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Immunoglobulin G-Class Mouse Monoclonal Antibodies to Major Brain Gangliosides[1]

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Mice genetically engineered to lack complex gangliosides are improved hosts for raising antibodies against those gangliosides. We report the generation and characterization of nine immunoglobulin G (IgG)-class monoclonal antibodies (mAbs) raised against the four major brain gangliosides in mammals. These include (designated as ganglioside specificity-IgG subclass) two anti-GM1 mAbs (GM1-1, GM1-2b), three anti-GD1a mAbs (GD1a-1, GD1a-2a, GD1a-2b), one anti-GD1b mAb (GD1b-1), and three anti-GT1b mAbs (GT1b-1, GT1b-2a, GT1b-2b). Each mAb demonstrated high specificity, with little or no cross-reactivity with other major brain gangliosides. Enzyme-linked immunosorbent assay (ELISA) screening against 14 closely related synthetic and purified gangliosides confirmed the high specificity, with no significant cross-reactivity except that of the anti-GD1a mAbs for the closely related minor ganglioside GT1aα. All of the mAbs were useful for ELISA, TLC immunooverlay, and immunocytochemistry. Neural cells from wild-type rats and mice were immunostained to differing levels with the anti-ganglioside antibodies, whereas neural cells from mice engineered to lack complex gangliosides (lacking the ganglioside-specific biosynthetic enzyme UDP-GalNAc:GM3/GD3 *N*-acetylgalactosaminyltransferase) remained unstained, demonstrating that most of the mAbs react only with gangliosides and not with related structures on glycoproteins. These mAbs may provide useful tools for delineation of the expression and function of the major brain gangliosides and for probing the pathology of anti-ganglioside autoimmune diseases.

Key Words: **glycosphingolipids; GM1; GD1a; GD1b; GT1b.**

Gangliosides, glycosphingolipids bearing one or more sialic acid residues, are expressed in all vertebrate tissues, but are the major sialoglycoconjugates of brain (1). Although variation in the sugar composition and sequence gives rise to dozens of distinct ganglioside structures (2), adult mammals and birds share the same set of four major brain gangliosides: GM1, GD1a, GD1b, and GT1b (boxed structures, Fig. 1). The goal of the current study was to raise high-affinity immunoglobulin G (IgG)[3]-class mouse monoclonal antibodies (mAbs) to these four major gangliosides for use in ganglioside detection *in vivo* and *in vitro* and for the

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[3] Abbreviations used: mAb, monoclonal antibody; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; PCR, polymerase chain reaction; TLC, thin-layer chromatography; GM3, II^3 NeuAc-LacCer; GD3, II^3 $(NeuAc)_2$-LacCer; GM2, II^3 NeuAc-GgOse3Cer; GA1, GgOse4Cer; GM1, II^3 NeuAc-GgOse4Cer; GD1a, IV^3 NeuAc,II^3 NeuAc-GgOse4-Cer; GD1b, II^3 $(NeuAc)_2$-GgOse4Cer; GT1b, IV^3 NeuAc,II^3 NeuAc-GgOse4Cer; GQ1b IV^3 $(NeuAc)_2$,II^3 $(NeuAc)_2$-GgOse4Cer; cisGM1, IV^3 NeuAc-GgOse4Cer; GM1α, III^6 NeuAc-GgOse4Cer; GD1α, IV^3 NeuAc,III^6 NeuAc-GgOse4Cer; GT1aα, IV^3 NeuAc,III^6 NeuAc,II^3 NeuAc-GgOse4Cer and GQ1bα, IV^3 NeuAc,III^6 NeuAc,II^3 $(NeuAc)_2$-GgOse4Cer; IgG, immunoglobulin G; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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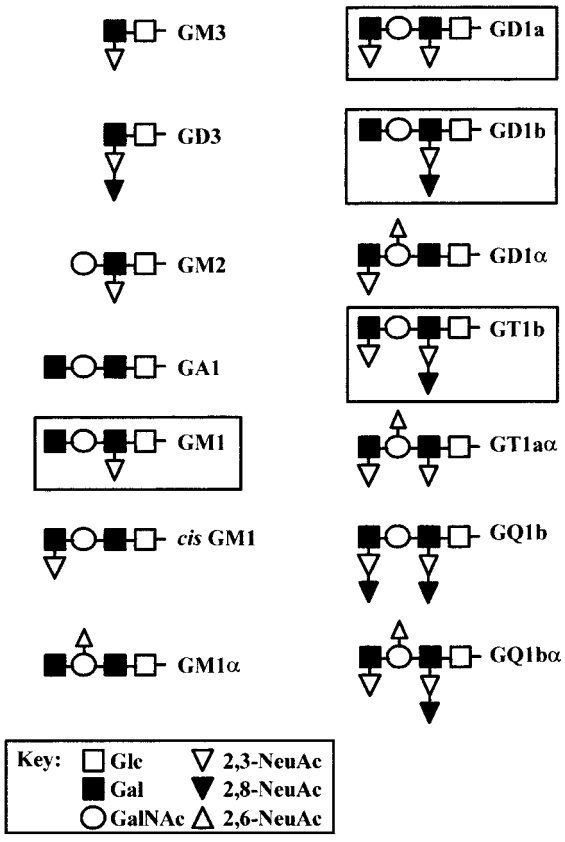

FIG. 1. Schematic representation of the ganglioside structures used in this study. Nomenclature is based on that of Svennerholm (12), with "α" designating a single α2-6-linked sialic acid attached to the GalNAc residue (33). The most abundant gangliosides in adult mammalian brain are boxed.

study of ganglioside function and anti-ganglioside autoimmune pathologies.

Since mammals and birds share the same major brain gangliosides, raising antibodies against these determinants has been a challenge, although Kotani *et al.* (3) and Ozawa *et al.* (4) raised mAbs against major brain gangliosides (primarily of IgM class) in C3H/HeN strain mice. In our previously published study (5), we demonstrated that wild-type mice injected with a multivalent GD1a immunoconjugate [GD1a–keyhole limpet hemocyanin (KLH)] mounted only a weak IgM response and no IgG response. In contrast, sibling mice genetically engineered to lack a key enzyme in major brain gangliosides biosynthesis (UDP-GalNAc:GM3/GD3 *N*-acetylgalactosaminyltransferase), and which fail to express any of the major brain gangliosides, mounted robust IgM and IgG anti-ganglioside responses to the same immunoconjugate (5). We speculated that wild-type mice, which express major gangliosides, have anergic or deleted ganglioside-reactive T-cell clones and therefore cannot mount a vigorous humoral immune response against "self" gangliosides, whereas the knockout mice, which do not express any of the major gangliosides, were free to mount such a response.

In our previous publication (5) we reported generation of a single monospecific anti-ganglioside mAb (Gg101, anti-GD1a IgG1). In the current paper we report the generation and characterization of eight additional monospecific anti-ganglioside mAbs, including IgG class antibodies against all four major brain gangliosides. The resulting suite of mAbs are intended as additional research tools to probe the expression and function of gangliosides, prominent determinants on nerve cell surfaces.

EXPERIMENTAL PROCEDURES

Materials. Purified gangliosides GM3, GD3, GM2, GA1, GM1, GD1a, GD1b, GT1b, and GQ1b were from Matreya (Pleasant Gap, PA) or Sigma Chemical Co. (St. Louis, MO); synthetic cisGM1 (GM1b), GM1α, GD1α, GT1aα, and GQ1bα were the kind gifts of Dr. M. Kiso, Gifu University (6–11). Ganglioside structures are shown in Fig. 1; nomenclature is based on that of Svennerholm (12). Mariculture KLH and Freund's adjuvants (complete and incomplete) were from Pierce (Rockford, IL). Mice lacking a functional gene for UDP-GalNAc:GM3/GD3 *N*-acetylgalactosaminyltransferase (*GalNAcT* (−/−) mice) were prepared using gene knockout technology as described previously (13). The genotypes of offspring were determined using PCR as described (14).

Ganglioside–KLH conjugates. Covalent conjugates of gangliosides and KLH were prepared as described (15). Gangliosides were specifically oxidized at the 4–5 double bond of the sphingosine moiety using ozone, introducing a single aldehyde at the original sphingosine 4-carbon and releasing a long chain fatty aldehyde. The resulting ganglioside aldehyde was covalently linked to KLH via reductive amination, resulting in a stable secondary amine linkage between the ganglioside and the protein. KLH conjugates of GM1, GD1a, GD1b, and GT1b were prepared using nearly identical conditions. Specifics for GM1 are given below, as an example.

GM1 (2 mg) was dissolved in methanol (2 ml) and chilled to dry-ice temperature in a glass reaction vessel. Ozone was generated from oxygen using a benchtop generator (Model GE60/FM100, Ozone Services, Burton, BC, Canada) at a power setting of "1" and a flow setting of "1/4." The output from the ozone generator was bubbled through the dry-ice-chilled ganglioside solution for 5 min, and then nitrogen was bubbled into the solution for an additional 5 min to disperse excess ozone. Dimethyl sulfoxide (100 μl) was added and the solution stirred at dry-ice temperature for 30 min and then at ambient temperature for an additional

90 min. The solution was transferred to a 13 × 100-mm thick-walled screw-capped glass tube and solvents were evaporated under vacuum. The fatty acid aldehyde was removed by hexane extraction. Hexane (4 ml) was added and the tube was sonicated for 5 min in a bath-type sonicator. The tube was then centrifuged (200*g*, 5 min), and the clear supernatant was carefully removed and discarded. Remaining hexane was evaporated from the pellet under a stream of nitrogen, and the resulting pellet was dissolved in 200 μl of phosphate-buffered saline (10 mM phosphate buffer, 100 mM sodium chloride, pH 7.2). KLH (2 mg in 200 μl of phosphate-buffered saline) and recrystallized sodium cyanoborohydride (1.75 mg, freshly prepared in 175 μl phosphate-buffered saline) were added. The reaction was stirred at 42°C for 48 h with the addition of an additional 1.75 mg of sodium cyanoborohydride after 24 h. The resulting conjugate was dialyzed against phosphate-buffered saline and quantified by UV spectroscopy. Thin-layer chromatography followed by sialic-acid-specific staining (16) revealed sialic acid remaining at the origin, indicating covalent conjugation of the ganglioside. Quantification of protein-bound sialic acid by acid release followed by HPLC/pulsed amperometric detection (17) in a test sample revealed ~60% efficiency of ganglioside conjugation (based on initial total ganglioside added).

Immunization and hybridoma generation. Young adult *GalNAcT* (−/−) mice, which lack complex gangliosides, were used for all immunizations. These mice were engineered to carry a deletion in the gene coding the ganglioside-specific glycosyltransferase UDP-GalNAc:GM3/GD3 β-4-*N*-acetylgalactosaminyltransferase (GM2/GD2 synthase) as described previously (5, 13). The resulting gene-deleted mice failed to express the major brain gangliosides GM1, GD1a, GD1b, or GT1b, instead expressing GD3 and GM3. Immunization, fusion, and hybridoma production were performed essentially as described by Harlow and Lane (18). On day 1 and day 8 of the protocol, knockout mice received an intraperitoneal injection of 50 μg of ganglioside–KLH conjugate in 300 μl of phosphate-buffered saline:complete Freund's adjuvant (1:1). On days 13, 14, and 15 mice were injected intravenously (tail vein) with 100 μl of phosphate-buffered saline containing 10 μg of the same ganglioside–KLH conjugate. On day 16, splenocytes were collected and fused with P3x653(1183) nonsecreting mouse myeloma cells using polyethylene glycol, and the resulting hybridomas were plated in microwells in an enriched HAT selection medium [per 100 ml; 67 ml Dulbecco's modified Eagle medium (DMEM); 10 ml NTCT-109 medium; 1 ml "HIPO" concentrate (1 M Hepes, 20 U/ml insulin, 0.35 μl/ml β-mercaptoethanol, 15 mg/ml oxalacetic acid, 5 mg/ml sodium pyruvate); 1 ml 200 mM glutamine; 1 ml HAT concentrate (10 mM sodium hypoxanthine, 40 μM aminopterin, and 1.6 mM thymidine); and 20 ml fetal bovine serum] on feeder layers of freshly prepared peritoneal macrophages collected from littermates primed by intraperitoneal injection of incomplete Freund's adjuvant 4 days before the fusion.

After 10 days of undisturbed growth, the wells were screened for anti-ganglioside antibody production via ELISA (see below). Positive wells with a single apparent colony were recloned twice by limiting dilution on macrophage feeder layers using the enriched selection medium described above. The final hybridoma clones were grown in a simplified growth medium (per 100 ml; 88 ml DMEM; 1 ml "HIPO" concentrate (see above); 1 ml 200 mM glutamine; 10 ml fetal bovine serum). Secreted IgG mAbs were purified from cell culture supernatants via affinity chromatography using immobilized goat anti-mouse IgG agarose (Sigma, No. A6531).

Anti-ganglioside ELISA. Microplate wells were adsorbed with phospholipid/cholesterol monolayers containing gangliosides as described (19). Briefly, 96-well polystyrene multiwell plates (No. 9017, Corning Life Sciences, Acton, MA) were washed with butanol and ethanol and then air-dried. The desired amount of ganglioside in storage solvent [chloroform:methanol:water (4:8:3)] was evaporated to dryness and redissolved in an ethanol solution containing 1 μM phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) and 4 μM cholesterol (Sigma). An equal volume of water was then added, the solution was mixed, and 50-μl aliquots were distributed into each well of the solvent-washed 96-well plates. The plates were placed uncovered in a fume hood for 90 min to allow ethanol evaporation and lipid adsorption, after which the gangliosides remained stably adsorbed in aqueous solutions. Ganglioside-adsorbed plates were washed three times with water and then used immediately or stored (filled with water) for up to 24 h before use. Plates were blocked for 30 min at 37°C with 200 μl/well of ELISA buffer (Dulbecco's phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin). Subsequent procedures were performed at room temperature. Culture supernatant or purified antibody diluted in ELISA buffer was added at a volume of 50 μl/well. After 90 min, the plates were washed with PBS, and 50 μl/well of alkaline phosphatase-conjugated secondary antibody, diluted 1:500 in ELISA buffer, was added. Secondary antibody was goat anti-mouse IgG, Fc-specific (No. 115-055-008, Jackson ImmunoResearch, West Grove, PA). After 45 min the plates were washed with PBS and assay buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5), and then substrate (2 mg/ml *p*-nitrophenyl phosphate in assay buffer (100 μl/well) was added. The absorbance at 405 nm was determined using a microplate reader (Benchmark, Bio-Rad, Hercules, CA). Anti-gan-

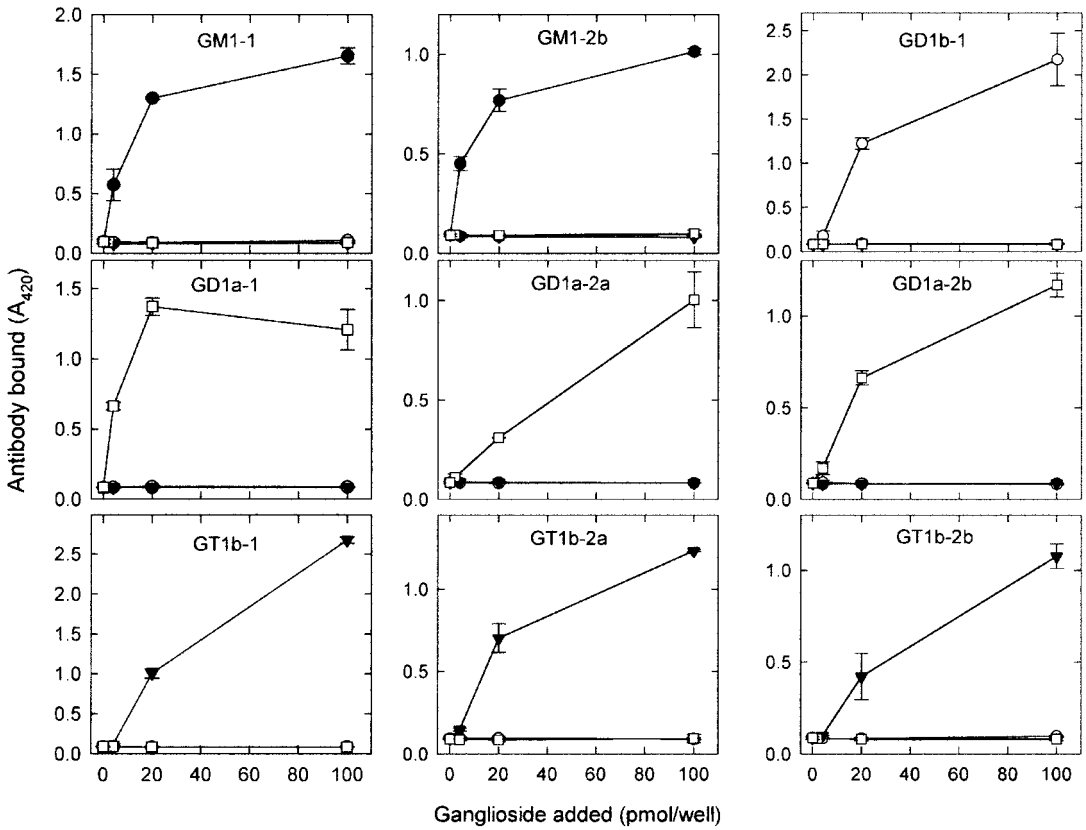

FIG. 2. Specificity of binding of anti-ganglioside mAbs to the major brain gangliosides. The four major brain gangliosides GM1 (●), GD1a (□), GD1b (○), and GT1b (▼) were adsorbed to microwell dishes, along with phosphatidylcholine and cholesterol, at the indicated ganglioside concentrations (pmol/well added). Binding of the indicated mAbs was determined by ELISA using hybridoma supernatants as described in the text. Data are means ± SD for duplicate determinations.

glioside antibody subclasses in serum were determined using rat anti-mouse subclass-specific antibodies (PharMingen, San Diego, CA) and alkaline phosphatase-conjugated donkey anti-rat IgG tertiary antibodies (Jackson ImmunoResearch).

TLC immune overlay. TLC immune overlay was performed as described (16). A mixture of gangliosides GM1, GD1a, GD1b, and GT1b (125 pmol each) was spotted on replicate silica gel TLC plates (0.2 mm layer thickness, Cat. No. 5644-5, EM Science, Gibbstown, NJ) and resolved using chloroform:methanol:aqueous 0.25% KCl (60:35:8). After development, the plates were thoroughly dried then immersed in 2 mg/ml polyisobutylmethacrylate in hexanes for 30 s. The plates were dried, sprayed with PBS, and then immersed in ELISA buffer for 1 h to inhibit nonspecific antibody binding. The plates were drained of ELISA buffer, overlaid with hybridoma culture supernatants diluted 1:5 with ELISA buffer, and then incubated 3 h at ambient temperature in a humidified chamber. After incubation with primary antibodies, the plates were washed three times with PBS and overlaid with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific) at 0.6 μg/ml in ELISA buffer. After 1 h at ambient temperature, the plate was washed three times with PBS and once with developing buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Finally, plates were immersed in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (1 and 0.5 mg/ml respectively in developing buffer). Upon appearance of purple bands, plates were washed with water, dried, and images acquired with a scanner. Companion plates were treated with resorcinol/HCl for chemical detection of gangliosides (16).

Immunocytochemistry. Whole cerebella were dissected from 4- to 5-day-old Sprague–Dawley rats, the meninges were removed under a dissecting microscope, and pooled cerebella were enzymatically dissociated using a papain dissociation system (Worthington Biochemicals, Freehold, NJ). The resulting cell population contained primarily cerebellar granule neurons and astroglia. Cells were plated at 50,000 per well in poly-D-lysine-coated 96-well plates in culture medium consisting of MEM (Invitrogen, Carlsbad, CA) supple-

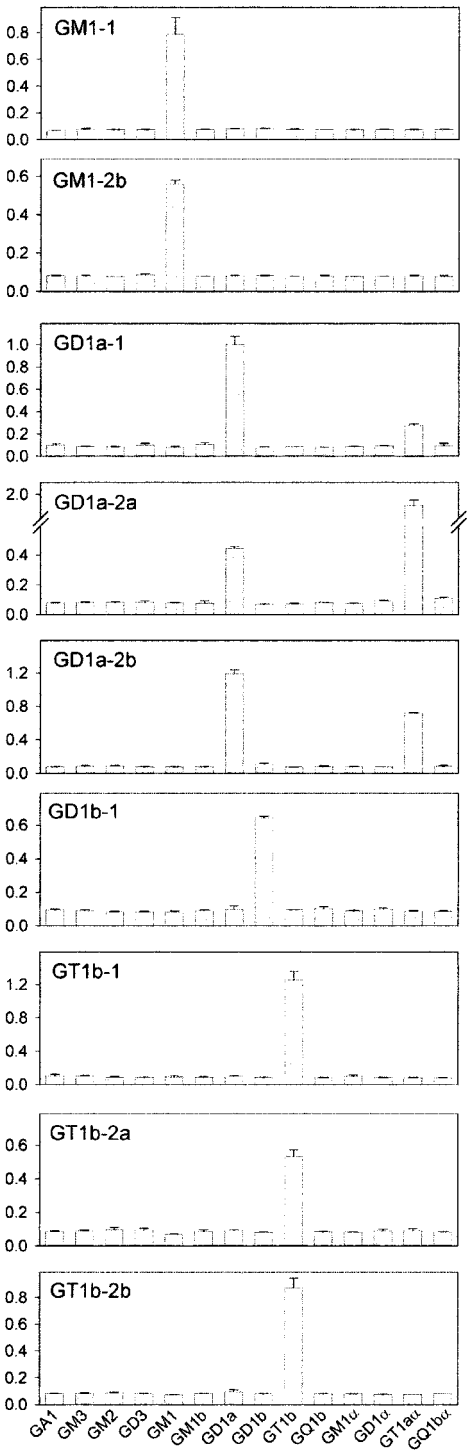

FIG. 3. Specificity of binding of anti-ganglioside mAbs to closely related ganglioside structures (see Fig. 1 for schematic structures and nomenclature). Fourteen different glycosphingolipids were adsorbed to microwell dishes, along with phosphatidylcholine and cholesterol, at 25 pmol/well (ganglioside added). Binding of the indicated mAbs was determined by ELISA using hybridoma supernatants or purified antibodies diluted in ELISA buffer to produce an A_{405} of ~0.5–1 for its primary ganglioside antigen 30 min after addition of the substrate. Data are the mean ± SD for duplicate determinations.

mented with 25 mM Hepes, 25 mM KCl, 1.5 g/l $NaHCO_3$, 10% heat-inactivated equine serum (Hyclone, Logan, UT), 5% fetal bovine serum (Hyclone), penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM). After 48 h of culture, cells were fixed using 4% paraformaldehyde in PBS, incubated with PBS containing 2.5% BSA, and 100 mM glycine for 45 min at ambient temperature. To immunostain, cells were first incubated in a 1:50 dilution of hybridoma culture supernatant in PBS/2.5% BSA/100 mM glycine for 1 h, washed, incubated in a 1:100 dilution of secondary antibody (CY3-labeled goat anti-mouse IgG, Fc-specific, Jackson ImmunoResearch) in the same buffer, and washed, and images were captured using a Nikon Eclipse microscope fitted with a CCD camera. For clarity, images are presented as gray scale, inverted intensity (black on white).

Cerebellar cells were also collected from *GalNAcT* (−/−) mice and their heterozygous siblings. Individual cerebella were dissected from 8-day-old mice, dissociated separately, and plated in 96-well dishes as described above for rat cerebella. The phenotype of each mouse was subsequently determined by extracting gangliosides from the remainder of each brain (20), resolving by thin-layer chromatography, and staining with resorcinol reagent (16). Genotypes were subsequently reconfirmed by PCR using tail-derived DNA as template (14). Cerebellar cells from heterozygotes (which express all major brain gangliosides) and sibling knockout mice (which express GD3 and GM3, but no GM1, GD1a, GD1b, or GT1b) were immunostained as follows. The growth medium was removed, the wells were washed with DMEM containing 10 mM Hepes and 4.6 mM $NaHCO_3$, and a 1:5 dilution of hybridoma supernatant in Hepes-buffered DMEM containing 5 mg/ml BSA premixed with CY3-labeled secondary antibody (goat anti-mouse IgG, Fc-specific, 1:100) was added. After 1 h the wells were washed with Hepes-buffered DMEM and then PBS, the cells were fixed with 4% paraformaldehyde in PBS, and then fluorescent images were collected as described for rat cerebellar cells (above).

RESULTS

Binding specificities of IgG-class anti-ganglioside monoclonal antibodies. Mice engineered to lack complex gangliosides are improved hosts for generation of anti-ganglioside antibodies (5). Using ganglioside–KLH conjugates as antigens we generated hybridomas secreting monoclonal IgG-class anti-ganglioside antibodies to each of the major brain gangliosides: GM1, GD1a, GD1b, and GT1b. High-affinity anti-ganglioside antibodies of multiple IgG isotypes were obtained, resulting in nine different clones, which we designate based on the ganglioside antigen, followed by a hy-

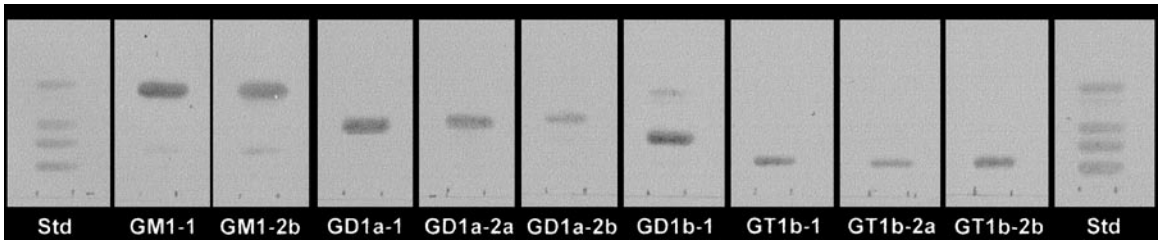

FIG. 4. TLC immunooverlay staining of major brain gangliosides by anti-ganglioside mAbs. A mixture of the four major brain gangliosides was spotted at the origin of replicate TLC lanes. After development, the plate was cut into replicate sections, which were either stained with resorcinol/HCl reagent to reveal the migration of the four gangliosides ("Std" lanes, top to bottom: GM1, GD1a, GD1b, GT1b) or immunostained with the indicated anti-ganglioside mAb, as described in the text.

phen, followed by the IgG isotype (1, 2a, or 2b). Thus, the nine hybridomas are GM1-1, GM1-2b, GD1a-1, GD1a-2a, GD1a-2b, GD1b-1, GT1b-1, GT1b-2a, and GT1b-2b. Ganglioside binding specificities of the nine antibodies were determined by ELISA as a function of the concentration of ganglioside for each of the four major brain gangliosides (Fig. 2). In every case, binding to the antigen ganglioside was readily detectable at 20 pmol/well or less, whereas no binding was detected to any of the nonantigen gangliosides at concentrations up to 100 pmol/well. Subsequently, we screened the ganglioside binding specificities of the nine antibodies to 14 closely related ganglioside structures (Fig. 3, see structures in Fig. 1). None of the antibodies bound significantly to any other of the closely related gangliosides, with the exception of anti-GD1a antibody binding to GT1aα.

All three anti-GD1a antibodies (GD1a-1, GD1a-2a, and GD1a-2b) bound to varying extents to the closely related synthetic ganglioside GT1aα (see Fig. 1), which is akin to GD1a, but with one additional sialic acid at the III^6 position (bound α2,6 to the GalNAc residue). The three anti-GD1a antibodies varied in their affinity for GT1aα. GD1a-1 bound fivefold better to GD1a than to GT1aα and GD1a-2b bound twofold better to GD1a, whereas GD1a-2a bound fivefold better to GT1aα. No other ganglioside supported detectable binding of any of the anti-GD1a antibodies. In using the anti-GD1a antibodies, their cross-reactivity with GT1aα must be considered, although GT1aα is expressed at only 0.12%

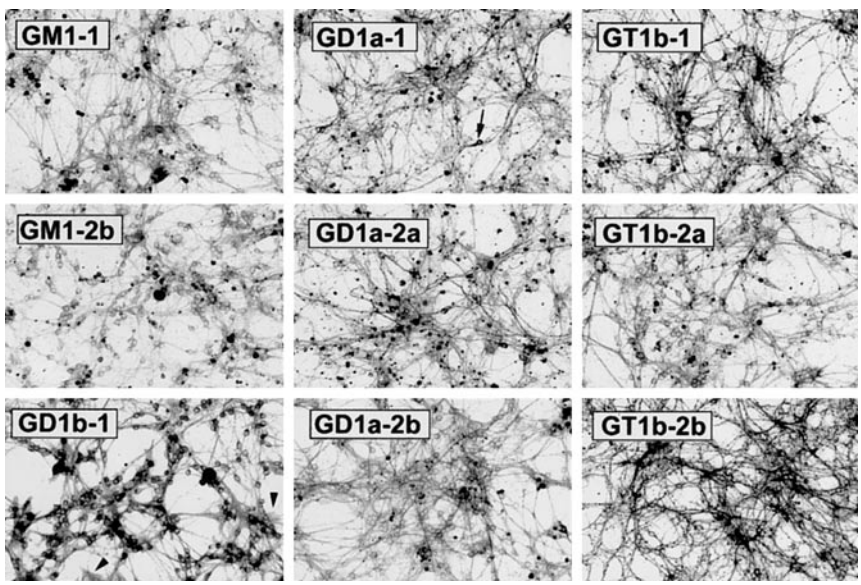

FIG. 5. Immunocytochemical staining of rat cerebellar cells with anti-ganglioside mAbs. Cerebella were dissected from newborn (4- to 5-day-old) rats, and the cells were dissociated using proteolytic enzymes and plated in 96-well tissue culture plates. After 48 h of culture, the cells were fixed and immunostained using the indicated primary mAb and a CY3-labeled secondary antibody as described in the text. Fluorescent images (red on black), which were captured electronically with a CCD camera, are presented as the reverse gray scale (black on white) for clarity. The mixed cell populations consist of neurons (arrow in panel GD1a-1) with round cell bodies and long extensions and glial cells (arrowheads in panel GD1b-1), which are larger and flat and often underlie the neurons.

the level of GD1a in the brain (21). In contrast to the anti-GD1a antibodies, none of the anti-GT1b antibodies bound to GQ1bα, which is akin to GT1b, but with an additional sialic acid at the III^6 position. This may indicate that addition of the III^6 substituent alters the conformation of the other sugars in GT1b sufficiently to abrogate antibody binding, whereas the same substituent may not significantly alter the conformation of the other sugars in GD1a.

All nine anti-ganglioside mAbs were useful in TLC overlay detection of gangliosides. When tested against a panel of major brain gangliosides, anti-GD1a and anti-GT1b antibodies showed no cross-reactivity. In contrast, the two anti-GM1 antibodies showed minor cross-reactivity with GD1b, and the anti-GD1b antibody showed minor cross-reactivity with GM1. The minor cross-reactivity may be based upon the shared "Galβ3GalNAcβ4" terminus of GM1 and GD1b (see Fig. 1). The detection of minor cross-reactivity of anti-GM1 and anti-GD1b antibodies upon TLC immune overlay but not ELISA (compare Figs. 2 and 4) may be due to the tendency of gangliosides to concentrate in narrow bands upon TLC in this system, resulting in a higher density, or it may be due to differences in the conformation of the oligosaccharide portion of the gangliosides in the two systems.

Ganglioside immunocytochemistry. Rat cerebellar granule cells cultured for 2 days *in vitro* express a low concentration of total gangliosides (74 pmol/10^6 cells) of which GT1b (34%), GD1a (28%), GD1b (12%), and GM1 (4%) are components (22). All nine anti-ganglioside mAbs effectively immunostained paraformaldehyde-fixed rat cerebellar cells (Fig. 5). The cultures contained neurons, which have small round cell bodies and long neurites and astroglia, which are larger, flatter, and typically underlie neurons. Anti-GT1b antibodies stained neurites intensely whereas anti-GD1b stained neuronal cell bodies and astroglia (arrowhead, Fig. 5), as well as neurites. Anti-GD1a antibodies stained some of the neurons in the population intensely (arrow, Fig. 5) and others lightly, whereas anti-GM1 antibodies stained neuronal cell bodies and neurites lightly.

To determine whether the anti-ganglioside mAbs cross-react with shared saccharide structures on glycoproteins (e.g., NeuAc α3 Gal β3 GalNAc), cerebellar granule cells were cultured from newborn *GalNAcT* (−/−) mice. The deleted *N*-acetylgalactosaminyltransferase is not involved in glycoprotein glycosylation, but is essential for biosynthesis of the major brain gangliosides (13). As shown in Fig. 6, all three anti-GT1b mAbs failed to bind above background levels to any cells from these mice, confirming their ganglioside specificity. The same result was found using all three of the anti-GD1a antibodies and the anti-GD1b antibody

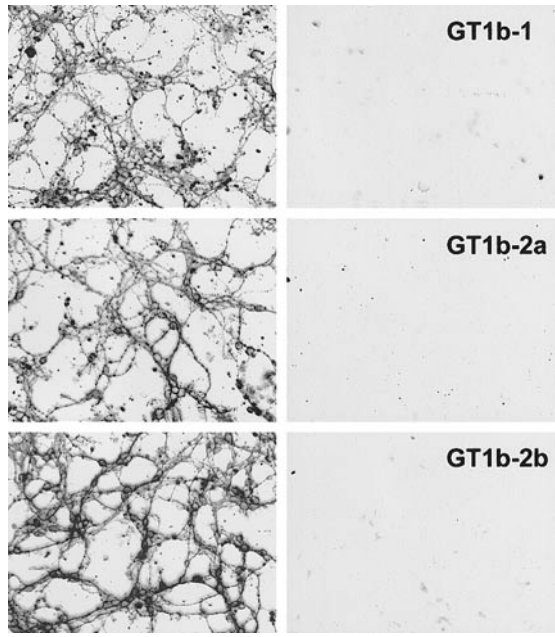

FIG. 6. Anti-ganglioside mAb staining of cerebellar cells from genetically modified mice. Cerebella were dissected from sibling *GalNAcT* (+/−) and *GalNAcT* (−/−) mice, and the cells were dissociated and cultured as described in the text. Major brain gangliosides are expressed in the heterozygote, but not the null mutant, where only GM3 and GD3 are expressed (data not shown). Matched heterozygote (left panels) and knockout (right panels) cerebellar cell cultures were immunostained identically with the indicated anti-GT1b mAb followed by CY3-labeled secondary antibody, as described in the text. Fluorescent images (red on black) captured with a CCD camera under identical conditions are presented as the reverse gray scale (black on white) for clarity. Hoffman contrast microscopy of the same fields revealed similar cell densities (data not shown).

(data not shown). Binding of the anti-GM1 antibodies to the wild-type mouse cerebellar granule cells was too weak to allow a clear determination concerning their specificity for wild-type versus *GalNAcT* (−/−) cells.

DISCUSSION

High-affinity, high-specificity antibodies against the major brain gangliosides (GM1, GD1a, GD1b, and GT1b) have been difficult to raise (23), perhaps because these structures are shared across mammalian and avian species (24, 25). Nevertheless, IgM-class mAbs against major brain gangliosides (and an IgG3-class mAb against GD1b) developed in C3H/HeN strain mice (4, 3, 26) have been useful in ganglioside immunohistochemical localization as well as functional studies (e.g., 27, 28). The discovery that mice genetically engineered to lack complex gangliosides survived to adulthood (29) and mounted a vigorous IgG-class antibody response to ganglioside GD1a conjugated to KLH (5) provided the potential to generate a new suite of high-

affinity IgG-class mAbs against the four major brain gangliosides. This potential was realized in the current study with the isolation of hybridomas secreting high-affinity and high-specificity IgG-class mAbs against GM1, GD1a, GD1b, and GT1b. With the recent discoveries that gangliosides may be involved in signal transduction via membrane rafts (30, 28) and that certain human autoimmune neuropathies involve anti-ganglioside immune responses (31, 32), the research utility of anti-ganglioside antibodies will likely increase. The suite of IgG-class mAbs reported here is intended to provide additional tools to probe ganglioside expression and function and ganglioside-related pathologies.

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