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ARTICLE

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Selective Depletion of Neuropathy-Related Antibodies from Human Serum by Monolithic Affinity Columns Containing Ganglioside Mimics

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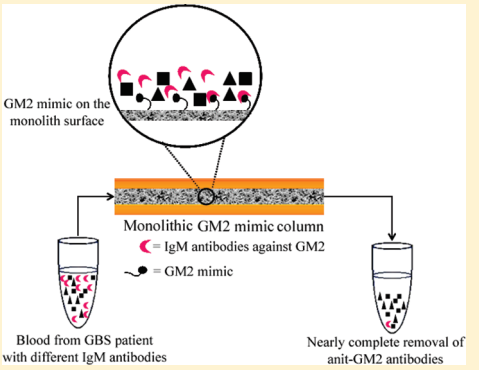
Supporting Information

ABSTRACT: Monolithic columns containing ganglioside GM2 and GM3 mimics were prepared for selective removal of serum anti-ganglioside antibodies from patients with acute and chronic immune-mediated neuropathies. ELISA results demonstrated that anti-GM2 IgM antibodies in human sera and a mouse monoclonal anti-GM2 antibody were specifically and selectively adsorbed by monolithic GM2 mimic columns and not by blank monolithic columns or monolithic GM3 mimic columns. In control studies, serum antibodies against the ganglioside GQ1b from another neuropathy patient were not depleted by monolithic GM2 mimic columns. Fluorescence microscopy with FITC-conjugated anti-human immunoglobulin antibodies showed that the immobilized ganglioside mimics were evenly distributed along the column. The columns were able to capture ~95% of the anti-GM2 antibodies of patients after only 2 min of incubation. A monolithic column of 4.4 μL can deplete 28.2 μL of undiluted serum. These columns are potential diagnostic and therapeutic tools for neuropathies related to anti-ganglioside antibodies.



1. INTRODUCTION

Antibodies against human peripheral nerve gangliosides are frequently encountered in various forms of immune-mediated neuropathies and may be directly involved in nerve damage.[1] An example of such a neuropathy is the Guillain−Barré syndrome (GBS) in which half of the patients display significant levels of serum antibodies against various types of gangliosides. GBS is an acute postinfectious polyneuropathy, characterized by a rapidly progressive muscle weakness with a potentially devastating disease course. More than 20% of patients develop respiratory insufficiency requiring artificial ventilation at an intensive care unit. Overall the mortality is 5%, and at least 20% develop a sustained disability. *Campylobacter jejuni* is the most frequent cause of preceding infection, especially in the most severe forms of GBS.[2] *C. jejuni* expresses lipooligosaccharides (LOS) on its surface, with carbohydrate moieties that are identical to gangliosides present in human neural cell membranes. This molecular mimicry can result in the production of antibodies against LOS during infection that cross-react with gangliosides. Antibodies to gangliosides are also found in patients with chronic forms of neuropathy, such as the paraproteinemic polyneuropathies. These forms of neuropathy are characterized by a progressive, incurable limb weakness usually leading to severe disability. At present no anti-ganglioside antibody specific treatment for these patients is available, although removal of the anti-ganglioside antibodies by selective immunoadsorption would be a rational approach.

Gangliosides are glycolipids containing a carbohydrate moiety with one or more sialic acid groups and a nonpolar ceramide unit by which gangliosides are anchored in cell membranes. Gangliosides are found abundantly in the human nervous system.[3] The most widely known gangliosides are GM1, GM1b, GM2, GQ1b, GalNAc-GD1a, GT1a, and GD1a.[1,2,4] Antibodies in sera from patients with neuropathies bind to the extracellular exposed carbohydrate moieties of these gangliosides. Synthetic carbohydrate mimics of these gangliosides could be used to capture anti-ganglioside antibodies from the blood, as a specific treatment for these neuropathies. Synthetic ganglioside mimics possess various advantages over the natural gangliosides, which are usually

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purified from bovine brain. These advantages include a higher purity, tunable chemical properties, higher stability, possibly higher affinity, improved bioavailability, and no involvement of animals or risk of bovine-transmitted infections.[5,6] Recently, Pukin et al. synthesized analogues of various gangliosides (GM1, GM2, and GM3) in which alkyl spacers with different terminal functional groups (alkene, alkyne, and azide) replace the natural nonpolar ceramide moiety,[7] as well as di-, tetra-, and octavalent derivatives of GM2 and GM1 gangliosides.[8]

Figure 1 depicts the chemical structures of the ganglioside mimics GM1, GM2, and GM3. Serum antibodies from neuropathy patients showed a similar high affinity to these ganglioside mimics compared to the bovine brain-derived gangliosides.[7]

A range of synthetic GM1 mimics with a modified oligosaccharide part has been examined for binding the human anti-GM1 antibodies in solution inhibition and immunoadsorption (Sepharose columns) studies.[9] It was found that the naturally derived GM1os (GM1 that lacks the ceramide aglycone) was superior to all investigated mimics. Thus, we focused on the genuine ganglioside analogues with an authentic carbohydrate structure and a functionalized aglycone part.

Carbohydrates with various functional groups have already been used as ligands, e.g., for microarrays on gold, silicon, and glass surfaces,[10−19] nanoparticles,[20] and carbon nanotubes[21] and in monolithic columns.[22−24] Monolithic supports have the advantage of a high surface density of reactive moieties that can bind oligosaccharides, which translates to a high loadability.[25] Since this capacity is much higher than what could ever be obtained in wall-coated microchannels, this will increase the sensitivity in diagnostic applications. Flow-through applications would allow in principle the depletion of anti-ganglioside antibodies from blood as a rational treatment of GBS and other forms of immune-mediated neuropathies or more generally, diseases where binding between a receptor and a pathogenic molecule is involved.

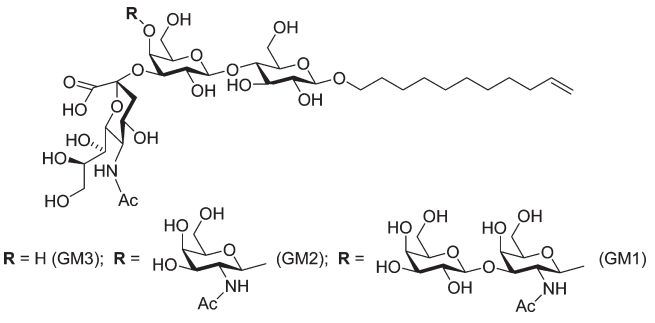

Figure 1. Structures of ganglioside mimics GM3, GM2, and GM1.

We focused on obtaining a proof of principle that monolithic ganglioside mimic columns can efficiently capture antibodies from patients' serum samples and mouse monoclonal antibodies. This study is schematically summarized in Figure 2. Anti-GM2 antibodies were selected as the target, and thus, GM2 was chosen as ligand and attached via a 10-undecenyl spacer to a monolithic column. To determine the efficiency and selectivity of these affinity columns, we used sera obtained from a patient with acute GBS (P1, patient 1) and a patient with chronic monoclonal gammopathy related polyneuropathy (P2, patient 2), both with high titers of IgM antibodies against GM2, and a mouse monoclonal antibody binding to GM2 (EM5). IgM antibodies against GM2 are found in various forms of immune-mediated neuropathy and in other disorders including patients with a human immunedeficiency virus 1 (HIV) infection, in which the anti-GM2 antibody level has prognostic relevance.[26,27]

2. RESULTS AND DISCUSSION

2.1. Depletion of IgM Antibodies Using Monolithic GM2 Mimic Columns. The monolithic GM2 mimic column was used to deplete anti-GM2 IgM antibodies from serum samples from the neuropathy patients P1 and P2. Figure 3A depicts the anti-GM2 IgM activity determined by ELISA in sera before and after exposure to this column, indicating that these antibodies were successfully depleted. The decrease of the IgM concentration was ∼97.5% in P1 (50 times diluted) and ∼92% in P2 (100 times diluted). Still, ∼78% of IgM antibodies in P1 were depleted when the serum dilution went from 1:50 to 1:5. This shows that a 4.4 μL monolithic column (250 μm i.d., 9 cm long) can deplete 28.2 μL of undiluted serum, which corresponds to a capacity of 6.4 μL of undiluted serum per microliter of column volume. The kinetics of the adsorption are fast, as the residence time of the serum in the columns was less than 2 min. Because of the strong binding of the serum anti-GM2 IgM to the GM2 mimic in the

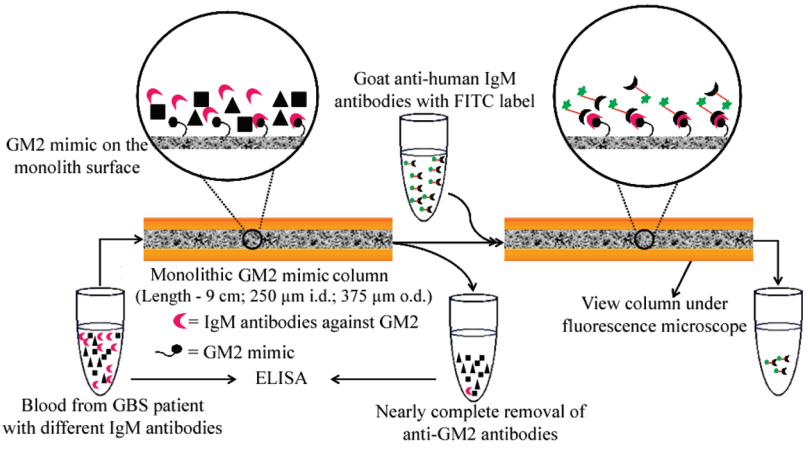

Figure 2. Schematic overview of the incubation studies with serum samples from patients with neuropathy and monolithic GM2 mimic columns to demonstrate selective depletion of serum anti-GM2 antibodies. The binding was measured by ELISA. The homogeneous distribution of captured antibodies along the column was measured by fluorescence microscopy and fluorescence conjugated anti-human IgM antibodies.

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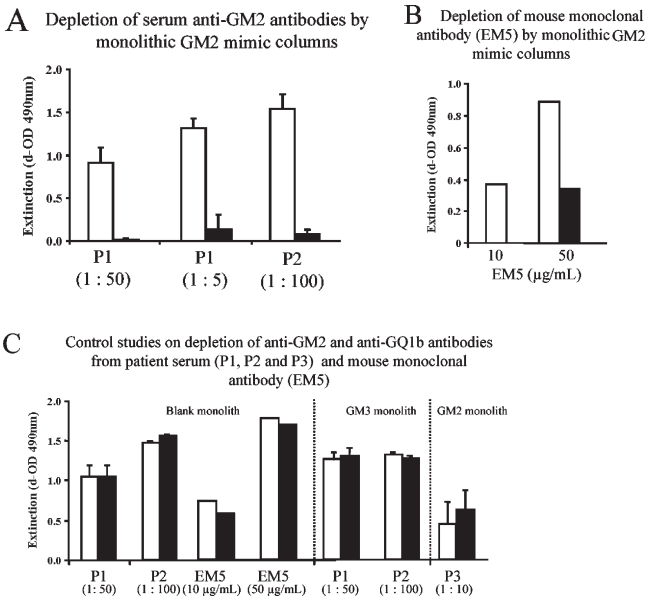

Figure 3. (A) Depletion of serum anti-GM2 antibodies by monolithic GM2 mimic columns. Serum anti-GM2 IgM antibody concentration was determined by ELISAs in samples before (white bars) and after (black bars) exposure to the column. Serum from patient P1 with GBS was tested in two dilutions (1:50 and 1:5). Serum from patient P2 was tested in one dilution (1:100). (B) Depletion of the mouse anti-GM2 monoclonal antibody EM5 (10 and 50 μg/mL) by monolithic GM2 mimic columns. The EM5 antibody concentration was determined by ELISAs in samples before (white bars) and after (black bars) exposure to the column. (C) Control studies to confirm the specificity of interaction between anti-GM2 antibodies and GM2 mimics in the monolithic columns: (left panel) no depletion of anti-GM2 IgM antibodies from sera from patient P1 and P2 after infusion into blank monolithic columns; (middle panel) no depletion of anti-GM2 IgM antibodies in sera from patients P1 and P2 after infusion into monolithic GM3 mimic columns; (right panel) no depletion of anti-GQ1b IgM antibodies in serum from a neuropathy patient after infusion into monolithic GM2 mimic columns. Serum anti-ganglioside IgM antibody (GM2 + GQ1b) and mouse monoclonal antibody (EM5) concentrations were determined by ELISAs in samples before (white bars) and after (black bars) exposure to the column.

column, the bound antibodies could not be washed off the column with binding buffer solution.

To further demonstrate the specificity and capacity of these columns to capture anti-GM2 antibodies, mouse monoclonal IgM antibodies (EM5) binding to GM2 were infused (see Figure 3B).[28] Studies with a dilution series of EM5 in concentrations up to 10 μg/mL showed that no residual activity to GM2 in the flow-through fraction was found after a single 2 min incubation with a monolithic GM2 mimic column. This column depleted more than 50% of the EM5 antibodies present at 50 μg/mL EM5 (Figure 3B). No depletion of EM5 (concentrations up to 50 μg/mL) was observed using blank monolithic columns (see Figure 3C).

Figure 3C depicts the ELISA data from three sets of control experiments to further confirm the specificity of the interaction between serum anti-GM2 antibodies and GM2 mimics in monolithic columns. First, sera from patients P1 and P2 were infused into blank monolithic columns (i.e., without GM2 mimics). No decrease in anti-GM2 IgM activity was observed, confirming that these antibodies bind to GM2 mimics in the columns only (left panel, Figure 3C). Second, both sera were also infused into monolithic GM3 mimic columns. These act as a control antigen since the anti-GM2 antibodies in the patient sera did not bind to GM3 mimics in ELISA. No depletion of serum anti-GM2 antibodies was observed (middle panel, Figure 3C). Third, a serum sample from patient P3 with GBS with IgM antibodies to the ganglioside GQ1b (and no antibodies to GM2) was infused into the monolithic GM2 mimic column (right panel, Figure 3C). The anti-GQ1b antibody activity in serum from patient P3 was not reduced after incubation with the monolithic GM2 mimic column, indicating that no nonspecific binding of antibodies occurs by the monolithic GM2 mimic column.

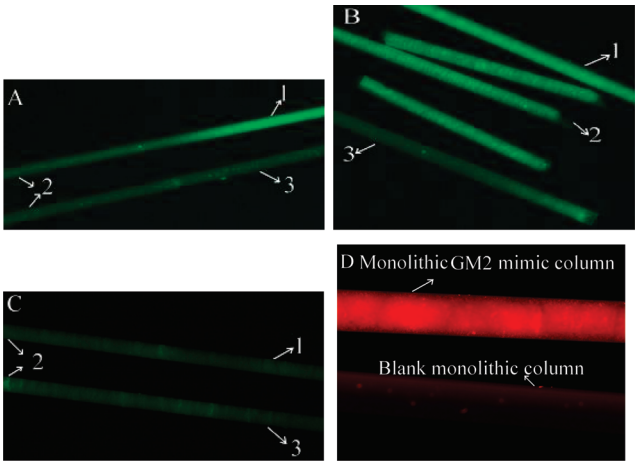

Figure 4. Fluorescence microscopy images of monolithic GM2 mimic columns infused with either serum or mouse monoclonal antibody and subsequently treated with relevant fluorescent labeled anti-human (FITC) or anti-mouse (Alexa Fluor 594) immunoglobulin: (A) serum from patient P1 (1:50 dilution); (B) serum from patient P1 (1: 5 dilution); (C) no serum treatment; (D) mouse monoclonal antibody (EM5) incubated with blank monolithic columns and monolithic GM2 mimic columns; (1) initial part of column; (2) middle of column; (3) end of column.

2.2. Fluorescence Microscopy Results. To define the homogeneity of the distribution of GM2 mimics in the monolithic columns and to visualize the IgM adsorption by these columns, staining studies were performed with FITC-conjugated anti-human IgM antibodies after infusion of patient serum samples into the columns. Both blank and monolithic GM2 mimic columns were first infused with serum P1 at two different dilutions (1:50 and 1:5). After prolonged washing and then staining with FITC-conjugated anti-human IgM antibodies, the columns were viewed under a fluorescence microscope. The recorded images are shown in Figure 4A–C.

Staining of the monolithic GM2 mimic columns with FITC-conjugated goat anti-human IgM showed a significant and homogeneous labeling after infusion with serum from patient P1, and the fluorescence was visible over a greater length of the column after incubation with the 1:5 dilution than with the 1:50 dilution (Figure 4A and Figure 4B, respectively). This indicates that most of the serum antibodies are immediately bound after introduction into the columns as long as free GM2 mimics remain available. The fluorescence images are in full agreement with the previously discussed results in ELISA showing depletion of serum anti-GM2 IgM activity after incubation with these columns (see Figure 3A). A monolithic GM2 mimic column not incubated with patient serum as a control did not show any staining, confirming that the

Table 1. Patients' Clinical Diagnosis and Serum Antibody Specificity, Class, and Titer

	antibody			
patient code	class	titer	antibody reactivity to gangliosides	diagnosis
P1	IgM	1600	GM2	GBS
P2	IgM	51200	GM2	polyneuropathy and monoclonal gammopathy
P3	IgM	200	GQ1b	GBS
HV	IgM		none	healthy control

FITC-conjugated anti-human IgM antibodies do not bind to the column (Figure 4C).

Next a blank monolithic column (i.e., no GM2 present) and a monolithic GM2 mimic column were infused with mouse monoclonal antibodies (EM5), followed by treating both the columns with Alexa Fluor 594 conjugated anti-mouse IgM antibody. The obtained fluorescence images are depicted in Figure 4D. This clearly indicates that the mouse monoclonal antibodies are successfully captured only by the monolithic GM2 mimic column. This result is in full agreement with the ELISA data (see Figure 3B and Figure 3C).

As additional controls, a blank monolithic column and a monolithic GM3 mimic column were infused with sera P1 (1:50) and P2 (1:100) and a monolithic GM2 mimic column was infused with serum P3 (1:10). Afterward they were stained with FITC-conjugated goat anti-human IgM antibodies to detect bound serum antibodies in the column (see Supporting Information). In none of these cases significant staining was observed. These results prove that no IgM antibodies were bound to any of the columns (blank monolith, monolithic GM3 mimic, and monolithic GM2 mimic), and they are in full agreement with the ELISA data shown in Figure 3C. Thus, the columns are highly selective, as many other IgM antibodies must have been present in these serum samples. The overall results demonstrate both the efficiency and selectivity of monolithic GM2 mimic columns to capture anti-GM2 IgM antibodies from serum samples containing antibodies against gangliosides. The long-term stability of the columns was also checked, as this is of prime importance for any future diagnostic or therapeutic use. The antibody capturing efficiency remained intact even after 2 years of storage in a dry state (results not shown).

Ganglioside mimics with an alkene-terminated spacer can be successfully incorporated in monolithic capillary affinity columns in a single step by in situ polymerization. Fluorescence microscopy images showed an even distribution of GM2 molecules along the entire column. Per microliter of GM2 column 6.4 μL of undiluted serum containing IgM antibodies (titer 1600) could be depleted after an incubation time of less than 2 min. GM2 columns specifically deplete IgM antibodies against GM2 and not IgM antibodies against the closely related ganglioside GQ1b or other IgM antibodies from a healthy volunteer. Both blank monolithic columns and monolithic GM3 mimic columns did not exhibit any nonspecific adsorption of IgM antibodies. Overall, these affinity monolithic columns can be used to deplete IgM antibodies specifically from serum samples of patients suffering from GBS. This proof of principle with GM2 opens up interesting possibilities for the development of new forms of diagnostics and even treatments for GBS and related neuropathies with neurotoxic anti-ganglioside antibodies. For diagnostic purposes, one could envisage trapping, staining with fluorescent anti-human antibodies followed by elution and detection to achieve rapid, selective, and sensitive detection of various GBS-related antibodies.

3. EXPERIMENTAL SECTION

3.1. General Synthetic Methods. Fused-silica capillaries of 250 μm i.d. and 375 μm o.d. were obtained from Polymicro Technologies (Phoenix, AZ, U.S.). Anhydrous sodium hydrogen phosphate, bovine serum albumin (BSA), and *O*-phenylenediamine tablets were purchased from Sigma Aldrich, The Netherlands. Hydrogen peroxide, citric acid, and natural GM2 ganglioside derived from bovine brain were purchased from Merck, The Netherlands. Mouse monoclonal antibody (EM5) was kindly provided by Prof. Hugh Willison, Department of Neurology, University of Glasgow, U.K. Alexa Fluor 594 goat anti-mouse IgM was purchased from Invitrogen, The Netherlands. Natural GQ1b was purchased from Sanbio, The Netherlands. Serum samples (see Table 1) were provided by the Erasmus MC (Rotterdam, The Netherlands) with titers as indicated in the table. Gangliosides (GM2 and GM3) with a 1-undecenyl spacer were synthesized as described before.[7] Gas tight syringes (500 μL) were purchased from Alltech, The Netherlands. Syringe pumps (Harvard 11 PicoPlus, dual syringe) were purchased from VWR International, The Netherlands. (+)-*N*,*N*-Diallyltartardiamide (DATD), 2-hydroxyethyl methacrylate (HEMA), piperazinediacrylamide (PDA), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPA) were purchased from Sigma Aldrich, The Netherlands.

3.2. Enzyme-Linked Immunosorbent Assay (ELISA). ELISAs were used to measure the IgM antibody binding to GM2 and GQ1b in serum samples. The wells of a 96-well plate (Nunc, Maxisorp) were treated either with ethanol or with 300 pmol of ganglioside dissolved in ethanol. These solutions were subsequently allowed to evaporate to dryness. All the wells were then blocked with a solution of PBS (phosphate buffered saline, pH 7.8, 200 μL per well) containing 1% (w/v) of BSA for 2 h at room temperature and a further 2 h at 4 °C. The plates were then emptied by flicking and incubated overnight at 4 °C. The serum samples were diluted, initially 1:100, in PBS−1% BSA, and added to four wells, two coated with GM2 or GQ1b and two with ethanol only.

The following day, the plates were washed with an automated ELISA washer (Elx50, Bio-Tek, U.K.) with PBS solution and filled with a solution of peroxidase-conjugated secondary antibodies (Jackson Immuno Research Labs) diluted to 1:2500 in PBS−1% BSA for 1.5 h at room temperature. After the plates were washed once again with the PBS solution, they were developed by adding 100 μL of substrate solution in citrate buffer (pH 5.0−5.2). The substrate solution was prepared by dissolving one (5 mg) *O*-phenylenediamine tablet in 6.0 mL of 4 mM citric acid solution, 6.5 mL of 8 mM anhydrous sodium hydrogen phosphate solution, and 12.5 mL of Milli-Q water. Immediately prior to use 12.5 μL of 30% hydrogen peroxide solution was added to the above substrate solution. The reaction in the well was stopped by the addition of 100 μL of 2 M hydrochloric acid, and the optical densities (OD) were read in an automated reader at 490 nm. The mean ODs of the blank (ethanol-coated) wells were subtracted from the mean ODs of the GM2-coated wells to obtain a specific OD.[5,6]

3.3. In Situ Preparation of Monolithic Ganglioside Mimic Columns. A fused silica capillary of 100 cm total length was activated with 3-(trimethoxysilyl)propyl methacrylate. Subsequently an affinity monolithic column was prepared as described earlier.[24] In short, a solution of ganglioside mimics (GM2 or GM3, 5 mg) in methanol (30 μL) was added to a mixture of HEMA (30 μL), ammonium sulfate (8 mg), DATD (20 mg), and PDA (16 mg) in 250 μL of phosphate buffer, pH 7.0, in an Eppendorf tube and mixed well, followed by deaeration for 5 min. Subsequently, the initiator AMPA (10 μL, 10% v/v in water) was added. The monolith solution was then sucked into the acrylate terminated capillary using vacuum, and both ends of the capillary were sealed with a gas chromatography septum. The column was placed in an oven (65 °C) for 12 h, which resulted in a monolithic ganglioside mimic column. The column was washed with water for 2 h at 2 μL/min.

3.4. Antibody Depletion of Serum Samples. A three-step procedure to capture antibodies (IgM) from serum samples was followed. First the columns were washed with PBS buffer (pH 7.8) for 1 h at 1.4 μL/min. Next various serum dilutions (P1 (1:50 and 1:5), P2 (1:100), P3 (1:10), HV (1:10)) in PBS buffer (pH 7.8)) were prepared, and in each case an amount of 180 μL was infused into the columns at 1 μL/min during 3 h. Subsequently the columns were washed again with PBS buffer for 1 h at 1.4 μL/min. All solutions were infused into the columns with a 500 μL syringe and a syringe pump. The collected samples were analyzed with ELISA.

3.5. Mouse Monoclonal Antibody (EM5) Depletion. A three-step procedure to capture mouse monoclonal antibodies (EM5) was as follows. First the columns were washed with PBS buffer (pH 7.8) for 1 h at 1.4 μL/min followed by infusion of 120 μL of EM5 (10 μg/mL or 50 μg/mL) in PBS buffer (pH 7.8) for 2 h at 1 μL/min. Finally the columns were washed again with PBS buffer for 1 h at 1.4 μL/min.

3.6. Control Experiments. Three sets of control experiments were performed to demonstrate that (a) IgM antibodies against GM2 in sera P1 and P2 could only be depleted by monolithic GM2 mimic columns and not by either a blank monolithic column or a monolithic GM3 mimic column, (b) monolithic GM2 mimic columns deplete IgM antibodies against GM2 only and not IgM antibodies against GQ1b nor various other species of antibodies, and (c) mouse monoclonal antibody (EM5) could specifically be depleted by a monolithic GM2 mimic column and not by a blank monolithic column.

3.7. Fluorescence Microscope (FM) Measurements. A stereofluorescence microscope and Olympus IX 51 microscope were used to carry out FM measurements. The homogeneous distribution of IgM antibodies along the monolithic ganglioside mimic columns was tested using FITC conjugated goat anti-human IgM. First, the column was washed with PBS buffer (pH 7.8) for 30 min at 1 μL/min. Subsequently, the column was treated with FITC conjugated goat anti-human IgM solution for 1 h at 1 μL/min and finally washed with PBS buffer (pH 7.8) for 45 min at 1 μL/min. The stained columns were evaluated using a fluorescence microscope. In the case of a blank monolithic column and a monolithic GM2 mimic column infused with mouse monoclonal antibody (EM5), the columns were treated with PBS buffer (pH 7.8) for 1 h at 1.4 μL/min followed by treatment with 120 μL Alexa Fluor 594 goat anti-mouse IgM (1:100 dilution) for 2 h at 1 μL/min. The columns were immediately washed with PBS buffer (pH 7.8) for 85 min at 1.4 μL/min and then viewed with a fluorescence microscope.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Four fluorescence microscopy images of various blank experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AMPA, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; BSA, bovine serum albumin; DATD, (+)-*N*,*N*-diallyltartardiamide; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GBS, Guillain−Barré syndrome; HEMA, 2-hydroxyethyl methacrylate; HIV, human immunodeficiency virus 1; HV, healthy human volunteer; i.d., internal diameter; LOS, lipooligosaccharides; OD, optical density; PDA, piperazinediacrylamide; P1, patient 1; P2, patient 2; P3, patient 3

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