linked sialic acids by themselves do not support MAG–Fc binding under conditions where α2,3-linked sialic acids support binding (15). This finding is confirmed in the present study (compare GM1b and GM1α, Fig. 4). (*ii*) Chol-1 gangliosides are thought to be restricted to cholinergic neurons, whereas myelination is not restricted to axons on the basis of neurotransmitter phenotype; this raises the possibility that cholinergic neurons have a privileged association with MAG and myelin (although there are no published data addressing whether Chol-1 gangliosides are expressed on axolemma subjacent to myelin). Alternatively, Chol-1 gangliosides may be related to other quantitatively minor MAG-

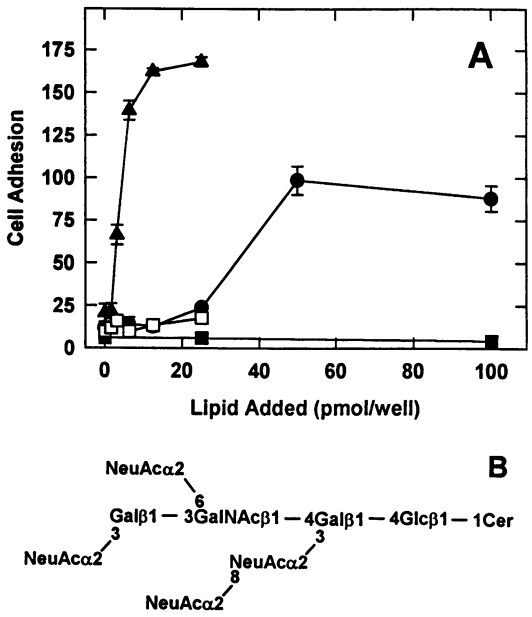

FIG. 6. Enhanced support of MAG-transfected COS cell adhesion by GQ1bα. (*A*) MAG-mediated cell adhesion. Microwells were adsorbed with various amounts of GQ1bα (▲), GT1b (●), GQ1b (□), or GD3 (■). Adhesion of MAG-transfected COS cells was performed as described for Fig. 2. Cell adhesion is reported as LDH activity (ΔA_{340} per min × 10^3). Values are expressed as the mean ± SE of quadruplicate determinations. (*B*) Structure of GQ1bα.

binding gangliosides that are more broadly distributed and have yet to be discovered.

Considered together, the ganglioside binding data in Fig. 4 indicate that an α2,3-*N*-acetylneuraminic acid residue on the terminal galactose of a gangliotetraose core is the primary determinant for MAG binding, but that additional sialic acid residues at each of the two other known sites of sialylation (α2,6 to the GalNAc and α2,3 to the internal Gal) contribute meaningfully to increased binding affinity. The lack of any MAG-mediated cell adhesion to other, closely related ganglioside structures indicates a level of ganglioside-binding specificity rivaled only by cholera toxin, which binds to GM1 (35, 36). The specificity of MAG binding and the sensitivity of that binding to a mAb directed against a neuronal cell-adhesion epitope implicate gangliosides as physiologically relevant mediators of MAG–neuron interactions and perhaps of MAG-mediated control of neurite outgrowth.

Cell-surface carbohydrates have long been posited to act as cell–cell recognition molecules (37). The discovery of the selectin family (38) and more recently the sialoadhesin (I-type lectin) family of carbohydrate-binding cell adhesion molecules (15, 16) confirms the importance of carbohydrate–protein binding in cell–cell interactions and provides new opportunities for development of therapeutic agents based on carbohydrates as cell-adhesion inhibitors. Detailed knowledge of the ganglioside determinants that support MAG recognition may provide opportunities for intervention in the control of neurite outgrowth and myelination.

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