Published in final edited form as:

Eur J Neurol. 2013 January ; 20(1): 62–70. doi:10.1111/j.1468-1331.2012.03767.x.

Antibodies to heteromeric glycolipid complexes in multifocal motor neuropathy

Francesc Galban-Horcajo F, M.Res.¹, Amanda M. Fitzpatrick, MBBS¹, Andrew J. Hutton, B.Sc.¹, Siobhan M. Dunn, B.Sc.¹, Gabriela Kalna, PhD², Kathryn M. Brennan, MBBS¹, Simon Rinaldi, MBBS¹, Robert K. Yu, Ph.D.³, Carl Goodyear, Ph.D¹, and Hugh J. Willison, MBBS¹

¹Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Science, Glasgow Biomedical Research Centre, University of Glasgow, Glasgow G12 8TA UK

²The Beatson Institute for Cancer Research, University of Glasgow, Glasgow, G61 1BD

³Institute of Molecular Medicine and Genetics, Georgia Health Sciences University, Augusta, GA 30912-2697

Abstract

Background—Measurement of anti-GM1 IgM antibodies in multifocal motor neuropathy (MMN) sera is confounded by relatively low sensitivity that limits clinical usefulness. Combinatorial assay methods, in which antibodies reactive to heteromeric complexes of 2 or more glycolipids are being increasingly applied to this area of diagnostic testing.

Methods—A newly developed combinatorial glycoarray able to identify antibodies to 45 different heteromeric glycolipid complexes and their 10 individual glycolipid components was applied to a randomly selected population of 33 MMN cases and 57 normal or disease controls. Comparison with an enzyme-linked immunosorbent assay (ELISA) was conducted for selected single glycolipids and their complexes.

Results—By ELISA, 22/33 MMN cases had detectable anti-GM1 IgM antibodies, whereas 19/33 MMN samples were positive for anti-GM1 antibodies by glycoarray. Analysis of variance (ANOVA) revealed that of the 55 possible single glycolipids and their 1:1 complexes, antibodies to the GM1:galactocerebroside (GM1:GalC) complex were most significantly associated with MMN, returning 33/33 MMN samples as positive by glycoarray and 29/33 positive by ELISA. Regression analysis revealed a high correlation in absolute values between ELISA and glycoarray. Receiver operator characteristic (ROC) analysis revealed insignificantly different diagnostic performance between the two methods, although at the lower end of sensitivity, the glycoarray appeared slightly advantageous by identifying antibodies in 4 ELISA-negative samples.

Conclusions—The use of combinatorial glycoarray or ELISA increased the diagnostic sensitivity of anti-glycolipid antibody testing in this cohort of MMN cases, without significantly affecting specificity, and may be a useful assay modification for routine clinical screening.

Keywords

neuropathy; autoantibody; glycolipid; ganglioside; GM1; galactocerebroside

Address for correspondence: Professor Hugh J. Willison, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Science, Glasgow Biomedical Research Centre, Room B330, 120 University Place, Glasgow, G12 8TA, Scotland. Tel: 44 (0) 141 330 8384; Fax 44 141 201 2993; hugh.willison@glasgow.ac.uk.

Introduction

Antibodies to GM1 ganglioside were first identified in multifocal motor neuropathy (MMN) sera by Pestronk and colleagues almost 25 years ago (Pestronk et al., 1988). Since then, extensive studies have examined the sensitivity and specificity of anti-GM1 IgM antibody detection in MMN, related disorders and control populations (Adams et al., 1991; Kornberg et al., 1994; Lewis et al., 1982; Parry and Sumner, 1992; Pestronk, 1991), using a range of assay methodologies (Alaadini and Latov, 2000; Bech et al., 1994; Carpo et al., 1999; Chabraoui et al., 1993; Conrad et al., 2007; Escande-Beillard et al., 2002; Pukin et al., 2011; Ravindranath et al., 1994; Willison et al., 1999). Although no uniform consensus on methodology has been achieved, in part due to differences in defining patient populations and assay reproducibility, it is widely accepted that IgM antibodies to GM1 do occur in a significantly higher proportion of MMN cases compared with control groups (Baumann et al., 1998; Nobile-Orazio et al., 2005). The clinical utility of antibody testing and its predictive value in clinical course and treatment responsiveness remain debated.

One long-standing consideration in assay design has been varying the antigen composition to include 'accessory' lipids that might enhance or attenuate the detection of anti-GM1 antibody binding. Many studies have shown that accessory lipids or liposomal GM1 preparations markedly affect anti-GM1 antibody detection (Willison et al., 1994). Pestronk previously detected enhanced MMN antibody binding to GM1 in the presence of galactocerebroside (GalC) (Pestronk et al., 1997), and Greenshields observed inhibition of anti-GM1 binding to GM1 in the presence of GD1a using MMN-derived human monoclonal antibodies (Greenshields et al., 2009; Paterson et al., 1995), a finding subsequently confirmed in a clinical cohort (Nobile-Orazio et al., 2010).

The recent observations by Kaida on ganglioside complexes has led to renewed interest in the roles of accessory lipids and glycolipids in influencing antibody binding to GM1 (Kaida and Kusunoki, 2010; Kaida et al., 2004). To investigate this phenomenon, we have screened MMN sera using a recently developed combinatorial glycoarray, in which highly diverse repertoires of heteromeric complexes of lipids and glycolipids can be readily examined for enhanced or attenuated antibody binding (Rinaldi, 2009 360 /id; Rinaldi, 2010 1979 /id; Brennan, 2011 10 /id). Here we report the analysis of heteromeric complex binding of sera from a cohort of patients fulfilling diagnostic criteria for MMN, mostly undergoing treatment in our local clinical centre, and compare the performance of this glycoarray with conventional ELISA methodology.

2. Materials and Methods

2.1. Sera and clinical data

Serum samples were collected from 33 MMN patients, 25 of whom are currently attending our local Neurology clinic. All 33 cases were identified from the Scottish population by local neurologists and neurophysiologists, 22 cases of whom had undergone screening for a clinical trial of a complement inhibitor (Fitzpatrick et al., 2011). Electrophysiology studies were reviewed, and all subjects in the MMN group fulfilled electrodiagnostic criteria as defined by EFNS guidelines (van Schaik et al., 2006). Clinical data were collected from patients hospital case notes, including age of onset, site of onset (nerve territory, side), presence of lower limb or sensory involvement (at any time). Antibody-negative MMN cases had not shown reactivity against GM1 by ELISA (see below). Other neurological disease (OND) sera (n=30) comprised other neuropathies (n=6), multiple sclerosis (n=4), motor neurone disease (n=3), chronic fatigue syndrome (n=2), non-organic or undiagnosed neurological disorder (n=6), encephalopathy (n=1), cerebrovascular disease (n=1), optic neuritis (n=1), viral meningitis (n=1), migraine (n=2), headache (n=1), idiopathic

intracranial hypertension (n=2). Healthy control (HC) sera were obtained from 27 volunteers. The study was approved by the West of Scotland Research Ethics Committee.

2.2. ELISA

The ELISA method from the Glasgow Diagnostic Neuroimmunology Laboratory was used (Willison et al., 1999). Briefly, polystyrene plates (Immulon 2HB) were coated with ganglioside (GM1, GM2, GD1a, GD1b, GT1b, GQ1b, GA1, sulphatide and globoside at 200ng per well) in methanol to evaporation, then blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at 4°C. For heteromeric complexes comprising a 50:50 ratio of 2 glycolipids, 100ng of each glycolipid was admixed in methanol by sonication (3minutes), and a total of 200ng of glycolipid mixture applied per well. Sera were diluted in 0.1% BSA/PBS, and 100µL applied to duplicate wells at 1/100, 1/500, 1/2500, 1/12,500 dilutions for 12 h at 4°C. After washing, peroxidase-labeled anti-human IgM antibody (diluted 1/3000) was applied for 1 h at 4°C. Detection was performed with *o*-phenylenediamine dihydrochloride and the reaction terminated with 50µL of 4M H₂SO₄. Optical density (OD) was detected at 492 nm using an automated plate reader (Ascent Multiscan, Labsystems, USA). Background (methanol only coated wells) OD values were subtracted to give final OD values. OD values >0.1 at 1/500 dilution were considered positive, based on previous assay validation data(Willison et al., 1999).

2.3. Combinatorial glycoarray

Screening of antibody binding by combinatorial array was performed as previously described(Rinaldi et al., 2009;Rinaldi et al., 2010) using the following lipids and glycolipids and their 1:1 heteromeric complexes: GM1, GM2, GD1a, GT1b, GA1, galactocerebroside (GalC), 3-sulphated galactosylceramide (sulphatide, sulph), sulphated glucuronyl paragloboside (SGPG), sialosyl-lactoneotetraosylceramide (LM1) and phosphatidylserine (PS). All lipids were obtained from Sigma, UK or Avanti Polar Lipids, Alabaster, AL, except SGPG (isolated from bovine cauda equina(Yu et al., 1994)) and LM1 (kindly provided by Dr J Mansson, Goteborg). Briefly, complexes were created by mixing equal volumes of the component glycolipid solutions and sonicating for 3 minutes. An ATS4 TLC autosampler (Camag) was used to apply 0.1µl per spot of single glycolipid or glycolipid complex at 100µg/ml in methanol to PVDF membranes affixed to glass slides. Arrays were blocked in 2% w/v bovine serum albumin / phosphate buffered saline (BSA/PBS), then incubated with sera diluted 1 in 100 in 1% BSA/PBS. After washing, rabbit anti-human IgG horse radish peroxidase conjugated secondary antibody was applied at 1:30,000. Binding was detected by enhanced chemiluminescence (ECL+, Amersham/GE Healthcare). Exposure time was 1 minute. Radiographs were digitized by flatbed scanning and spot intensity calculated using TotalLab image analysis software (Nonlinear Dynamics), expressed as intensity units (IU). From 10 individual lipids, a total of 45 heteromeric lipid complexes are achieved, each duplicated in a mirror image against a diagonal control line of methanol. Representative examples of arrays are shown in Supplementary Figure 1.

2.4. Statistical methods

Receiver operator characteristic (ROC) analysis was performed in MedCalc software using Hanley & McNeil methodology with 95% confidence intervals. The areas under the curve were analyzed assuming a likelihood ratio of LR+2.0 and LR-0.5, corresponding to an area under the curve of 0.75 (MedCalc software). Intensity unit values from glycoarrays were used to produce heatmaps which underwent hierarchical clustering (HCL) and Pearson correlation for distance metric selection (MeV software). Correlation studies for upper limit of normal (ULN) calculation including Wilcoxon signed rank test for non-parametric data were calculated using Minitab 15 software. Box and whisker plots are set within 5–95%

intervals. The remaining significance tests and graphic representations were produced using GraphPad Prism 5 software.

3. Results

Application of the glycoarray method to diagnostic testing in MMN and controls

Sera were first screened for anti-GM1 IgM antibodies by ELISA using our previously established threshold for optical density (OD) to define positivity, where a serum is regarded positive when $OD \geq 0.1$ at 1/500 dilution. {Willison, 1999 61 /id}. By these criteria, two thirds (22/33) of MMN cases were anti-GM1 IgM antibody positive (Table 1). There were no significant differences in clinical features between the anti-GM1 positive and negative cases. Patients typically presented with multifocal distal upper limb motor symptoms.

All MMN and control samples were then screened by glycoarray comprising 10 single lipids and their 45 possible 1:1 complexes, with 9 typical patient arrays appearances being shown in Figure S1, alongside 2 controls. As the glycoarray had not previously been systematically applied to an MMN population, there was no pre-determined upper limit of normal range (ULN) prior to the start of this study. Two proposed methods for determining ULN were compared. Firstly, the median and 95% confidence interval of GM1 spot intensity signal in the healthy control population was calculated by Wilcoxon signed rank test (as data were not normally distributed) giving an estimated median of 460 intensity units (IU), with a 95% confidence interval of 341 to 654 IU. Secondly, as the correlation coefficient for ELISA and glycoarray was high (0.78) (Figure S2, Panel A), the regression equation was used to directly calculate the glycoarray value equivalent to 0.1 OD units on ELISA, yielding an ULN for the glycoarray of 4365 IU. This latter value (4365 IU) was used as the cut-off value for positivity for GM1 and all other single lipids and heteromeric complexes studied in the glycoarray. Using this cut-off value, 19/33 MMN samples were positive for anti-GM1 IgM by glycoarray. 3 of the samples positive for anti-GM1 IgM by ELISA were negative when screened by glycoarray (Figure S2, Panel A).

Receiver operator characteristic analysis of combinatorial glycoarray data

Digitised array data from all MMN cases, OND and healthy controls were subjected to cluster analysis that yielded a heat map comprising spot intensities for 40/55 glycolipids and glycolipid complexes amongst cases and controls (Figure 1A). For the remaining 15 targets, no detectable array signal was obtained from any of the samples (cases or controls), and these were excluded from further analysis. Spot intensities were categorised as either positive (>4365 AU) or negative (<4365 AU) and each of the 40 remaining glycolipid and glycolipid complexes were then individually subjected to ROC analysis, comparing MMN cases ($n=33$) with the combined OND and healthy control groups ($n=57$). The areas under the ROC curve (AUC, used as a summary measure of diagnostic accuracy) were ranked according to value (Figure 1B). Through this process, the best performing glycolipid complex combinations for diagnostic accuracy were identified as GM1, GA1 and GM2, all in complex with GalC. Another high performing complex, GM1:SGPG (ranked 3rd), was not considered further in this study as SGPG is scarcely available and thus impractical for routine diagnostic use in most laboratories. ROC graphs for GM1, GA1 and GM2, individually and in complex with GalC are shown in Figure 1C, and all the individual sample intensity values in Figure 1D. Examining these data, GM1:GalC is the best performing complex for diagnostic sensitivity and specificity in this MMN population. ANOVA also revealed GM1:GalC as the lipid complex most significantly differentiating the MMN group from the combined control group ($p=6.4 \times 10^{-17}$).

Comparative analysis of ELISA and glycoarray data for MMN samples

ELISA and glycoarray values for GM1 and GM1:GalC complex were determined to have high correlation coefficients by regression analysis (Figures S2 and 2). Thus, comparison of ELISA and glycoarray for determining anti-GM1 IgM Ab yields a correlation coefficient of 0.78 (Figure S2, A). The correlation coefficients for GM1 versus GM1:GalC (Figure S2, B–C) were also high, whether assaying by ELISA or glycoarray (0.92 and 0.72 respectively). Focusing on samples at the lower end of the assay range in ELISA, 7/11 anti-GM1 Ab negative MMN samples were either very weakly or borderline positive for GM1:GalC complexes (Figure S2, B). Using the glycoarray in the same comparative analysis between GM1 and GM1:GalC complex (Figure S2,C), average IU values were in general higher for GM1:GalC than for GM1 alone, and all 33 MMN samples were GM1:GalC positive (i.e. above the 4365 IU threshold), compared with 19/33 for GM1 alone. Data from more detailed examination of the GM1:GalC complex is shown in Figure 2. Using the assay cut-off criteria described above and including all control samples (n=57), ROC analysis was used to assess the overall sensitivity and specificity of the glycoarray and ELISA techniques and showed no significant difference in diagnostic performance ($p=0.59$) (Figure 2A). Examining the data sets for individual MMN sera from both assays (Figure 2B), the correlation coefficient is 0.57. Four MMN samples negative for anti-GM1:GalC complex IgM antibodies by ELISA were positive by glycoarray (Figure 2C, an expansion of bottom left corner of Figure 2B). The corresponding glycoarrays for these 4 samples are shown in Figure 2D. In each of these 4 samples, reactivity with other glycolipids or complexes are also seen, including GM2, GM2:GalC, GA1 and GA1:GalC.

Analysis of glycoarray data in the control population

From visual observation of the heat map for the array data (Figure 1A, red being positive, black intermediate and green negative), it is clear that IgM antibodies to certain individual glycolipids and their complexes are present in a proportion of both OND and normal controls in addition to MMN samples, GalC:SGPG being prominent in this respect. Individual data values in control samples for 6 antigens shown in Figure 1D indicate that for some glycolipids sensitivity is low but specificity is high (e.g. GM2), whereas for others (e.g. GA1:GalC) high sensitivity is offset by lower specificity, as summarised in the ROC analyses (Figure 1B). Quantified examples for GM1 and GM2 in complex with GalC, sulfatide or SGPG are shown in Table 2, A. Evaluation of these findings is, by definition, dependent upon the statistical methodology used for setting assay thresholds; thus had we used the upper 95% confidence interval of the median control value as the ULN in glycoarray, 25/33 of the MMN population would be positive for anti-GM1 antibodies, equating to 76%, as opposed to 19/33 (58%), when the ULN of 4365 was applied. By corollary, lowering the threshold for sensitivity also decreases the specificity.

Since the GM1:GalC complex assessed by glycoarray appears from this study to be a highly sensitive marker for MMN, careful attention was paid to the 4 positive samples for this complex in the control population (datapoints as shown in Figure 1, D). These data are plotted for the OND group (n=30) in Figure S3B, for both ELISA and glycoarray in comparison with data for GM1 alone. Of the 4 OND samples, one was positive for both GM1 and GM1:GalC by both ELISA and glycoarray (sample 1). The remainder were either negative for GM1 by ELISA (samples 2, 3, 4) and glycoarray (samples 3, 4) or negative for GM1:GalC by ELISA (samples 3, 4). The glycoarray for sample 1 is shown in Figure S3C and shows a range of antibodies to single glycolipids and complexes in addition to GM1 and GM1:GalC, most notably GA1 and GA1:GalC. Clinically, sample 1 was catalogued as having ‘idiopathic peripheral neuropathy’ but on review of the clinical notes had presented with multifocal upper limb motor and sensory symptoms affected ulnar and radial nerves with median nerve motor conduction block in a forearm segment. When previously assayed

for anti-GM1 IgM by ELISA as part of routine diagnostic workup, this serum sample contained anti-GM1 antibodies just below the ULN for the assay, and thus had been reported as negative at the time. In retrospect, this patient may have fulfilled diagnostic criteria for MMN or Lewis-Sumner syndrome but this was not evaluated further, and the sample was retained in the OND group.

Analysis of enhancing and attenuating complexes in MMN samples

A characteristic of the glycoarrays as described here are the readily observable patterns of enhancement and attenuation seen with different complexes, in comparison with antibody binding intensity to the single lipid (Figure 3 and S1). Thus, the median signal intensity of antibody binding to GM1 in the MMN group was 13394 IU (IQR 5213 – 28801). When GM1 is in complex with other glycolipids, the signal intensity may increase (complex enhanced), decrease (complex attenuated) or remain unchanged (complex independent). These data are quantified in the MMN group for GM1, GM2 and GA1 in complex with the 9 other antigens spotted in this glycoarray, where the single glycolipid signal intensity is set to zero for each sample (Figure 3). Illustrative arrays from individual patients are shown in the adjacent panels. From these data sets, it is evident that GalC always provides the greatest complex enhancement for GM1, GA1 and GM2. Thus for GM1 in complex with GalC, the mean intensity of the GM1:GalC complex was significantly higher (paired t-test, $p=0.008$) by 8797 IU (95% CI, 2538 – 15056), compared with the intensity of the sum of each single spot (GM1 single + GalC single). A similar pattern was observed for GM2 and GA1. Other enhancing glycolipids for GM1, GM2 and GA1 are sulfatide and SGPG, a finding supported by data shown in Table 2. By contrast the complex-inhibiting glycolipids for antibody binding to GM1, GM2 or GA1 were LM1, GD1a, and GT1b.

4. Discussion

This study demonstrates that a combinatorial glycoarray provides a useful investigative tool for detecting IgM antibodies to GM1, related glycolipids and their complexes in the diagnostic work-up of patients with suspected multifocal motor neuropathy. The glycoarray correlates well with the standard method of anti-GM1 IgM antibody detection by ELISA, which is used widely in clinical diagnostic labs. The specific advantage of using the glycoarray is the ease with which very small smaller amounts of sera and lipids can be used to simultaneously screen for antibodies to large numbers of different lipid complexes and single lipids. Whilst ELISA remains an excellent technique for detecting antibodies to single or complexed lipids present in serum at medium to high titre, our data suggest that the glycoarray method may be more sensitive at the lower end of the detection range.

This study provides strong support for the long-standing observation by Pestronk and colleagues that a one-to-one complex of GM1:GalC constitutes a very sensitive antigen for screening MMN sera (Pestronk et al., 1997). Indeed, in this cohort of 33 MMN cases, all sera were reactive against the GM1:GalC complex in glycoarray screening, including those that were not reactive to either GM1 or GalC alone. In addition, 4 cases whose sera were negative for antibodies to the GM1:GalC complex by ELISA, were positive by glycoarray. These findings need to be viewed cautiously until the overall conclusions can be validated in other cohorts of MMN cases and appropriate controls. Although the 33 cases from our national area were randomly selected for inclusion in this survey, referral bias to both our diagnostic neuroimmunology laboratory and clinical service may have been a factor in increasing the proportion of antibody positive cases. The principle clinical point emerging from this study is that the diagnostic yield of the standard anti-GM1 antibody ELISA can be improved upon through use of the combinatorial glycoarray.

The finding of antibodies to GM1 in complex with other glycolipids influences our ideas about the immunopathogenesis of MMN by providing further support for an antibody-mediated autoimmune hypothesis. What remains unknown is whether any complexes that are capable of binding antibody exist in the living nerve environment, and where they might be localised. Equally, inhibitory complexes, such as GM1:GD1a, may play important roles in attenuating antibody binding and subsequent tissue injury, as has been shown experimentally (Greenshields et al., 2009). Further information about this will come from isolating anti-complex antibodies for use in more detailed pathogenesis studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Reference List

- Adams D, Kuntzer T, Burger D, Chofflon M, Magistris MR, Regli F, Steck AJ. Predictive value of anti-GM1 ganglioside antibodies in neuromuscular diseases: a study of 180 sera. *J Neuroimmunol.* 1991; 32:223–230. [PubMed: 2033117]
- Alaedini A, Latov N. Detection of anti-GM1 ganglioside antibodies in patients with neuropathy by a novel latex agglutination assay. *J Immunoassay.* 2000; 21:377–386. [PubMed: 11071254]
- Baumann N, Harpin M-L, Marie Y, Lemerle K, Chassande B, Bouche P, Meininger V, Yu RK, Leger J-M. Antiglycolipid antibodies in motor neuropathies. *Ann N y Acad Sci.* 1998; 845
- Bech E, Jakobsen J, Orntoft TF. Elisa-type titertray assay of IgM anti-GM1 autoantibodies. *Clin Chem.* 1994; 40:1331–1334. [PubMed: 8013109]
- Carpo M, Allaria S, Scarlato G, Nobile-Orazio E. Marginally improved detection of GM1 antibodies by Covalink ELISA in multifocal motor neuropathy. *Neurology.* 1999; 53:2206–2207. [PubMed: 10599811]
- Chabraoui F, Derrington EA, Mallie-Didier F, Confavreux C, Quincy C, Caudie C. Dot-blot immunodetection of antibodies against GM1 and other gangliosides on PVDF-P membranes. *J Immunol Methods.* 1993; 165:225–230. [PubMed: 8228272]
- Conrad K, Schneider H, Ziemssen T, Talaska T, Reinhold D, Humbel RL, Roggenbuck D. A new line immunoassay for the multiparametric detection of antiganglioside autoantibodies in patients with autoimmune peripheral neuropathies. *Ann N y Acad Sci.* 2007; 1109:256–264. [PubMed: 17785314]
- Escande-Beillard N, David MJ, Portoukalian J, Pouget J, Azulay JP, Bernard D, Boucraut J. A sensitive flow cytometry method for anti-GM1 antibodies detection. *J Neuroimmunol.* 2002; 125:163–169. [PubMed: 11960653]
- Fitzpatrick AM, Mann CA, Barry S, Brennan K, Overell JR, Willison HJ. An open label clinical trial of complement inhibition in multifocal motor neuropathy. *J Peripher Nerv Syst.* 2011; 16:84–91. [PubMed: 21692905]
- Greenshields KN, Halstead SK, Zitman FM, Rinaldi S, Brennan KM, O'Leary C, Chamberlain LH, Easton A, Roxburgh J, Pediani J, Furukawa K, Goodyear CS, Plomp JJ, Willison HJ. The neuropathic potential of anti-GM1 autoantibodies is regulated by the local glycolipid environment in mice. *J Clin Invest.* 2009; 119:595–610. [PubMed: 19221437]
- Kaida K, Kusunoki S. Antibodies to gangliosides and ganglioside complexes in Guillain-Barre syndrome and Fisher syndrome: Mini-review. *J Neuroimmunol.* 2010
- Kaida K, Morita D, Kanzaki M, Kamakura K, Motoyoshi K, Hirakawa M, Kusunoki S. Ganglioside complexes as new target antigens in Guillain-Barre syndrome. *Ann Neurol.* 2004; 56:567–571. [PubMed: 15389898]
- Kornberg AJ, Pestronk A, Bieser K, Ho TW, McKhann GM, Wu HS, Jiang Z. The clinical correlates of high-titer IgG anti-GM1 antibodies. *Annals of Neurology.* 1994; 35:234–237. [PubMed: 8109905]
- Lewis RA, Sumner AJ, Brown MJ, Asbury AK. Multifocal demyelinating neuropathy with persistent conduction block. *Neurology.* 1982; 32:958–964. [PubMed: 7202168]

- Nobile-Orazio E, Cappellari A, Priori A. Multifocal motor neuropathy: current concepts and controversies. *Muscle Nerve*. 2005; 31:663–680. [PubMed: 15770650]
- Nobile-Orazio E, Giannotta C, Briani C. Anti-ganglioside complex IgM antibodies in multifocal motor neuropathy and chronic immune-mediated neuropathies. *J Neuroimmunol*. 2010; 219:119–122. [PubMed: 20006388]
- Parry GJ, Sumner AJ. Multifocal motor neuropathy. *Neurol Clin*. 1992; 10:671–684. [PubMed: 1501600]
- Paterson G, Wilson G, Kennedy PGE, Willison HJ. Analysis of anti-GM1 ganglioside IgM antibodies cloned from motor neuropathy patients demonstrates diverse v-region gene usage with extensive somatic mutation. *J Immunol*. 1995; 155:3049–3059. [PubMed: 7673721]
- Pestronk A. Motor neuropathies, motor-neuron disorders, and antiglycolipid antibodies - invited review. *Muscle & Nerve*. 1991; 14:927–936. [PubMed: 1658646]
- Pestronk A, Choksi R, Blume G, Lopate G. Multifocal motor neuropathy: Serum IgM binding to a GM1 ganglioside-containing lipid mixture but not to GM1 alone. *Neurology*. 1997; 48:1104–1106. [PubMed: 9109910]
- Pestronk A, Cornblath DR, Ilyas AA, Baba H, Quarles RH, Griffin JW, Alderson K, Adams RN. A treatable multifocal motor neuropathy with antibodies to GM1 ganglioside. *Ann Neurol*. 1988; 24:73–78. [PubMed: 2843079]
- Pukin AV, Jacobs BC, Gillen AP, Gilbert M, Endtz HP, van BA, Visser GM, Zuillhof H. Detection of Antibodies in Neuropathy Patients by Synthetic GM1 Mimics. *Glycobiology*. 2011
- Ravindranath MH, Ravindranath RMH, Morton DL, Graves MC. Factors affecting the fine specificity and sensitivity of serum antiganglioside antibodies in elisa. *Journal Of Immunological Methods*. 1994; 169:257–272. [PubMed: 7510761]
- Rinaldi S, Brennan KM, Goodyear CS, O'Leary C, Schiavo G, Crocker PR, Willison HJ. Analysis of lectin binding to glycolipid complexes using combinatorial glycoarrays. *Glycobiology*. 2009; 19:789–796. [PubMed: 19349623]
- Rinaldi S, Brennan KM, Willison HJ. Heteromeric glycolipid complexes as modulators of autoantibody and lectin binding. *Prog Lipid Res*. 2010; 49:87–95. [PubMed: 19735674]
- van Schaik IN, Bouche P, Illa I, Leger JM, Van den Bergh P, Cornblath DR, Evers EM, Hadden RD, Hughes RA, Koski CL, Nobile-Orazio E, Pollard J, Sommer C, Van Doorn PA. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. *Eur J Neurol*. 2006; 13:802–808. [PubMed: 16879289]
- Willison HJ, Paterson G, Kennedy PG, Veitch J. Cloning of human anti-GM1 antibodies from motor neuropathy patients. *Ann Neurol*. 1994; 35:471–478. [PubMed: 8154875]
- Willison HJ, Veitch J, Swan AV, Baumann N, Comi G, Gregson NA, Illa I, Zielasek J, Hughes RA. Inter-laboratory validation of an ELISA for the determination of serum anti-ganglioside antibodies. *Eur J Neurol*. 1999; 6:71–77. [PubMed: 10209353]
- Yu RK, Yoshino H, Yamawaki M, Yoshino JE, Ariga T. Subcellular Distribution of Sulfated Glucuronyl Glycolipids in Human Peripheral Motor and Sensory Nerves. *J Biomed Sci*. 1994; 1:167–171. [PubMed: 11725022]

Figure 1. Quantitative and statistical analysis of glycoarray data. Panel A. Heat map showing the values (logarithmic scale) of antibody binding intensity to the 40/55 single glycolipids and complexes that returned a detectable signal for 33 MMN patient sera, 27 healthy controls and 30 ONDs. The 15 lipids or lipid complexes that returned no signal in any samples are excluded from the heat map. The minimal value assigned to any sample was set at 3.6 IU, corresponding to $\log_{10}$ ULN (4365 AU). Thus, pale green corresponds to antibody negative signals (<4365 AU), darker green and black to mid-range signals and red to high value signals. Panel B. ROC analysis was applied to rank signals by sensitivity/specificity ratio as a summary measure of assay performance, the highest values representing the best performing glycolipids and lipid complexes. Black bars highlight datasets plotted in Panels C and D. Panel C. Individual ROC curves for 3 single glycolipids (GM1, GM2 and GA1) and their complexes with GalC. Panel D. Individual glycoarray intensity values for MMN cases and all controls against 3 single glycolipids (GM1, GM2 and GA1) and their complexes with GalC.

Figure 2.

Comparative data of ELISA and glycoarray performance for MMN serum binding to GM1:GalC. Panel A. ROC curves show high performance for the two methods, that are insignificantly different ($p=0.59$). Panel B. Correlation analysis shows a coefficient of 0.57 between the 2 methods. Panel C. Magnified view of ELISA-negative values ($n=4$) that were positive by glycoarray. Panel D. Individual glycoarray blots of the 4 ELISA-negative, glycoarray-positive MMN samples shown in Panel C.

Figure 3.

Patterns of antibody binding to GM1 (Panel A), GA1 (Panel C) and GM2 (Panel E) for MMN sera showing complex enhancement and/or attenuation with other glycolipids. Data are shown as box and whisker plots (median and interquartile ranges) depicting signal intensities above (complex enhancement) or below (complex attenuation) the intensity value for the single glycolipid which is set at zero. Panels B (GM1), D (GA1) and F (GM2) show representative glycoarrays from individual patients binding to each of the three glycolipids. For all MMN cases (n=33), datasets were derived in duplicate from the boxed coordinates comprising each of the single glycolipids and the 9 possible complexes. Most MMN sera bind to more than one complex; thus in Panels B and D, the 2 MMN sera are binding

strongly to GM1:GalC and GA1:GalC; and in Panel F, the MMN serum is binding strongly to GM2:GalC and GA1:GalC. Other complex reactivities are also often seen (e.g. sulphatide:GalC and SGPG:GalC in Panels B and F) but were not subjected to further enhancement and attenuation analysis.

Table 1

Clinical features of MMN patients included in the study

	GMI antibody negative (n=11)*	GMI antibody positive (n = 22)	p-value
Age (median years)	56 (49 – 70)	58 (51 – 69)	0.92
M:F ratio	4.5 : 1	3.4 : 1	0.76
Age at onset (median years)	44.5 (32 – 50)	43 (32 – 54)	0.87
Disease duration (median years)	19 (5 – 28)	11 (3.5 – 27)	0.49
LL involved	55.5%	57.9%	0.91
SS involved	30.0%	31.6%	0.93
dcMAP reduced	78.0%	65.0%	0.49
CB absent (%)	0.0%	4.8%	0.44
CB probable (%)	33.3%	33.3%	1.00
CB definite (%)	66.7%	61.9%	0.80

* Anti-GM1 antibody testing was performed by ELISA

Table 2

Sensitivity and specificity values for GM1,GM2,and representative complexes.

	MMN	Control	Sensitivity	Specificity
GM1	19	2	19/33 (58%)	2/57 (96.5%)
GM1:Galc	33	4	33/33 (100%)	4/57 (93%)
GM1:Sulph	22	3	22/33 (67%)	3/57 (95%)
GM1:SGPG	24	6	24/33 (73%)	6/57 (89.5%)

	MMN	Control	Sensitivity	Specificity
GM2	6	0	6/33 (18%)	0/57 (100%)
GM2:GALC	28	6	28/33 (85%)	6/57 (89.5%)
GM2:Sulph	21	2	21/33 (64%)	2/57 (96.5%)
GM2:SGPG	26	6	26/33 (79%)	6/57 (89.5%)