Solid phase immunoadsorption for therapeutic and analytical studies on neuropathy-associated anti-GM1 antibodies

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Autoimmune neuropathies including Guillain-Barré syndrome are frequently associated with anti-GM1 ganglioside antibodies. These are believed to play a pathogenic role and their clearance from the circulation would be predicted to produce therapeutic benefit. This study examines the conditions required for effective immunoadsorption of anti-GM1 antibodies using glycan-conjugated Sepharose as a matrix. In solution inhibition studies using a range of GM1-like saccharides in conjunction with mouse and human anti-GM1 antibodies, the whole GM1 pentasaccharide β-Gal-(1-3)-β-GalNAc-(1-4)-(α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc was the favored ligand for maximal inhibition of antibody-GM1 interactions in comparison with monosaccharides, Gal-(1-3)-β-GalNAc-βOME, and synthetic GM1 mimetics. Immunoadsorption studies comparing binding of mouse monoclonal anti-GM1 antibodies to GM1-Sepharose and β-Gal-(1-3)-β-GalNAc–Sepharose confirmed the preference seen in solution inhibition studies. GM1-Sepharose columns were then used to adsorb anti-GM1 immunoglobulin G and immunoglobulin M antibodies from human neuropathy sera. Anti-GM1 antibodies subsequently eluted from the columns often showed a striking monoclonal or oligoclonal pattern, indicating that the immune response to GM1 is restricted to a limited number of B-cell clones, even in the absence of a detectable serum paraprotein. These data support the view that immunoadsorption plasmapheresis could potentially be developed for the acute depletion of serum anti-GM1 antibodies in patients with neuropathic disease, and also provide purified human anti-GM1 antibodies for analytical studies.

Key words: antibody/GM1 ganglioside/immunoadsorption/ neuropathy/treatment

Introduction

GM1 ganglioside is a glycosphingolipid widely synthesized throughout the body, where it plays an important modulating role in a diverse range of biological functions (Tettamanti 2004). In addition to its normal role, GM1 acts as the intestinal receptor for bacterial toxins including Escherichia coli heat-labile enterotoxin and Vibrio cholerae toxin (Spangler 1992) and acts as a peripheral nerve target antigen for anti-GM1 autoantibodies in human autoimmune neuropathies including Guillain-Barré syndrome (GBS) (Willison and Yuki 2002). In this latter role, evidence from human and animal studies indicates that anti-GM1 antibodies play a major pathogenic role in initiating autoimmune injury (Willison 2005). Antibodies such as these are historically termed “anti-ganglioside” antibodies, although they can potentially recognize the oligosaccharide component linked to any lipid or protein support, including bacterial liposaccharides (Bowes et al. 2002).

Strategies aimed at inhibiting GM1-antibody interactions or immunoadsorbing GM1 antibodies could be useful in a variety of physiological, pathological, and therapeutic settings. For example, they could be used either as soluble inhibitors of antibody-GM1 interactions or, if immobilized on solid matrices, as immunoadsorption tools for analytical studies or for therapy. A similar strategy has recently been applied to neuropathy-associated anti-GQ1b antibodies (Willison et al. 2004).

In this study, we have used mouse and human neuropathy-associated anti-GM1 antibodies and oligosaccharides containing GM1-like structures to investigate these ligand–antibody interactions. The critical sugar residues required for anti-GM1 antibody binding to GM1 have not been clarified. We therefore examined a range of natural and synthetic GM1-like oligosaccharides varying in size and configuration, including GM1 mimics prepared as glycomic inhibitors of cholera toxin (Bernardi et al. 2000; Arosio et al. 2004). Since cholera toxin binds to GM1 via its two terminal sugars, galactose (Gal) and carboxyl group of sialic acid, these were retained during the design of these mimics, along with the GalNAc residue. These mimics might therefore also be suitable for binding antibodies that recognize either the Gal(1-3)GalNAcβ(1-4)[Neu5Acα(2-3)]βGal- or Galβ(1-3)GalNAc- of GM1, the two most common binding patterns seen in human autoantibodies. Glycomimetics such as these are more straightforward to chemically synthesize than the whole GM1 oligosaccharide, and can be more metabolically stable. They
can also be conjugated to various supports via the nonsugar scaffold.

In order to establish general principles, we first examined two prototypic immunoglobulin G (IgG) anti-GM1 mouse monoclonal antibodies (mAbs): one that appears monospecific for GM1 and another that is reactive with the terminal Galβ(1-3)GalNAc disaccharide common to GM1, asialoganglioside GM1 (Ga1) and GD1b as this latter pattern of reactivity is typical of human neuropathy-associated anti-GM1 autoantibodies (Willison and Yuki 2002). We then applied the findings obtained using mouse mAbs to an inhibition and immunoadsorption study of human IgG and immunoglobulin M (IgM) anti-GM1 antisera from patients with acute and chronic neuropathies, including GBS.

Results

Properties of mouse anti-GM1 mAbs DG1 and DG2

The ganglioside-binding characteristics of IgG mAbs DG1 and DG2 were analyzed by enzyme-linked immunosorbent assay (ELISA) against a panel of gangliosides and Ga1 (GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, GT1a and GQ1b). Both mAbs reacted with GM1 with similar maximal binding values: 0.5 μg/mL for DG1 and 0.4 μg/mL for DG2. DG1 is monospecific for GM1 (Figure 2A), whereas DG2 binds GM1, Ga1, and GD1b (Figure 2B), suggesting that the epitope for DG2 comprises the nonreducing Galβ(1-3)GalNAc moiety common to these three glycolipids.

Heavy (H) and light (L) immunoglobulin variable (V) region sequencing determined the variable region gene usage of DG1 and DG2 and established that they have distinct clonal origins, containing different immunoglobulin heavy-chain and light-chain gene rearrangements. Heavy-chain variable regions of both mAbs are encoded by the same V_{H}-J_{558} gene family member, GN2 (DG1, 87% nucleotide identity; DG2, 92% nucleotide identity), and the same J_{H} segments (both J2), but use different D regions (DG1, FL16; DG2, QS2). Thus, DG1 and DG2 are not differentially class-switched variants of each other.

Defining the oligosaccharide epitope in GM1 required for mouse monoclonal anti-GM1 antibody binding

The minimum oligosaccharide requirement for anti-GM1 antibody binding was assessed in solution inhibition studies, using component oligosaccharides and synthetic GM1-mimics (Figure 3). Gal, sialic acid, GalNAcβ(1-3)Gal, GM2 oligosaccharide produced by metabolically engineered E. coli (EGM2-OS), and 3 did not inhibit the binding of either antibody to GM1 or Ga1 (data for monosaccharides not shown).

The GM1 monospecific mAb DG1 was inhibited from binding GM1 by the complete GM1 pentasaccharide, either cleaved from the native ganglioside (GM1-OS, 1 mM, 83.5%) or when produced in E. coli (EGM1-OS, 1 mM, 87.8%), as would be expected (Figure 3A). DG1 interaction with GM1 was not inhibited by the synthetic GM1 mimics, 1, 2, or 3, which bind cholera toxin, suggesting that the primary interaction of this antibody with GM1 is not solely mediated by the carboxyl group of sialic acid, as this group is retained in these mimics. Importantly, and consistent with its mono-specificity for GM1, DG1 binding to GM1 was also not inhibited by the terminal disaccharide of GM1, Galβ(1-3)GalNAc (4) (Figure 3A).

DG2 mAb binds GM1, Ga1, and GD1b that have, in common, the nonreducing Galβ(1-3)GalNAc moiety. DG2 was assessed for soluble oligosaccharide inhibition in competition with solid phase Ga1 and GM1 (Figure 3B and C). Both GM1-OS and EGM1-OS fully inhibited binding to both GM1 and Ga1. No inhibition of DG2 binding to GM1 or Ga1 was observed with 3. However, 1, a structure very similar to 3 and that displayed a similar affinity for cholera toxin, inhibited binding to both GM1 and Ga1 (61.5% and 86.7%, respectively), suggesting that despite sharing the same carboxyl group, the cyclohexylmethyl group on 3 must significantly interfere with antibody access to the Galβ(1-3)GalNAc disaccharide. The GM1 mimic 2, comprising divalent 1 linked by a dendrimeric core, inhibited DG2 binding to both GM1 (63.9%) and Ga1 (96.6%) at a similar level to that achieved with the monomer structure 1.

In contrast to the situation with DG1, 4 (Galβ(1-3)GalNAcβOMe) was able to partially inhibit the binding of DG2 to both GM1 and Ga1 (43.6% and 74.7%, respectively), as predicted by the specificity of DG2 for Galβ(1-3)GalNAc-bearing glycolipids. However, the inhibition achieved by 4 was not as high as that seen with the intact GM1 oligosaccharides, GM1-OS and EGM1-OS, suggesting that even for a promiscuously Galβ(1-3)GalNAc-reactive anti-GM1 antibody, the whole GM1 pentasaccharide is a favored ligand in comparison to the nonreducing disaccharide.

Defining oligosaccharide inhibitors of human anti-GM1 antisera binding to GM1

In order to establish whether the principles observed for mouse mAbs DG1 and DG2 also applied to human neuropathy-associated antibodies, three representative anti-GM1 IgG and three anti-GM1 IgM antisera (termed Patient 1–Patient 6; P1–P6) were examined in solution inhibition studies with EGM1-OS, Galβ(1-3)GalNAcβOMe (4) and irrelevant control oligosaccharides. All six sera were fully or partially inhibited from binding to GM1 by EGM1-OS (P1, 93.2%; P2, 55.2%; P3, 56.7%; P4, 31.8%; P5, 27.6%; P6, 100%, at 1 mM oligosaccharide) and not by EGM2-OS or Gal (Figure 4). For all human antisera tested, inhibition of binding to GM1 was more efficiently achieved with EGM1-OS than with 4 (P1, 12.8%; P2, 13%; P3, 37.9%; P4, 11.1%; P5, 21.1%; P6, 91.8%), irrespective of the degree to which the antisera-binding depended upon the terminal Galβ(1-3)GalNAc epitope, as judged by reactivity with GM1, Ga1, and GD1b (Table I).

Adsorption of anti-GM1 antibodies by GM1 and Galβ(1-3)GalNAc affinity columns

Imunoaffinity principles could be used for both analytical and therapeutic approaches to human neuropathies associated with antiglycan antibodies. To investigate this, oligosaccharide–Sepharose conjugates were assessed for human anti-GM1 antibody immunoadsorption from disease-associated antisera using the two major oligosaccharides identified through the solution-inhibition studies—the whole GM1 pentasaccharide (GM1-OS) and the more weakly inhibiting
Galβ(1-3)GalNAc terminal disaccharide. In preparation for the human serum trials, firstly, the mouse mAb DG2 that binds GM1, GA1, and GD1b was assessed for binding to GM1–Sepharose and Gal β(1-3)GalNAc–Sepharose. As successful binding would depend on a sufficient affinity for the immobilized ligand and also on favorable association and dissociation rates, these were first measured by surface plasmon resonance (SPR). DG2 antibody-binding fragment (Fab) interaction with GM1 did not fit to a 1:1-binding model and, consequently, kinetic data could not be obtained. Therefore, the dissociation equilibrium constant ($K_D$, $3 \times 10^{-7}$ M), association rate constant ($k_a$, $2.17 \times 10^3$ M$^{-1}$ s$^{-1}$) and dissociation rate constant ($k_d$, $7.9 \times 10^{-4}$ s$^{-1}$) were measured for the interaction with GA1. These values were in a similar range to other anticarbohydrate antibodies that were successfully purified by immunoaffinity techniques (Lee et al. 1998).

After application of DG2 (100 μg) to the GM1–Sepharose and Galβ(1-3)GalNAc–Sepharose columns and washing with...
phosphate-buffered saline (PBS), bound antibody was eluted at pH 4. Column profiles obtained with each column were strikingly different (Figure 5). For the Galβ(1-3)GalNAc–Sepharose column, DG2 gradually eluted in the wash buffer, indicating that it was not binding with sufficient affinity to be fully retained by the column under normal physiological conditions. In contrast, DG2 was fully retained by GM1–Sepharose and did not elute in the wash buffer, being retained until eluted at pH 4.0. By way of control for nonspecific binding, DG2 was applied to TGM2–Sepharose and showed no binding (results not shown). These data suggest that, even for an antibody that binds three Galβ(1-3)GalNAc-bearing glycolipids GM1, GA1, and GD1b in solid phase ELISA, GM1–Sepharose appears superior to Galβ(1-3)GalNAc–Sepharose for immunoabsorption, as was also found for the solution inhibition studies (Figure 3).

In view of the above, GM1–Sepharose was selected for absorption trials with six human anti-GM1-containing antisera, P1–P6. Antisera were applied to a GM1–Sepharose column and, by way of negative control, to an irrelevant...
oligosaccharide–Sepharose column (GD1a–Sepharose). Anti-GM1 antibodies from all six sera were retained by GM1–Sepharose and could be eluted at either pH 4 or pH 10 (Figure 6). None of P1–P6 bound to GD1a–Sepharose (data not shown). One of the anti-GM1 IgG antisera (P2, Figure 6B) gradually eluted off the GM1–Sepharose column in the neutral pH wash buffer, as had been seen with the mouse mAb DG2 on Gal-GalNAc–Sepharose (Figure 5A), whereas other antibodies appeared more stably bound at neutral pH, as had been seen with DG2 on GM1–Sepharose (Figure 5B).

### Table I. Antibody titres to GM1, GA1, and GD1b in serum from six neuropathy cases

<table>
<thead>
<tr>
<th>Serum</th>
<th>Diagnosis</th>
<th>Isotype</th>
<th>Antibody titre (endpoint dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GBS</td>
<td>IgG</td>
<td>&gt;1/12 500  &gt;1/12 500  &gt;1/12 500</td>
</tr>
<tr>
<td>P2</td>
<td>GBS</td>
<td>IgG</td>
<td>&gt;1/12 500  &lt;1/100  &lt;1/100  &lt;1/100</td>
</tr>
<tr>
<td>P3</td>
<td>CMAN</td>
<td>IgG</td>
<td>1/6000  &gt;1/12 500  1/2400</td>
</tr>
<tr>
<td>P4</td>
<td>MMN</td>
<td>IgM</td>
<td>1/2500  1/12 000  1/170</td>
</tr>
<tr>
<td>P5</td>
<td>MMN</td>
<td>IgM</td>
<td>1/2100  &lt;1/100  &lt;1/100  &lt;1/100</td>
</tr>
<tr>
<td>P6</td>
<td>MMN</td>
<td>IgM</td>
<td>1/4000  1/12 500  1/400</td>
</tr>
</tbody>
</table>

GBS, Guillain-Barré syndrome; CMAN, chronic motor axonal neuropathy; MMN, multifocal motor neuropathy.

Oligosaccharide–Sepharose column (GD1a–Sepharose). Anti-GM1 antibodies from all six sera were retained by GM1–Sepharose and could be eluted at either pH 4 or pH 10 (Figure 6). None of P1–P6 bound to GD1a–Sepharose (data not shown). One of the anti-GM1 IgG antisera (P2, Figure 6B) gradually eluted off the GM1–Sepharose column in the neutral pH wash buffer, as had been seen with the mouse mAb DG2 on Gal-GalNAc–Sepharose (Figure 5A), whereas other antibodies appeared more stably bound at neutral pH, as had been seen with DG2 on GM1–Sepharose (Figure 5B).

### Analysis of human anti-GM1 antibodies retrieved from GM1–Sepharose columns

In order to provide evidence in support of the antibody-specific nature of the GM1–Sepharose column, we then examined the clonal characteristics of the eluted anti-GM1
antibody populations from P1 to P6. Eluted fractions were pooled, dialyzed, and concentrated, then subjected to isoelectric focusing (IEF) followed by Western blot probed for human IgG (P1–P3) or IgM (P4–P6). Spectrotypes for the GM1–Sepharose affinity-purified antibody fractions from P1 to P6 were compared with the IEF patterns in whole sera (Figure 7). As normally expected, whole serum samples (lanes S) contain polyclonal immunoglobulins and focus as diffuse background interspersed with some discrete monoclonal and oligoclonal bands. In contrast, antibodies eluted from the GM1 affinity columns (lanes E) tended toward highly discrete clontypes, in some cases appearing monoclonal (P1) or dominated by one or two major clones (P2). In IEF gels, mAbs typically appear as ladders due to charge microheterogeneity with any one antibody species. These data provide evidence that the GM1–Sepharose columns do not simply absorb all immunoglobulins from the sera in a nonspecific manner, but bind specific GM1-reactive populations of antibodies.

Discussion

These studies provide preclinical evidence that antigen-specific immunoabsorption could be developed as a treatment for anti-GM1 antibody-associated neuropathies. We first conducted solution inhibition studies using a range of glycans in conjunction with two distinctly specific anti-GM1 antibodies to ascertain the structure of the most widely effective inhibitor of anti-GM1 antibody–glycan interactions. From these studies, we conclude that the whole GM1 pentasaccharide is superior to other structures tested here. Although the terminal disaccharide of GM1 (Galβ(1–3)GalNAc) was sufficient in some cases to both bind and inhibit a proportion of anti-GM1 antibodies, greater inhibition of binding was observed with the full GM1 pentasaccharide. Solution inhibition and structural data suggest that carbohydrate epitopes recognized by antibodies generally consist of di- to tetrasaccharides (Feizi et al. 1979; Gooi et al. 1981; Kabat et al. 1982; Cylgser et al. 1991; Bundle et al. 1994; Zdanov et al. 1994; Villeneuve et al. 2000; Vyas et al. 2002; Calaresu et al. 2003; Nguyen et al. 2003; Ramsland et al. 2004; van Roon et al. 2004), including epitopes on gangliosides (Pichla et al. 1997; Krengel et al. 2004). The largest proportion of the binding energy arises for the recognition of di- to tetrasaccharide epitopes, although extended epitopes may contribute relatively modest free energy gains (Bundle 1998). It is therefore unlikely that the whole pentasaccharide is fully engaged in the antigen-binding site, but the terminal Galβ(1-3)GalNAc disaccharide and internally Gal-linked sialic acid form a more favorable conformation in native GM1 for antibody binding than other structures tested.

The above view is supported by the results obtained using the GM1 oligosaccharide mimics developed for cholera toxin inhibition studies (1 and 3) that lack sialic acid but retain the correctly oriented carboxyl group, albeit in a less rigid structure (Lanne et al. 1994; Merritt et al. 1994; Lanne et al. 1999). In contrast to cholera toxin (Bernardi et al. 2000), the GM1 monospecific mAb DG1 was unable to bind these GM1 mimics, suggesting a more specific requirement for the whole native sialic acid residue. As would be expected, because of its lower dependence on the presence of sialic acid, the Galβ(1-3)GalNAc reactive mAb, DG2, did bind 1 and 3 to some extent, but it offered no advantage over native GM1 oligosaccharide. Based on the data from these two mouse mAbs, it seems unlikely that the natural GM1 oligosaccharide could be easily improved upon. Now that the GM1 oligosaccharide can be readily produced biosynthetically and is here shown to be immunologically competent; producing the large amounts required for therapeutic columns is achievable (Antoine et al. 2003) and particularly attractive if they can be assembled as dendrimers or other multivalent glycoclusters (Fort et al. 2005).

One prospect arising from the identification of blocking glycans identified in solution-inhibition studies would be the use of soluble oligosaccharide inhibitors as therapy. Delivered orally or intravenously to patients, these might block anti-GM1 autoantibodies from binding self-GM1, or
enhance antibody clearance through other mechanisms. Although this approach appears possible in vitro as described here, high levels of antibody–glycan inhibition were only achieved with at least 50 μM oligosaccharide and such compounds would be complex to develop for clinical use. The use of multivalent ligands might overcome some of the limitations of monovalent oligosaccharides (Landers et al. 2002; Nagahori et al. 2002; Schengrund 2003; Arosio et al. 2004), but could introduce other problems, such as circulating immune complex formation.

The more attractive therapeutic application of GM1-like oligosaccharides is their use in extracorporeal immunoadsorption (Willison et al. 2004). Similar strategies have been successfully applied to blood group and other carbohydrate antigens that mediate transplant rejection, including the α-Gal epitope (Galα(1-3)Galβ(1-4)GlcNAc) attached to an inert matrix (Bannett et al. 1987; Sablinski et al. 1995; Taniguchi et al. 1996; Lambrits et al. 1998; Xu et al. 1998). These anti-α-Gal IgG antibodies have similar affinities to the mouse anti-GM1 antibody used in the immunoadsorption studies described here (KD approximately 10^{-6}–10^{-7} M) (Lee et al. 1998). Furthermore, since many neuropathy-associated anti-GM1 antibodies are IgM, there would be an additional gain in binding due to avidity seen with multivalently displayed antigen.

We were surprised that the GM1 pentasaccharide was a greatly superior ligand in both solution inhibition and immunoadsorption studies compared with the Galβ(1-3)GalNAc disaccharide, especially for antibodies that bind GM1, GA1, and GD1b, as is the case with many human sera and with the mAb, DG2. One caveat of the immunoadsorption matrices used here is that in GM1–Sepharose, the Galβ(1-3)GalNAc epitope is supported by the Gal–Glc core that would function as a spacer. Since the spacer is lacking in Galβ(1-3)
GalNAc–Sepharose, this could potentially sterically inhibit DG2 from engaging its epitope. Epitope density is also an important factor for affinity matrices: although the Galβ(1-3) GalNAc–Sepharose has up to 6-fold lower molar content of epitope than the GM1–Sepharose, this in itself is unlikely to fully account for the superiority of the latter. From a practical perspective, GM1–Sepharose columns successfully bound and removed at least a proportion of anti-GM1 antibodies from the sera of all six patients tested, suggesting that a therapeutic column incorporating the whole GM1 oligosaccharide should be effective for most anti-GM1 antibodies. It is possible that sera containing complex populations of multiple antibodies with different specificity and affinity for GM1-like oligosaccharides might bind differentially to column matrices and there might be situations in which complete clearance cannot be achieved. We have already previously that the binding of serum anti-ganglioside antibodies may be weaker at 37°C compared with 4°C (Willison and Veitch 1994). If necessary, it would be straightforward to include a phase into a therapeutic procedure where the patients’ sera is cooled prior to immunoadsorption, and then rewarmed.

The ability to isolate and analyze anti-GM1 antibodies from patients’ serum by immunoaffinity purification using GM1–Sepharose offers an important opportunity to examine their hitherto unknown properties in detail. Thus, an unexpected finding arising from this study was the observation that anti-GM1 IgG and IgM antibodies can be monoclonal or oligoclonal, either when arising as an acute immune response to infections that precede GBS, including Campylobacter jejuni, or when occurring chronically as long-lived IgM antibodies in chronic neuropathies, even in the absence of a detectable serum IgM paraprotein.

Materials and methods

Purification and characterization of murine anti-ganglioside IgG

Two anti-GM1 mAbs (DG1 and DG2) were cloned from GalNAc transferase knockout mice immunized with C. jejuni HS19 (GM1(–)GT1α(–)) lipopolysaccharide [OH4384 isolate; Aspinall et al. (1994)], which bears a GM1-like oligosaccharide (DG1) or GM1 liposomes (DG2), respectively, using previously described immunization and hybridoma techniques (Goodyear et al. 1999; Bowes et al. 2002). MAbS were purified from tissue culture supernatants using HiTrap Protein A (DG2, IgG3) or Protein G (DG1, IgG2b) affinity columns (Amersham Pharmacia Biotech, UK), dia lyzed against PBS, pH 7.2, and quantitated using standard ELISA and polyacrylamide gel electrophoresis techniques. DG2 Fabs were prepared from purified IgG using an ImmunoPure Fab preparation kit (Pierce, Rockford, IL), and Protein A chromatography. The kinetics of DG2 Fab interaction with GA1 was measured by SPR using a Biacore 2000 (Biacore AB, Uppsala, Sweden), following a previously described protocol (Boffey et al. 2005). Hybridoma VH and VL genes were cloned into pGEM-T Easy vector as previously described (Boffey et al. 2004). Sequences were analyzed using Genejockey and numbering is from IMGT, http://imgt.cines.fr (Lefranc 2003).

Human serum samples

Human sera from acute and chronic neuropathy cases were studied subject to local ethical committee approval for use of patient material. Six anti-GM1 patient samples (P1–P6) were selected that tested positive for IgM (3) or IgG (3) anti-GM1 antibodies, at titres ranging from 1/2500 to 1/12 500 (Table I) by ELISA, performed as previously described (Willison and O’Hanlon 1999). Two patients (P1, P2) had typical acute motor axonal forms of GBS, one patient (P3) had a chronic motor axonal neuropathy (CMAN) (Gorson et al. 1999), and three patients (P4–P6) had the typical features of multifocal motor neuropathy.

Natural and synthetic oligosaccharide structures

Oligosaccharide structures were sourced commercially (Neu5Ac and Gal, Sigma, Poole, Dorset, UK; β-GalNAc-(1-3)-β-Gal-, Dextra Laboratories, UK) or produced in house. The oligosaccharide of GM1 (GM1-OS) was prepared by ozonolysis of GM1 ganglioside followed by alkaline fragmentation (Wiegangt and Bucking 1970). Structural characterization was performed using nuclear magnetic resonance spectroscopy. This oligosaccharide and the oligosaccharide of GM2 were also produced metabolically in engineered E. coli (EGM1-OS and EGM2-OS, respectively) (Antoine et al. 2003). The structures of the synthetic GM1 mimics—1, 2, 3 and 4 (Galβ(1–3)GalNAc, methyl glycoside), which were synthesized during the design of binding partners for cholera toxin (Bernardi et al. 2000; Arosio et al. 2004)—are shown in Figure 1. These pseudo-GM1 compounds contain a nonsugar scaffold (cyclohexanediol) and lack the sialic acid moiety, but retain the carboxyl group in the correct orientation for cholera toxin binding. Structures 1 and 3 have the sialic acid replaced by a hydroxyacid mimic—with a methyl group (lactic acid) and a cyclohexylmethylene group, respectively. Structure 2 is a dimer of 1 connected by a dendrimeric core based on the 3,5-di(2-aminoethoxy)benzoic acid repeating unit.

Oligosaccharide inhibition ELISA

Inhibition of mouse mAbs and human antisera binding to GM1 and GA1 by natural and synthetic oligosaccharides was investigated by ELISA. MAbs or antisera were diluted in 0.1% (w/v) bovine serum albumin (BSA) in PBS and used at concentrations/dilutions equivalent to half-maximal binding, as determined by titration in ELISA. Stock solutions of oligosaccharides were prepared in distilled water to 5–10 mg/mL and further diluted in 0.1% (w/v) BSA in PBS. Equal volumes of mAb or antisera and oligosaccharide were mixed prior to application to a standard ganglioside ELISA (50 μL/well). Percentage inhibition was calculated by comparison to the binding of mAb/antisera alone (100%). All assays were performed in duplicate and repeated at least 3 times.

Ozonolysis and conjugation of ganglioside oligosaccharide to Sepharose

Ozone was generated from oxygen using a bench top generator (Model OL80W, Ozone Services, Burton, BC, Canada) and ozonolysis performed as previously described to produce ganglioside oxidized at the carbon 4–5 double bond of sphingosine (Wiegangt and Bucking 1970). The reaction was

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monitored by thin-layer chromatography. The aldehyde group was then covalently linked to Sepharose (EH Sepharose 4 Fast Flow resin, Pharmacia Biotech, UK) by reductive amination in the presence of sodium cyanoborohydride (Sigma, UK). Briefly, ozonolyzed GM1 or GD1a was suspended in deionized water (dH2O), pH 4.5, and Sepharose added to yield 3 μmol of GM1 oligosaccharide/mL of Sepharose, assuming full incorporation. Sodium cyanoborohydride (0.1 M), prepared in dH2O, pH 4.5, was then added and mixed for 72 h. The oligosaccharide–Sepharose matrix was finally washed in 3 cycles each of low pH (0.1 M acetate/0.5 M NaCl, pH 4.0) then high pH (0.1 M Tris–HCl/0.5 M NaCl, pH 8.0) buffers, followed by 1 cycle of dH2O and 1 cycle of PBS. The terminal trisaccharide of GM2 conjugated to Sepharose (TGM2–Sepharose) was prepared as previously described (Andersen et al. 2004). The terminal disaccharide of GM1, Galβ(1-3)GalNAc, conjugated to Sepharose (0.6 μmol/mL Sepharose) was purchased from Lectinity (Moscow, Russia).

Oligosaccharide–Sepharose immunoadsorption columns

Oligosaccharide–Sepharose conjugates (1 mL) in glass columns were equilibrated with 50 mL of PBS. MAb (100 μg in 1 mL PBS) or sera (neat, 1 mL) were applied to the column, and allowed to flow through. The first 1 mL of flow-through (bed volume, comprising mainly PBS) was discarded to prevent a dilution effect. The next 1 mL of flow-through was cycled 5 times through the column, and then left at 4 °C overnight. The column was then washed with PBS and collected in 1 mL fractions. Bound mAb–antisera were eluted in 1 mL fractions of 0.1 M glycine–NaOH, pH 10.0, 8.0, 6.0, or 4.0, containing 1 M NaCl. The columns were regenerated immediately afterwards with 3 cycles of 2–3 mL of alternating high pH (0.1 M Tris–HCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffers, and then equilibrated in PBS.

IEF and Western blotting of immunoadsorption column fractions

Eluted fractions from patients’ sera separated by the GM1–Sepharose column were pooled and concentrated to approximately 100 μL. IEF of the immunoglobulins was performed using 0.5 mm agarose gels with a pH gradient composed of 0.25 mL of Pharmalyte 5-8 and 1 mL of Pharmalyte 3-10 (Pharmacia Diagnostics, Uppsala, Sweden) using standard apparatus and reagents, followed by transfer to nitrocellulose and Western blotting as previously described (Willison et al. 1994). IgMs were analyzed both in native form and after reduction with 1% 2-mercaptoethanol for 30 min before focusing in order to optimize resolution.

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Conflict of interest statement

None declared.

Abbreviations

BSA, bovine serum albumin; Cer, ceramide; EGM1-OS, E. coli produced GM1 oligosaccharide; EGM2-OS, the oligosaccharide of GM2 produced by metabolically engineered E. coli; ELISA, enzyme-linked immunosorbent assay; Fab, antibody-binding fragment; Gal, galactose; GalNAc, N-acetylgalactosamine; GBS, Guillain-Barre syndrome; Glc, glucose; GlcNAc, N-acetylglucosamine; GM1, II′Neu5Ac GgOse4Cer, β-Gal-(1-3)-β-GalNAc-(1-4)[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1-OS, GgOse4, β-Gal-(1-3)-β-GalNAc-(1-4)[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-Glc; IEF, isoelectric focusing; IgG, immunoglobulin G; IgM, immunoglobulin M; kₐ, association rate constant; Kᵤ, dissociation equilibrium constant; k₅, dissociation rate constant; mAbs, monoclonal antibodies; Neu5Ac, N-acetylmuramic acid; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; VH, immunoglobulin heavy chain variable region; VL, immunoglobulin light chain variable region.

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