Synthetic disialylgalactose immunoadsorbents deplete anti-GQ1b antibodies from autoimmune neuropathy sera

Hugh J. Willison,1 Kate Townson,1,2 Jean Veitch,1 Judith Boffey,1 Neil Isaacs,2 Soren M. Andersen,3 Ping Zhang,3 Chang-Chun Ling3 and David R. Bundle3

1Clinical Neurosciences Division, University of Glasgow
Department of Neurology, Southern General Hospital, and
2Department of Chemistry, University of Glasgow,
Glasgow, Scotland, and 3Department of Chemistry,
University of Alberta, Edmonton, Alberta, Canada

Summary
Acute and chronic autoimmune neuropathies, including Guillain-Barre syndromes (GBS) are often characterized by the presence of autoantibodies that react with neural gangliosides. Evidence from human and animal studies indicates that anti-ganglioside antibodies play a primary neuropathogenic role, and their rapid elimination from the circulation through specific immunoadsorption therapy thus has the potential to ameliorate the course of the disease. Here we have tested this therapeutic principle in the Miller Fisher variant of GBS that is associated serologically with acute phase anti-GQ1b ganglioside immunoglobulin G (IgG) antibodies, and in chronic ataxic neuropathies associated with persistently elevated immunoglobulin M (IgM) antibodies that react with GQ1b, GD3 and other disialylated gangliosides. Human and mouse anti-GQ1b IgG and IgM antibodies may also react with GD3, suggesting the shared terminal disialoside epitope could be involved in antibody binding. We thus synthesized the terminal trisaccharide, NeuAc(α2-8)NeuAc(α2-3)Gal common to GQ1b and GD3, and conjugated it to bovine serum albumin (BSA). This disialylgalactose glycoconjugate (DSG-BSA) binds anti-GQ1b antibodies in 32/58 (55%) human sera containing IgG or IgM anti-GQ1b antibodies at titres up to 1/130 000; it also binds a wide range of mouse monoclonal anti-GQ1b and -GD3 antibodies. When conjugated to Sepharose as mock therapeutic immunoaffinity columns, the immobilized trisaccharide (DSG-Sepharose) eliminates anti-GQ1b antibodies from positive sera in proportion to their level of binding to DSG-BSA. Oligosaccharide-specific immunoadsorption therapy thus provides a new therapeutic approach to anti-GQ1b antibody-associated syndromes that could be applied to clinical practice. Furthermore, modification of the immobilized oligosaccharide epitopes to incorporate other glycan structures may allow this approach to be adapted to other forms of autoimmune neuropathy associated with uniform anti-glycolipid antibody profiles.

Keywords: peripheral neuropathy; gangliosides; autoantibodies; immunoadsorption; therapy

Abbreviations: AMAN = acute motor axonal neuropathy; BSA = bovine serum albumin; DSG = disialylgalactose; DSL = disialyllactose; DS = disialic acid; ELISA = enzyme-linked immunoborbert assay; GBS = Guillain-Barre syndrome; IgG = immunoglobulin G; IgM = immunoglobulin M; IVIg = intravenous immunoglobulin; MFS = Miller Fisher syndrome; mAb = monoclonal antibody; PBS = phosphate-buffered saline; OND = other neurological disease; TGM2 = terminal trisaccharide of GM2.


Introduction
Guillain-Barre syndromes (GBSs) are the foremost cause of neuromuscular paralysis worldwide with a global incidence of ~1.5 in 100 000 (Hahn, 1998; Hughes et al., 1999). Onset can be rapid, leading to total paralysis within 48 h, or can evolve over several weeks. The Miller Fisher syndrome (MFS) variant of GBS has been widely studied because it
represents a well characterized clinical and serological phenotype, being associated with immunoglobulin G (IgG) antibodies to GQ1b and structurally related gangliosides in > 95% of cases (Chiba et al., 1992, 1993; Willison and O’Hanlon, 1999). These phenotypes, which comprise acute cranial and bulbar paralyses with ataxia in varying clinical combinations, have been referred to as ‘anti-GQ1b syndromes’ (Yuki, 1998; Willison and Yuki, 2002). The very close association of these syndromes with elevated serum anti-GQ1b antibodies and the absence of anti-GQ1b antibodies in control groups, along with a wealth of experimental evidence, strongly suggests that anti-GQ1b and related antibodies are the primary pathogenic mediators of the diseases with which they are associated (Willison and O’Hanlon, 1999; Willison and Yuki, 2002).

The motor axonal form of GBS termed acute motor axonal neuropathy (AMAN) can result in long-term disability because of permanent axonal transaction arising from axolemmal targeting of the immune attack (McKhann et al., 1991; Hafer-Macko et al., 1996; Ho et al., 1997). As for MFS, AMAN is also highly associated with IgG antibodies to a relatively restricted group of gangliosides including GM1, GD1a, GM1b and GalNac-GD1a (Yuki et al., 1990, 1996; Kusunoki et al., 1994; Kusunoki, 2000). The demyelinating form of GBS is less consistently associated with anti-glycolipid antibodies and the range of glycolipid specificities is wider (Willison and Yuki, 2002). The therapeutic window for GBS is considered to be short, and initiation of treatment within 14 days from onset is recommended, although delayed treatment up to 1 month from onset may also be effective. The current optimal treatment with intravenous immunoglobulin (IVlg) or plasma exchange only reduces illness severity by half (Hughes, 2002). Patients left severely disabled as a result of permanent axonal injury represent a major social and economic burden.

Chronic peripheral neuropathies also exist with similar autoantibodies but of the immunoglobulin M (IgM) class, including multifocal motor neuropathy with anti-GM1 antibodies, and chronic axitic neuropathy with monoclonal IgM anti-disialoside antibodies (Pestronk et al., 1988; Willison et al., 2001). Anti-disialoside antibodies most usually react with GQ1b, GT1a, GD3 and other gangliosides bearing the same disialic acid (DS) epitope. Treatment of these chronic cases is unsatisfactory, requiring chronic long-term therapy that is currently most often administered in the form of IVlg. There is a need to develop more effective, targeted immunotherapy for anti-ganglioside antibody associated GBS and chronic neuropathies. Plasma exchange removes total plasma proteins and immunoglobulins from the serum, but is non-selective and can only be applied for a limited duration without causing depletion of vital plasma components (Yang et al., 2002). IVlg has limited availability, high cost and potentially serious long-term risks.

In this study, we have considered the possibility that oligosaccharide-specific immunoadsorption therapy could be applied to GBS. Such a therapy would remove only the specific anti-glycolipid antibodies and allow all other blood components to be returned to the patient. Similar strategies have been previously and successfully applied to blood group and carbohydrate antigens that mediate transplant rejection (Bennett et al., 1987; Taniguchi et al., 1996; Lambrigts et al., 1998). We chose to examine this principle in the anti-GQ1b antibody associated syndromes because of their relative serological homogeneity and the availability of many IgG and IgM containing human sera from these cases through our clinical laboratory. We also had available large numbers of recently cloned mouse monoclonal anti-GQ1b antibodies (mAbs) with which to conduct detailed epitope mapping studies on candidate saccharides. First, we estimated and synthesized the predicted minimal epitope involved in anti-GQ1b antibody-antigen interactions as distalalagalactose (DSG), and then demonstrated that it bound a high proportion of anti-GQ1b positive sera and mAbs. Finally, we examined the capacity of DSG to deplete serum of anti-GQ1b antibodies when conjugated to Sepharose in immunoaffinity columns.

Material and methods

**Human serum samples**

Human sera from neuropathy cases were tested for anti-ganglioside antibodies in the Glasgow Neuroimmunology Laboratory. Positive anti-GQ1b IgG \( (n = 48) \) or IgM \( (n = 10) \) sera were identified and selected at random for further study under Southern General Hospital Ethics Committee guidelines, subject to linked anonymization. Clinical details beyond the referring diagnosis, typically described as MFS, GBS or a chronic neuropathy were not sought. These samples thus represent anti-GQ1b-antibody containing sera that on the basis of extensive clinical serological studies are invariably associated with acute MFS or chronic axitic neuropathy syndromes and their formes frustes (Yuki, 1998; Willison and Yuki, 2002). Routine immunoassays were conducted according to previously published methods (Willison et al., 1999). For this study, sera were included when the anti-GQ1b antibody titre was \( >1:1000 \) by end-point dilution analysis. Two control groups of samples were used. One group \( (n = 24) \) comprised serum samples from cases of suspected multiple sclerosis in whom CSF oligoclonal bands were detected. The ‘other neurological disease’ (OND) control group \( (n = 30) \) comprised patients with neurological symptoms whose serum and CSF were referred for oligoclonal band testing in whom the result was negative, and myasthenia gravis patients \( (n = 6) \) who were positive for serum acetylcholine receptor antibodies. Serum from the control groups \( (n = 54) \) did not contain any anti-GQ1b antibodies, consistent with the known very high specificity and sensitivity of the anti-GQ1b antibody test for MFS and related syndromes. Six sera containing anti-GQ1b IgG antibodies (G1–G6), two sera containing anti-disialoside (including GD3/GQ1b) IgM antibodies (M1, M2) and two sera containing anti-GM1 IgG antibodies (GM1-G1, GM1-G2), from whom sufficient sample volumes \( (>5 \text{ ml}) \) were available were selected for more detailed studies. By way of control for the authenticity of the conjugate of the terminal trisaccharide of GM2 (TG, M2, see below) with bovine serum albumin (BSA), serum samples from two cases of chronic motor neuropathy associated with anti-GM2, anti-GalNAc-GD1a and anti-GalNAc-GM1b IgM antibodies were studied (O’Hanlon et al., 2000).
Mouse mAbs

Mouse mAbs were cloned from ganglioside or Campylobacter jejuni lipopolysaccharide immunized strains of adult C3H/HeN, C57 black or GalNAc transferase knock-out mice, using previously described immunization and hybridoma techniques under a UK Home Office licence (Goodyear et al., 1999; Bowes et al., 2002). Antibody binding characteristics were calculated as half maximal binding values as previously described (Goodyear et al., 1999). Antibodies were characterized for isotype, subclass, and antibody concentrations and quantitated using standard enzyme-linked immunosorbent assay (ELISA) based techniques. Purification of mAbs from tissue culture supernatants was performed using HiTrap Protein A affinity columns (Amersham Pharmacia Biotech, UK) according to the manufacturer’s instructions. The anti-GD3 mAb, R24, was cultured and purified as above (Dippold et al., 1980). Following affinity chromatography, protein content was assayed by adsorption at 280 nm; A280/1.43 was used to calculate the IgG concentration (in mg/ml) and the purity was further checked by gel electrophoresis and mouse IgG subclass specific ELISAs.

Preparation of synthetic saccharides, BSA and Sepharose conjugates

Oligosaccharide structures are shown in Figs 1 and 2. Chemical synthesis was performed to prepare oligosaccharides 1–4, 6 and 7 (Fig. 2; S. Andersen, C.C. Ling, P. Zhang, K. Townsend, H.J. Willison, D.R. Bundle, unpublished results). Briefly, the three epiteopes NeuNAc(α2–8)NeuNAc(α2–3)Gal(β1–1)DSG, NeuNAc(α2–8)NeuNAc(α2–3)Gal(β1–1)DSL and NeuNAc(α2–3)GalNAc(β1–4)Gal(β1–1)DSL  were synthesized as the glycosides 1–3 of an 11 carbon tether. The terminal amino group of the tether was used to conjugate the ligands to BSA and NHS-activated Sepharose (Pharmacia) as illustrated for DSG in Fig. 3. An identical procedure was used to conjugate TGM2 and DSL to BSA and Sepharose. The degree of hapten incorporation on BSA was as follows: DSG–BSA, 6; DSL–BSA, 8; TGM2–BSA, 13. When trisaccharides 1 and 3 were coupled to NHS-activated Sepharose, hapten loadings of 0.65 mmol/ml and 0.89 mmol/ml of dry gel, respectively, were achieved. Oligosaccharides were also synthesized as methyl glycosides, NeuNAc(α2–8)NeuNAc(α2–3)Gal[β1–1]OCH3 (4, DSG), NeuNAc(α2–3)[GalNAc(β1–4)]Gal[β1–1]OCH3 (5, TGM2), and NeuNAc(α2–8)NeuNact2-OCH3 (7, DS). Galactose, sialic acid and DSL (5) were also purchased (Sigma, UK) for use in inhibition studies.

Ganglioside and oligosaccharide-BSA ELISAs

Binding of the mAbs to gangliosides and BSA-conjugates was evaluated by ELISA (Willison et al., 1999). Antigens were immobilized onto 96-well Immulon 2 HB microtiter plates (Dynatech, UK). Glycoconjugate-BSA coated plates were prepared at 10 μg/ml of BSA in bicarbonate buffer (100 μl per well), incubated overnight at 4°C and washed. Ganglioside structures with corresponding DSG, DSL and DS epitopes are shown in Fig. 4. GM1, GD1b, GT1b, GD3, GQ1b, GM2 (Sigma) dissolved in methanol (2 μg/ml) were added to wells (100 μl per well) and evaporated to dryness, then incubated at 4°C for a minimum of 1 h. Non-specific binding was blocked by incubating with 150 μl per well of 2% (w/v) BSA in phosphate-buffered saline (PBS) for 1 h at 4°C. The BSA was discarded, and serial dilutions of human sera or mAbs [in 0.1% (w/v) BSA in PBS] were added and incubated for 3 h (mAbs) or overnight (human sera) at 4°C, then washed in PBS (without detergent). Horseradish peroxidase conjugated anti-human or anti-mouse IgG or IgM (Sigma) diluted 1:3000 (anti-human) or 1:2000 (anti-mouse) in 0.1% (w/v) BSA in PBS was added and incubated for 1 h at 4°C. Plates were washed, and developed for 15 min at room temperature, then stopped with 4 M sulphuric acid. The optical density (OD) at 410 nm was determined, and the background OD from binding to antigen-free control wells (10 μg/ml BSA) was subtracted. Samples were always studied in duplicate and the mean OD calculated. Statistical analyses were conducted on three separately repeated experiments.

Competitive inhibition studies using free saccharides and solid phase ganglioside antigens

The inhibitory properties of unconjugated mono, di, tri and tetrasaccharides (structures are shown in Fig. 2) sharing structural

Fig. 1 The oligosaccharide components of gangliosides GQ1b, GD3 and GM2 as condensed structures. The relevant terminal disialosyl epitopes contained within each ganglioside are identified as DSG, DSL and DS.
components with gangliosides (Fig. 1) were investigated by inhibition ELISA, with the inhibitor in the liquid phase and the ganglioside in solid phase. The ELISA procedure described above was followed with additional steps. The half maximal concentration of purified mAb was identified from the standard binding curve to the appropriate ganglioside, and diluted in 0.1% (w/v) BSA in PBS. Sialic acid (NeuNAc, Sigma), disialoside (DS, methyl glycoside), galactose (Sigma), DSL (Sigma) and DSG were dissolved in deionized water to 10 mg/ml, and a titration series (10⁻⁶–10⁻³M) prepared by diluting in 0.1% (w/v) BSA in PBS. After blocking the ganglioside-coated ELISA plates, equal volumes of mAb and oligosaccharide were mixed and immediately added to the plates (50 μl per well). Inhibition of mAb binding to the appropriate ganglioside(s) was expressed as a percentage reduction in comparison to the binding of mAb in the absence of oligosaccharide. All assays were performed in duplicate and repeated at least three times for statistical analysis. The same procedure was applied to human serum G3, the half maximal titre being 1/400.

**DSG-Sepharose immunoadsorption columns**

DSG-Sepharose and TGM2-Sepharose (Sepharose 4 Fast Flow resin, Pharmacia Biotech) were prepared as described above. Sepharose
conjugate (1 ml) was added to a 10 ml glass column and equilibrated with 50 ml PBS, pH 7.2. After equilibration, the buffer was drained to the top of the resin and the column stopped. For human serum samples, 2 ml neat serum was added and the column allowed to flow. The first 1 ml of flow through, comprising the PBS in the bed volume intermixed with serum, was discarded to avoid any bias in subsequent analysis introduced by a dilution effect. Serum was recycled 4× through the column in 0.5 ml fractions over 10 min. After the 4th pass, 0.5 ml PBS was added and the first 1 ml eluate, comprising neat serum, was set aside (unbound fraction). The column was then washed with 30 ml PBS, which was collected in 1 ml fractions and monitored for anti-GQ1b antibody activity. This anti-GQ1b activity in the wash was undetectable by the 10th fraction. The bound material was eluted in 10×1 ml fractions with 0.1 M glycine/NaOH buffer, pH 10, containing 0.15 M NaCl and immediately neutralized with 0.1 M glycine/HCl, pH 1.5. The 10 eluted fractions were then assayed by titration analysis, starting with neat eluates, for anti-GQ1b antibody activity alongside the starting material and the flow through fractions on a single ELISA plate for each column in order to control for any inter-assay variability. The results presented represent the fraction containing the highest anti-GQ1b titre; this was always present in either the 2nd or 3rd fraction. All detectable antibody had eluted from the column by the 10th fraction. The column was then regenerated with 30 ml 0.05 M Na2HPO4 buffer, pH 12, containing 0.15 M NaCl, then washed with 50 ml of PBS and stored at 4°C until re-used. A single column was used for all the analyses and the binding capacity of the column was checked periodically by re-running a positive serum, G3 (see below); it was found to be constant throughout the study. The negative control column, comprising NeuNac(α2-3)GalNAc(β1–4)Gal(β1-Sepharose (TGM2-Sepharose), which was not expected to bind anti-GQ1b or -GD3 antibodies, was run using the same assay procedures. Plate-to-plate variations in ELISA plate performance and reagents account for any variation of starting titres observed in the analyses of individual column runs (Willison et al., 1999).

Six anti-GQ1b IgG samples, termed (G1–G6) and two anti-disialosyl IgM samples (M1, M2) were studied. Column fractions for G1–G6 and M1–M2 were monitored on GQ1b and GT1b coated ELISA plates, respectively. By way of control, one of each (G1 and M1) was studied on the TGM2-Sepharose column. As a further control, two anti-GM1 IgG containing sera were applied to the DSG-Sepharose column, with fractions monitored on GM1-coated ELISA plates.

The anti-GQ1b mAb, EG3 was also studied on both the DSG-Sepharose and TGM2-Sepharose columns. EG3 (1 mg) was applied to the column in 1 ml PBS, pH 7.4, and passed through the column 8× over 15 min, washed as above and eluted in 0.1 M glycine/NaOH buffer, pH 10, containing 1 M NaCl and immediately neutralized with 0.1 M glycine/HCl, pH 1.5, as above. Conditions were otherwise as described above.
Statistics
Quantitative ELISA data are expressed as the mean ± SEM.

Results
Binding of anti-GQ1b IgG antibody positive human sera to DSG-BSA conjugate and GD3 ganglioside
We first analysed by ELISA the binding of control sera and anti-GQ1b IgG antibody containing sera to the synthetic DSG-BSA conjugate. All sera in the two control groups (n = 54) were negative, with undetectable titres (<1/100) to DSG-BSA. In the screen of anti-GQ1b IgG antibody positive sera, 26 out of 48 (54%) bound at titres >1/100 to DSG-BSA, in a few cases at titres up to 1/100 000 (Fig. 4). There was no correlation between the anti-DSG and anti-GQ1b titres: thus the anti-DSG negative sera ranged in anti-GQ1b titres from 1/1000–1/100 000, and the anti-DSG positive sera similarly exhibited a broad range of titres that were independent of the anti-GQ1b antibody titre.

Anti-GQ1b positive sera were also screened for reactivity with the structurally related ganglioside GD3, since it contains the same terminal trisaccharide as DSG (Fig. 1). Twenty-one out of 48 (44%) anti-GQ1b positive sera bound detectably (titres >1/100) to GD3, with nine out of 48 (19%) sera at GD3 titres >1/1000. No correlative relationship was observed between the GD3 and GQ1b titres, or between the GD3 and DSG-BSA titres (data not shown). Thus, although occasional anti-GQ1b antibody containing sera were very strongly positive (titres >1/10 000) for GQ1b, GD3 and DSG-BSA, such an association was not observed for the majority of sera.
Binding of anti-GQ1b IgM antibody positive human sera to DSG-BSA conjugate

Ten sera containing anti-disialosyl antibodies from patients with chronic ataxic neuropathies, also termed CANOMAD (chronic ataxic neuropathy, ophthalmoplegia, M protein, agglutinins and disialosyl antibodies) were selected for assaying DSG-BSA binding from a previous study (Willison et al., 2001). These sera contain IgM paraproteins that are very broadly reactive with disialylated gangliosides, including GQ1b, GD3, GT1b and GD1b. All 10 reacted with GQ1b at titres ranging from 1/900 to 1/170 000, and nine out of 10 reacted with GD3 at titres ranging from 1/500 to 1/150 000. When screened for anti-DSG-BSA reactivity, six (60%) were positive at titres ranging from 1/450 to 1/180 000 (Fig. 4). As was the case for anti-GQ1b IgG antibodies, there were no apparent differences between the anti-DSG-BSA positive and anti-DSG-BSA negative sera with respect to their ganglioside reactivity profiles.

Binding of mouse anti-GQ1b/GD3 mAbs to DSG-BSA and DSL-BSA conjugates

The human sera studied contain complex mixtures of polyclonal anti-GQ1b antibodies with variable affinities for GQ1b and GD3, which might confound the interpretation of the binding studies described above. To more fully explore the discrete relationships between mAb binding to GD3, GQ1b and DSG-BSA, we selected 17 IgG and IgM antibodies from a panel of anti-disialylated ganglioside mAbs that varied in their specificity for disialylated gangliosides, ranging from mAbs that were either GD3 or GQ1b mono-reactive, to intermediate binders that reacted either preferentially or equally with both GD3 and GQ1b gangliosides (Fig. 5, upper panel). Three antibodies (EG2, EM4, EM9) were also included which are reactive with disialoside epitopes on the internal galactose (and not the external), and thus reacted with GQ1b, GT1b and GD1b, but not GD3: these we predicted should not bind to DSG-BSA. In addition to anti-disialoside mAbs cloned in-house, the commercially available mAb, R24, was included since it is a very widely studied anti-GD3/GQ1b antibody that has been developed for human cancer therapy (Dippold et al., 1980). These 17 antibodies were screened for reactivity with DSG-BSA (Fig. 5, lower panel). All categories of mAbs bound DSG-BSA with the exception of the three mAbs (EG2, EM4 and EM9) that are specific for internal disialoside epitopes. The general principle that GQ1b mono-specific mAbs are able to bind DSG-BSA, even when the same antibodies do not bind GD3, was thus demonstrated. By way of a control, none of the 17 mAbs bound TGM2-BSA in ELISA, with the exception of weak binding by EM4 (as expected from its half maximal value for GM2).
Four mAbs (CGG2, GD3 monoreactive; R24, GD3>GQ1b; EG1, GQ1b> GD3; EG3, GQ1b monoreactive) were selected to investigate the comparative level of binding to DSG-BSA, DSL-BSA and GQ1b/GD3 (Fig. 6). The titration curves show that these four mAbs bind DSG-BSA as efficiently as their known ganglioside epitopes over biologically relevant concentration ranges.

With respect to DSL-BSA binding, the GD3 mono-specific mAb, CGG2, exhibited the same half maximal binding as DSG-BSA. Both bi-specific GQ1b/GD3 mAbs, EG1 and R24, exhibited 10× lower half-max binding to DSL-BSA compared with DSG-BSA. The GQ1b monospecific mAb, EG3, did not bind DSL-BSA at all, despite binding well to DSG-BSA. These data suggest that the internal glucose residue common to DSL and GD3 does not favour GQ1b antibody binding, but does not interfere with GD3 antibody binding, as might be predicted from the known structures shown in Fig. 1.

**Identification of the minimal saccharide epitope required for anti-GQ1b/GD3 antibody binding**

The above studies demonstrated that all mouse anti-GQ1b and -GD3 mAbs and a significant proportion (~50%) of anti-GQ1b antibody containing human sera are efficient binders of

<table>
<thead>
<tr>
<th>mAb/sera</th>
<th>Half max [mAb]</th>
<th>Molar ratio (mAb : OS) at 1mM oligosaccharide</th>
<th>Inhibition of binding (%) at 1mM oligosaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGG2</td>
<td>17 µg/ml</td>
<td>1 : 10000</td>
<td>DSL: 0, DSG: 0, DS: 0, NeuNAc: 0</td>
</tr>
<tr>
<td>EG1</td>
<td>3 µg/ml</td>
<td>1 : 50000</td>
<td>20.3, 0, 0, 0</td>
</tr>
<tr>
<td>EG2</td>
<td>3 µg/ml</td>
<td>1 : 50000</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>EG3</td>
<td>10 µg/ml</td>
<td>1 : 15000</td>
<td>59.4, 90, 0, 0</td>
</tr>
<tr>
<td>R24</td>
<td>15 µg/ml</td>
<td>1 : 10000</td>
<td>62.1, 40.9, 0, 0</td>
</tr>
<tr>
<td>Serum G3</td>
<td>1/400 (dilution)</td>
<td>-</td>
<td>- 66.5, 22.5, 6.96</td>
</tr>
</tbody>
</table>

Fig. 7 **Upper panel**: Inhibition curves of three mAbs and one human anti-GQ1b positive sample (serum G3) with the soluble oligosaccharides, galactose, sialic acid, DS, DSG and DSL. The mAb/serum and the solid phase ganglioside antigen (GD3 or GQ1b) are indicated for each panel. (Error bars: mean ± SEM; n = 3 experiments). **Lower panel**: antibody characteristics and percentage inhibition at 1 mM oligosaccharide. CGG2/GD3 interaction was not inhibited by any oligosaccharide tested.
DSG-BSA, and do not require a larger saccharide epitope than NeuAc(α2–8)NeuAc(α2–3)Gal. In order to look more closely at the minimum epitope requirement for antibody binding, we conducted solution inhibition studies with a range of saccharides, comprising galactose, sialic acid (NeuAc), DS (as the methyl capped derivative that maintains an α configuration in its free form), DSG and DSL as shown in Figs 1 and 2. The five mAbs selected for the titration analysis described above (CGG2, EG1, EG2, EG3 and R24) were used at concentrations corresponding to their half maximal binding in inhibition studies over oligosaccharide concentrations ranging from $1 \times 10^{-6}$ to $1 \times 10^{-3}$ M (Fig. 7, lower panel).

As expected, the control antibody, EG2, which reacts with disialoside epitopes on the internal galactose of gangliosides and did not react with DSG-BSA, was not inhibited from binding GQ1b by any of the oligosaccharides tested (Fig. 7, lower panel). None of the mono- or disaccharides were able to inhibit any mAb binding to GD3 or GQ1b, indicating that DS alone (or any monosaccharides) is an insufficiently large or conformationally stable epitope to competitively interact with antibody in this assay.

The GQ1b monospecific antibody, EG3, was 90% inhibited from binding GQ1b by 1 mM DSG (Fig. 7, upper panel, top right). With respect to DSL, despite not binding DSL-BSA in solid phase ELISA, EG3 was 60% inhibited from binding GQ1b by 1 mM DSL in solution. This indicates that the internal glucose on DSL has less of an inhibitory effect on EG3 binding to the terminal DSG trisaccharide in solution than in solid phase. Possible explanations include the greater flexibility of glucose in solution with attendant loss of inhibitor power, or the often observed effect of lipid tether on saccharide activity (Hakomori, 1989; Lloyd et al., 1992; Lingwood, 1996).

The GD3 mono-specific mAb CGG2 could not be inhibited from binding GD3 by either DSG or DSL, despite binding well to both DSG-BSA and DSL-BSA in solid phase ELISA. This indicates a strong preference of this mAb for a fixed oligosaccharide rather than an oligosaccharide in solution. The bispecific mAb, EG1, was weakly inhibited from binding GD3 by DSL, but not from binding GQ1b, for which it has a higher half maximal binding value (Fig. 7, upper panel, top left). Similarly, R24, which binds GD3 better than GQ1b, was only inhibited by DSG and DSL from binding to its weaker ligand, GQ1b (Fig. 7, upper panel, bottom left). This indicates a hierarchy of inhibition consistent with the half maximal binding values.

One human anti-GQ1b IgG serum, G3 (titre to GQ1b, 1/9000; GD3, 1/400; DSG-BSA, 1/1200) was titrated in GQ1b ELISA to establish the dilution corresponding to half maximal binding (1/400) and then analysed for inhibition of GQ1b binding by soluble oligosaccharides in competition assays as described above (Fig. 7, upper panel, bottom right). The serum binding to GQ1b was almost 70% inhibited by DSG and also very weakly (~20%) inhibited by DS. Although we were restricted in scope by the limited availability of inhibitors and of serum samples with sufficiently steep titration curves for these studies, this demonstrates the principle that human anti-GQ1b antisera can also be inhibited by DSG oligosaccharide in solution in the same manner as the mouse mAbs described above.

**Adsorption of anti-GQ1b/GD3 antibodies on DSG-Sepharose immunoaffinity columns**

The above experiments provided the rationale for conjugating DSG to affinity column resins as part of the development of immunoabsorption therapy for anti-GQ1b antibody associated syndromes. To provide pre-clinical evidence for the validity of this approach, DSG-Sepharose columns were tested with the anti-GQ1b monospecific mAb, EG3, and with human anti-GQ1b positive sera, along with an appropriate control column and control sera.

EG3 (1 mg) was applied to the DSG-Sepharose column, followed by washing steps and elution. As a control for non-specific binding to the Sepharose column, TGM2-Sepharose was used. Both column profiles are shown in Fig. 8. EG3 clearly binds to, and can be eluted from the DSG-Sepharose
 Adsorption of human anti-GQ1b positive sera on DSG-Sepharose immunoaffinity columns

Anti-GQ1b IgG and IgM antibody positive sera were then tested for serum antibody depletion and column binding by application to both DSG-Sepharose and TGM2-Sepharose columns over a 10 min timescale; the results are shown in Table 1. Six anti-GQ1b IgG sera (G1–G6), two anti-disialosyl IgM sera (M1, M2) and two anti-GM1 IgG sera (GM1-G1, GM1-G2) were applied to DSG-Sepharose columns (all sera) or TGM2-Sepharose control columns (G1, M1). Data are displayed as anti-ganglioside antibody titres monitored by either GQ1b (G1–G6), GT1b (M1–2) or GM1 (GM1–G1–2) ELISA and measured in the starting material (START), the unbound flow through (FT) and the bound fraction that was subsequently recovered from the column by elution (ELUATE).

Table 1 DSG-Sepharose and TGM2-Sepharose affinity column data for human neuropathy sera

<table>
<thead>
<tr>
<th>Anti-GQ1b IgG</th>
<th>DSG titre</th>
<th>DSG-Sepharose column monitored on GQ1b ELISA</th>
<th>TGM2-Sepharose column monitored on GQ1b ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>START</td>
<td>FT</td>
<td>ELUATE</td>
</tr>
<tr>
<td>G1</td>
<td>70 000</td>
<td>13 000</td>
<td>450</td>
</tr>
<tr>
<td>G2</td>
<td>950</td>
<td>9 000</td>
<td>280</td>
</tr>
<tr>
<td>G3</td>
<td>1 200</td>
<td>2 400</td>
<td>680</td>
</tr>
<tr>
<td>G4</td>
<td>90</td>
<td>20 000</td>
<td>12 500</td>
</tr>
<tr>
<td>G5</td>
<td>70</td>
<td>180</td>
<td>100</td>
</tr>
<tr>
<td>G6</td>
<td>0</td>
<td>9 000</td>
<td>9 000</td>
</tr>
</tbody>
</table>

Monitored on GT1b ELISA

<table>
<thead>
<tr>
<th>Anti-GQ1b IgM</th>
<th>DSG titre</th>
<th>DSG-Sepharose column monitored on GT1b ELISA</th>
<th>TGM2-Sepharose column monitored on GT1b ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>START</td>
<td>FT</td>
<td>ELUATE</td>
</tr>
<tr>
<td>M1</td>
<td>9 600</td>
<td>28 000</td>
<td>30 000</td>
</tr>
<tr>
<td>M2</td>
<td>180 000</td>
<td>18 000</td>
<td>20 000</td>
</tr>
</tbody>
</table>

Monitored on GM1 ELISA

<table>
<thead>
<tr>
<th>Anti-GM1b IgG</th>
<th>DSG titre</th>
<th>DSG-Sepharose column monitored on GM1 ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>START</td>
<td>FT</td>
</tr>
<tr>
<td>GM1-G1</td>
<td>0</td>
<td>10 500</td>
</tr>
<tr>
<td>GM1-G2</td>
<td>0</td>
<td>1 500</td>
</tr>
</tbody>
</table>

Discussion

These data provide the first evidence that the small synthetic trisaccharide, DSG, being the terminal trisaccharide structure of QG1b and GD3, is the minimal epitope that can effectively bind to a significant proportion of human neuropathy associated anti-GQ1b antibodies. Furthermore, when conjugated to Sepharose as a solid support in an immunoabsorption column, DSG can substantially deplete serum of anti-GQ1b antibody when flowing through the column resin over very short periods of time (10 min). This supports the notion that column, but as expected, had no interaction with the TGM2-Sepharose column.
such columns, if manufactured on a larger scale, could be adapted for human therapeutic use in series with plasma exchange, a procedure in which the plasma is filtered over several hours. Alternatively, oligosaccharides could be coupled to modified dialysis filters that would obviate the requirement for plasma exchange, as has been demonstrated for removal of anti-A and anti-B blood group antigens (Hout et al., 2000). The extent to which upscaling of the immunoabsorption technique necessary to significantly lower the serum anti-GQ1b antibody titre for a patient is feasible remains to be determined.

In this series of 58 anti-GQ1b positive IgG and IgM sera, just over half bound to DSG. It is likely that further structural modification or enlargement of the DSG epitope might provide the additional features required to bind universally to anti-GQ1b antibodies. Addition of a glucose residue internal to the galactose (i.e. DSL as present on GD3) is not sufficient: our unpublished pilot studies conducted on an earlier series of GQ1b positive sera demonstrated that DSL-BSA bound a lower proportion of anti-GQ1b antisera than observed in the current study using DSG-BSA. We are currently synthesising the tetrasaccharide, NeuNAc(a2–8)NeuNAc(a2–3)Gal(b1–3)GalNAc(b1–), as comprises the terminal tetrasaccharide of GQ1b to determine whether this constitutes an improved antigen, in comparison with DSG or DSL.

Our data obtained from studies with mouse mAbs provides considerable support for the data we obtained with human sera. Because of the polyclonal nature of these human sera and the unknown amounts and affinities of specific antibody that they contain, the unlimited quantities of precisely defined mAbs we have generated offer great advantages. Thus, our mouse mAb data clearly show that monospecific anti-GQ1b mAbs (that do not bind GD3) can bind as well to DSG as GQ1b, and that this trisaccharide is the minimum size of epitope required for binding. Furthermore, for monospecific anti-GQ1b mAbs, DSG binding is in marked preference to DSL binding, and this preference becomes less significant as the specificity spreads in favour of GD3. Thus, GD3 monospecific mAbs bind DSG and DSL more equally.

The solution inhibition studies conducted with mAbs provided the important information that DSG was the minimum size of the epitope required for effective anti-GQ1b antibody binding, and supported our earlier unpublished data that DSL offered no additional advantage for anti-GQ1b antibodies. In order to conduct these inhibition studies, mAb concentrations that represent half maximal binding, occurring in the steepest part of the sigmoid shaped dilution curve, were identified. We have observed that many human sera containing anti-GQ1b antibodies exhibit rather linear decay in their dilution curves, possibly because of the polyclonal nature and variable affinities of the anti-GQ1b antibody response. Such serum samples are not suitable for conducting accurate competition studies with free oligosaccharides as described above, and we were only able to identify one such sample with a steep dilution curve (G3) which we had in sufficient quantities to conduct this study. Nevertheless, this demonstrated that the principle outlined with the mouse mAbs also applied to human anti-GQ1b antibodies.

Apart from identifying the minimum epitope requirements for antibody binding, another rationale for conducting the solution inhibition studies is that we considered the therapeutic possibility that soluble oligosaccharides delivered intravenously might block anti-GQ1b auto-antibodies, or inactivate them through immune complex formation, sequestration, or other mechanisms (Simon et al., 1998). The mouse mAb data described here, and the very limited human serum data presented, suggest that, at least in theory this might be possible. However the 10^4–10^5 fold molar excess of DSG oligosaccharide to mAb required to produce a ~50% blocking effect indicates that this is unlikely to be an effective therapy, unless an improved soluble ligand with higher affinity can be developed. One such strategy might be the use of soluble decavalent ligands tailored to match the spacing between individual arms of a pentameric IgM antibody, with each of the monomers possessing two antibody-binding sites (Kitov et al., 2000), as IgM antibody can be the most difficult to eliminate due to the low intrinsic affinity of each monovalent binding site. A potentially more attractive approach might be the attachment of such optimally spaced ligands to an affinity matrix. Soluble oligosaccharide therapy also raises substantial drug development and safety issues, which are unlikely to be as great a factor for extracorporeal immunoadsorption strategies (Simon et al., 1998). Potential safety issues that need to be considered and monitored in clinical situations include the possibility of local complement activation by column-bound antibody, and immune complex formation by antigen-antibody complexes (Deodhar et al., 2002; Pierson et al., 2002).

Anti-GQ1b antibody associated syndromes tend to be mild and self-limiting in comparison with other forms of GBS, and might not be universally viewed as important diseases to target for new therapy development. However, they provide a highly defined clinical, serological and immunopathological paradigm and thus an important platform from which to consider other anti-ganglioside antibody mediated forms of GBS, including AMAN. By synthesising alternative saccharides based on other ganglioside structures, the principles outlined here could be applied to the GM1, GD1a and GalNac-GD1a antibodies that characterize AMAN, and investigated using both newly developed murine mAbs and human sera. Indeed the GalNac[(α2–3)NeuAc][(α2–3)Gal-BSA and Sepharose conjugates, used in this study as the negative control antigen, are able to bind a proportion of anti-GM2 and anti-GalNac-GD1a antibodies, as would be predicted and we have also conducted successful pilot studies with GM1 and GD1a oligosaccharides. The very rapid removal of the transient, acute phase, complement-fixing anti-ganglioside antibodies by immunoabsorption plasma exchange described here may offer the best prospect for the development of the highly targeted immunotherapy needed in anti-GQ1b antibody syndromes and in the more severe and
disabling forms of GBS associated with anti-ganglioside antibodies.

Acknowledgements

This work was supported by grants 060349 and 065261 from the Wellcome Trust and by grant MOP 43968 from the Canadian Institutes of Health Research.

References


