Table 1
Summary of clinical features, serological findings, and treatment of neuralgic amyotrophy.

Patient	Age(y)/sex	Preceding infection	Clinical features	Nerve conduction study	Anti-ganglioside antibodies	Anti-CMV antibodies	Treatment	Outcome	Course
1	34/F	Fever and vomiting	Painful right arm neuropathy	Low F-wave persistence	IgM GalNAc-GD1a	IgG	IVIG	Improvement	Acute monophasic
2	26/F	Upper airway inflammation	Painful bilateral leg neuropathy	Low F-wave persistence	IgM GalNAc-GD1a	IgG	IVIG	Improvement	Acute monophasic
3	54/M	Fever	Painful left arm neuropathy	Low F-wave persistence	IgM GalNAc-GD1a and GM2	IgG	Nothing	Spontaneous remission	Acute monophasic
4	37/M	Unknown	Painful right arm neuropathy	Normal	IgG GalNAc-GD1a	IgG	IVIG	No change	Chronic

GalNAc-GD1a, *N*-acetylgalactosaminyl GD1a; IVIg, immunoglobulin infusion therapy.

was complicated by aseptic meningitis. Thus, IVIg was performed for only 3 days. The aseptic meningitis was resolved immediately after treatment was discontinued. Pain and muscular weakness were not improved, but no further aggravation was observed.

3. Discussion

The etiology of NA remains to be clarified. In some cases, however, preceding events, the monophasic course, and the good response to immunomodulative therapy implicate an autoimmune mechanism. Pathologic findings with brachial plexus neuropathy in which conspicuous mononuclear inflammatory infiltrates are observed surrounding epineurial and endoneurial vessels within the brachial plexus support an immune-mediated mechanism (Suarez et al., 1996). However, NA in the presence of herpes zoster virus infection has been reported (Ohtake et al., 1991; Ismail et al., 2009), suggesting viral reactivation may also be a cause of NA. In addition, NA sometimes occurs on a hereditary basis and with genetic abnormalities (Tsao et al., 2004; Kuhlenbäumer et al., 2005). Thus, NA should be considered as a syndrome that is caused by a variety of etiologies.

Here, we reported the case of four patients with NA who were positive for anti-GalNAc-GD1a antibody and who had a preceding infection. Three of the four patients had an antecedent infection. All patients had elevated titers of antibodies against GalNAc-GD1a. Two of the four patients responded to IVIg. A complication forced discontinuation of IVIg in one patient, but his symptoms had stopped progressing before discontinuation. The clinical features, serological findings, and treatment of NA are summarized in Table 1.

GalNAc-GD1a, which is a minor component of the gangliosides in the nervous system, was reported as a target antigen for the acute-phase serum antibody in GBS (Kusunoki et al., 1994). Further investigation showed that high titers of IgG antibodies to GalNAc-GD1a were closely associated with the pure motor variant of GBS (Kaida et al., 2000). In contrast, IgM anti-GalNAc-GD1a antibodies were grouped into two types. Those in the patients with antecedent CMV infection were frequently cross-reactive with GM2 and characterized by frequent facial and sensory deficits, whereas those in the patients with preceding gastrointestinal infections were specifically reactive with GalNAc-GD1a and characterized by motor GBS (Kaida et al., 2001). In addition, anti-GalNAc-GD1a antibodies were sometimes associated with multifocal motor neuropathy and chronic motor axonal neuropathy (Sugie et al., 1998; Kaji et al., 2000). The present investigation showed that some patients with NA exhibited anti-GalNAc-GD1a antibodies with or without cross-reactivity to GM2. We could not detect evidence of recent CMV infection in serological markers or antibodies cross-reactive to GalNAc-GD1a and GM2, however these findings might indicate that autoimmune mechanisms similar to GBS were involved in the pathogenesis of some cases of NA.

The pathogenic role of anti-GalNAc-GD1a antibodies is still unclear. The location of GalNAc-GD1a has been shown in the nodes of Ranvier, the paranodal regions, and in the small-diameter dorsal root fibers (Kaida et al., 2003). Anti-GalNAc-GD1a antibodies may function by binding to these sites and causing atrophy, weakness, and severe pain. Thus we hypothesized that anti-GalNAc-GD1a antibodies could cause part of NA. However, the binding of anti-ganglioside antibodies is known to be affected by the local environment that surrounds the ganglioside antigens and the avidity of the antibodies (Kaida and Kusunoki, 2010).

Future investigations are necessary to elucidate whether the anti-GalNAc-GD1a antibody is actually involved in the pathogenesis. In any case, anti-GalNAc-GD1a antibodies could be useful markers in NA to predict response to immune therapy, especially IVIg.

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Antibodies to LM1 and LM1-containing ganglioside complexes in Guillain–Barré syndrome and chronic inflammatory demyelinating polyneuropathy

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ABSTRACT

LM1 is localized in human peripheral nerve myelin. Antibodies to ganglioside complexes (GSCs) have been reported in Guillain–Barré syndrome (GBS). We investigated IgG antibodies to LM1 and two GSCs (GM1 and LMI, or GD1b and LM1) in the sera of each 40 patients with chronic inflammatory demyelinating polyneuropathy (CIDP) and GBS, using ELISA. We detected anti-LM1 antibody in five with GBS and seven with CIDP; anti-GM1/LM1 antibody in three with GBS and one with CIDP; and anti-GD1b/LM1 antibody in two with CIDP. Antibodies to LM1 and LM1-containing GSCs may be among the targets for autoimmunity in GBS and CIDP.

1. Introduction

Guillain–Barré syndrome (GBS) is an acute immune-mediated polyneuropathy that is often preceded by an infection. The acute-phase sera of approximately 60% of patients with GBS contain anti-ganglioside antibodies. Each ganglioside is uniquely localized. Therefore, each anti-ganglioside antibody may influence clinical features by specifically binding to the regions where the target ganglioside is localized. Although there are reports of antibodies to several antigens such as P0, PMP22, Gal-C, and sulfatide, a positive reaction is rarely detected in patients with chronic inflammatory demyelinating polyneuropathy (CIDP) (Meléndez-Vásquez et al., 1997; Yan et al., 2001; Allen et al., 2005).

Antibodies to ganglioside complexes (GSCs) have recently been reported in patients with GBS and Fisher syndrome (FS) (Kaida et al., 2004; Kaida et al., 2006; Kaida et al., 2007; Kaida et al., 2008; Kanzaki et al., 2008; Ogawa et al., 2009). These antibodies may recognize novel epitopes that are formed by two different gangliosides. Gangliosides are components of the lipid raft on the plasma membrane. Anti-GSC antibodies may cause neuropathy by binding to the GSCs formed by the clustered gangliosides on the lipid rafts. Among the anti-GSC antibodies, the IgG antibodies to the GD1a/GD1b complex and the GD1b/GT1b complex are associated with GBS in which a patient requires artificial ventilation (Kaida et al., 2007). IgG antibodies to the GM1/GalNAc-GD1a complex are associated with acute motor conduction block neuropathy (AMCBN), which is characterized by frequent conduction blocks (CBs) at the intermediate nerve segments and no sensory involvement (Kaida et al., 2008). IgM antibodies to GSCs have also been reported in patients with CIDP and IgM monoclonal gammopathy (Nobile-Orazio et al., 2010).

LM1 is a ganglioside localized in peripheral nerve myelin (Ogawa-Goto et al., 1992). It is a candidate target molecule in the demyelinating peripheral neuropathies. However, antibodies against LM1-containing GSCs have not been examined in patients with GBS or CIDP. Therefore, we investigated the activity of IgG antibodies to LM1 and LM1-containing GSCs (e.g., GM1/LM1 and GD1b/LM1) in patients with GBS and CIDP.

2. Materials and methods

2.1. The serum samples

Serum samples were obtained from 40 patients with GBS who were diagnosed in accordance with the established criteria (Asbury and Cornblath, 1990); 40 patients with CIDP who were diagnosed in accordance with the criteria proposed by European Federation of Neurological Societies and Peripheral Nerve Society (EFNS/PNS) (Joint Task Force of the EFNS and the PNS., 2005); 15 normal control subjects; and 40 patients with other neurological diseases (ONDs), which included 10 patients with motor neuron disease, four patients with Parkinson disease, three patients with spinocerebellar

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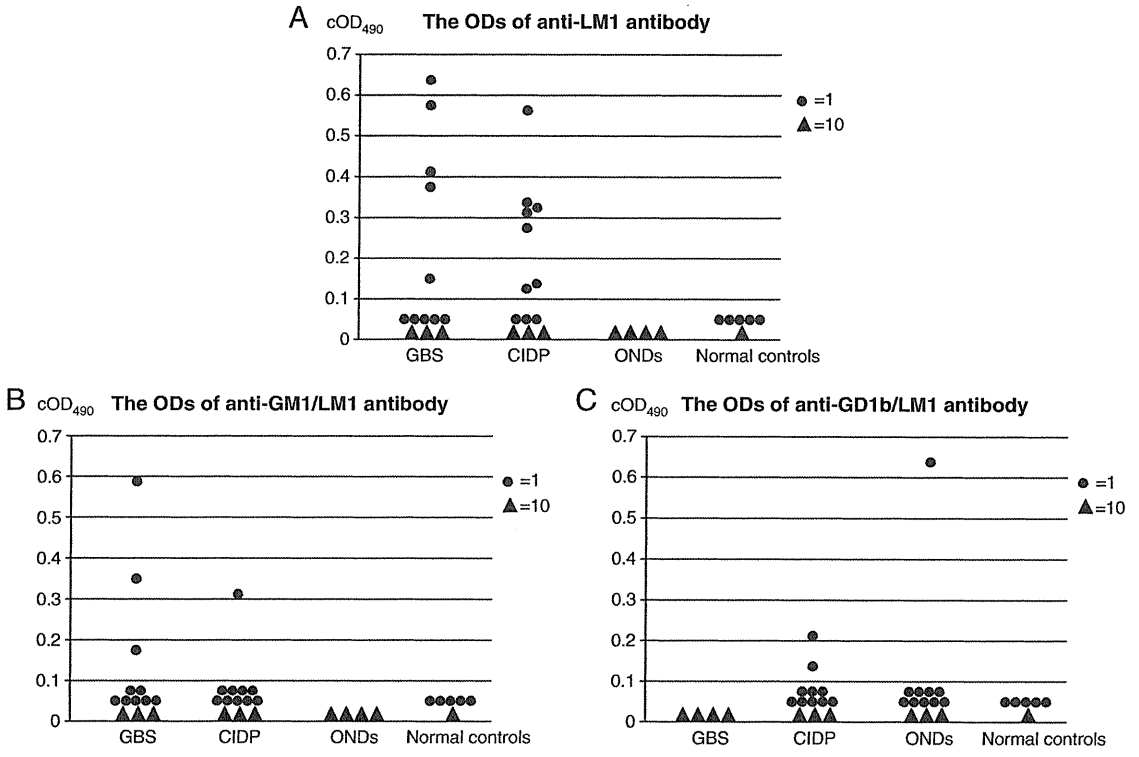

Fig. 1. The ODs of antibodies to LM1, GM1/LM1 and GD1b/LM1. Abbreviations: OD = optical density; CIDP = chronic inflammatory demyelinating polyneuropathy; GBS = Guillain–Barré syndrome; ONDs = other neurological diseases.

degeneration, two patients with multiple system atrophy, one patient with corticobasal degeneration, six patients with multiple sclerosis, four patients with neuromyelitis optica (NMO), eight patients with myasthenia gravis, and two patients with Lambert–Eaton syndrome. The sera from GBS patients were obtained in the acute phase (within 4 weeks after the onset of the disease) and the sera from CIDP patients were obtained in relapse. The sera from GBS and CIDP patients were collected before immunotherapy.

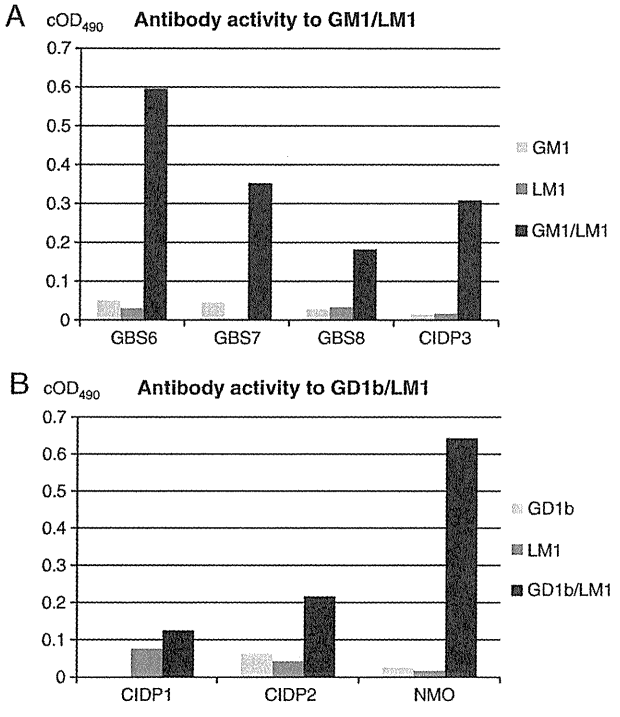

Fig. 2. The ODs of IgG antibodies to LM1-containing GSCs in antibody-positive patients. Abbreviations: OD = optical density; CIDP = chronic inflammatory demyelinating polyneuropathy; GBS = Guillain–Barré syndrome; NMO = neuromyelitis optica.

2.2. Enzyme-linked immunosorbent assay for detecting antibodies against gangliosides and LM1-containing ganglioside complexes

IgG antibodies to GM1, GD1b, LM1, the GM1/LM1 complex, and the GD1b/LM1 complex in the patients' sera were tested by enzyme-linked immunosorbent assay (ELISA), as previously reported (Kaida et al., 2004). A sample was considered positive for the presence of GM1 and GD1b antibodies when its optical density (OD) was greater than 0.1, as previously described (Kaida et al., 2004). A sample was considered positive for the presence of IgG antibodies to LM1 when its OD was greater than 0.12. (The OD was the value of the mean + 3 standard deviation [SD] of the ODs of 15 normal control subjects.) The antibodies to other gangliosides (e.g., GM2, GM3, GD1a, GD3, GT1b, GQ1b, and GalNAc-GD1a) were also examined in the same way, as previously described (Kaida et al., 2004). A sample was considered positive for the presence of antibodies to GM1/LM1 complex if one of the three criteria was fulfilled; 1) it was negative for antibodies to GM1 alone and LM1 alone and the ODs were greater than 0.12 in the wells that had been coated with both GM1 and LM1, 2) it was positive for antibodies to GM1 alone or LM1 alone and the ODs of the wells coated with both GM1 and LM1 were 0.2 higher than the ODs of the wells coated with GM1 alone and LM1 alone, 3) it was positive for antibodies to GM1 alone and LM1 alone and the ODs of the wells coated with both GM1 and LM1 were greater than the sum of the ODs of the wells coated with GM1 alone and those with LM1 alone. The same criteria were applied for the presence of antibodies to GD1b/LM1 complex.

2.3. The clinical and electrophysiological features of Guillain–Barré syndrome patients with anti-LM1 or LM1-containing GSCs IgG antibodies

Clinical and electrophysiological features were assessed in GBS patients with IgG antibodies to LM1 or LM1-containing GSCs. The patients were classified as having acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN), equivocal, or inexcitable based on the criteria of Ho et al (Ho et al., 1995).

This study was approved by the internal review board of Kinki University School of Medicine.

3. Results

3.1. Antibodies to LM1 or LM1-containing GSCs

The presence of anti-LM1 IgG antibody was positive in five patients with GBS and seven patients with CIDP. In patients with ONDs and in normal control subjects, anti-LM1 IgG antibody was not detected (Fig. 1). Anti-GM1/LM1 IgG antibody was detected in three patients with GBS and in one patient with CIDP. Anti-GD1b/LM1 IgG antibody was detected in two patients with CIDP and in one OND patient who had NMO (Fig. 2). IgG antibody to LM1 alone was not present in the patient who had antibodies to LM1-containing GSCs. No patient had antibodies to both GM1/LM1 and GD1b/LM1. The prevalence of LM1, GM1/LM1, and GD1b/LM1 antibodies in patients and in normal control subjects was shown in Table 1.

3.2. Clinical and electrophysiological features of GBS patients with anti-LM1 or anti-GM1/LM1 IgG antibodies

All the eight sera from anti-LM1 or -GM1/LM1 antibody-positive GBS patients were obtained within 4 weeks and five of those were within 2 weeks after the onset of disease. The GBS patients with either anti-LM1 (five patients) or anti-GM1/LM1 (three patients) antibodies were all men. Of the five patients positive for anti-LM1 antibody, two had an antecedent respiratory infection and one had an antecedent gastrointestinal infection, whereas two of the three patients positive for anti-GM1/LM1 antibody had antecedent gastrointestinal infections. Four of the five patients positive for anti-LM1 antibodies and one of the three patients positive for anti-GM1/LM1 antibodies were classified as having AIDP. The remaining three were classified as equivocal. IgG antibodies to GD1b were detected in three patients. Among them, two patients were positive for anti-GM1/LM1 antibody and one patient was positive for anti-LM1 antibody. IgG antibody to GalNAc-GD1a was detected in one patient who had anti-LM1 antibody. No antibodies to other gangliosides were detected in four patients (Table 2).

Table 1
The prevalence of LM1, GM1/LM1, and GD1b/LM1 antibodies in patients and in normal control subjects.

Patient	LM1	GM1/LM1	GD1b/LM1	Total
GBS	5/40 (12.5%)	3/40 (7.5%)	0/40 (0%)	8/40 (20%)
CIDP	7/40 (17.5%)	1/40 (2.5%)	2/40 (5%)	10/40 (25%)
ONDs	0/40 (0%)	0/40 (0%)	1/40 (2.5%)	1/40 (2.5%)
Normal controls	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)

Table 2
The clinical and electrophysiological features of anti-LM1 IgG antibody-positive or GM1/LM1 IgG antibody-positive patients with GBS.

Patient no.	Age/sex	Antecedent infection*	Involved cranial nerve(s)	Classification of NCS†	Presence of antibody to LM1 or GM1/LM1	Presence of antibodies to other gangliosides
GBS1	59/M	R	(−)	AIDP	LM1	(−)
GBS2	63/M	R	(−)	AIDP	LM1	(−)
GBS3	59/M	(−)	(−)	AIDP	LM1	GalNAc-GD1a
GBS4	47/M	GI	(−)	AIDP	LM1	GD1b
GBS5	43/M	(−)	(−)	Equivocal	LM1	(−)
GBS6	28/M	(−)	(−)	AIDP	GM1/LM1	GD1b
GBS7	31/M	GI	12	Equivocal	GM1/LM1	(−)
GBS8	36/M	GI	9, 10	Equivocal	GM1/LM1	GD1b

* GI, gastrointestinal infection; R, respiratory tract infection.
† NCS, nerve conduction study; AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy;

4. Discussion

LM1 is the predominant ganglioside in human peripheral nerve myelin. GM1 is also present in human myelin and is mainly contained in motor nerve myelin (Ogawa-Goto et al., 1992). There is some indication that GM1 is present in the nodes of Ranvier (Kusunoki et al., 1993). GD1b has been detected in human peripheral nerve myelin, predominantly in the sensory nerves (Ogawa-Goto et al., 1992). Immunolocalization of GD1b also has been reported in the paranodal myelin of human peripheral nerves and in the large neurons in the dorsal root ganglia (Kusunoki et al., 1993). In this study, we detected IgG antibody to LM1 in the sera of five of 40 patients (12.5%) with GBS and seven of 40 patients (17.5%) with CIDP, and we detected IgG antibodies to LM1-containing GSCs in the sera of three patients (7.5%) with GBS and three patients (7.5%) with CIDP. None of these antibodies were present in the normal controls or the patients with ONDs (except for one patient [2.5%] affected with NMO).

Four of five patients who were diagnosed with GBS and who had anti-LM1 IgG antibody were classified as having AIDP. Of the three GBS patients who were positive for anti-GM1/LM1 antibody, one was classified as having AIDP and the remaining two were equivocal. Considering that LM1 is distributed predominantly in the myelin of human peripheral nerve (Ogawa-Goto et al., 1992), anti-LM1 IgG antibody may be involved in the demyelinating process in AIDP and CIDP (Fredman et al., 1991; Ilyas et al., 1992; Yako et al., 1999; Harukawa et al., 2002; Susuki et al., 2002). Antibodies to LM1-containing GSCs may also cause demyelination by binding to the epitopes formed by two contiguous gangliosides in the plasma membrane.

IgG antibody to GD1b/LM1 was detected in one patient with NMO. In NMO, it is known that anti-AQP4 antibody and other antibodies associated with collagen diseases are frequently present. It is considered that humoral immunity is activated in NMO. LM1 is present in human microvascular endothelial cells that form the blood–brain barrier (BBB) (Kanda et al., 2004). It is therefore possible that the destruction of the BBB in patients with NMO may secondarily raise the serum level of the antibodies to LM1-containing GSCs. In contrast, all the sera from anti-LM1 or -GM1/LM1 antibody-positive patients with GBS were obtained in the acute phase, indicating that the antibodies were associated with the pathogenesis of GBS.

Target molecules have rarely been identified in patients with CIDP. The anti-ganglioside antibodies are usually associated with the AMAN form of GBS. Only a few gangliosides are known to be targeted by serum antibodies in patients with AIDP. The present investigation shows that LM1 and LM1-containing GSCs are among the target antigens in the sera of patients with CIDP and AIDP. Future studies involving a larger number of patients are necessary to elucidate how these antibodies cause demyelination in the peripheral nervous system.

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Anti-GM1/GD1a complex antibodies in GBS sera specifically recognize the hybrid dimer GM1–GD1a

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It is now emerging the new concept that the antibodies from some patients with Guillain–Barré syndrome (GBS) recognize an antigenic epitope formed by two different gangliosides, a ganglioside complex (GSC). We prepared the dimeric GM1–GD1a hybrid ganglioside derivative that contains two structurally different oligosaccharide chains to mimic the GSC. We use this compound to analyze sera from GBS patients by high-performance thin-layer chromatography immunostaining and enzyme-linked immunosorbent assay. We also synthesized the dimeric GM1–GM1 and GD1a–GD1a compounds that were used in control experiments together with natural gangliosides. The hybrid dimeric GM1–GD1a was specifically recognized by human sera from GBS patients that developed anti-oligosaccharide antibodies specific for grouped complex oligosaccharides, confirming the information that GBS patients developed antibodies against a GSC. High-resolution ^{1}H-^{13}C heteronuclear single-quantum coherence-nuclear overhauser effect spectroscopy nuclear magnetic resonance experiments showed an interaction between the IV Gal-H1 of GM1 and the IV Gal-H2 of GD1a suggesting that the two oligosaccharide chains of the dimeric ganglioside form a single epitope recognized by a single-antibody domain. The availability of a method capable to prepare several hybrid gangliosides, and the availability of simple analytical approaches, opens new perspectives for the understanding and the therapy of several neuropathies.

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Introduction

High titers of antibodies that recognize carbohydrate epitopes shared by mammalian and bacterial cell membranes can be found in human sera following bacterial infection and in the course of such neuropathies, as Guillain–Barré syndrome (GBS; Kusunoki et al. 2008), promoted by them, by targeting the nodes of Ranvier or motor nerve terminals (Willison and Yuki 2002; Van Doorn et al. 2008).

Gangliosides are widely used in thin-layer chromatography (TLC) immunostaining and in enzyme-linked immunosorbent assays (ELISAs) to recognize serum anti-carbohydrate antibodies. The specificity of anti-oligosaccharide antibodies is variable. Some sera recognize several oligosaccharides, linked to lipids and/or proteins, with different complexity, whereas others seem highly specific (Kaida, Kamakura, et al. 2008). This not necessarily represents the antibody–antigen interaction properties in vivo as proved by the non-constant capability of anti-oligosaccharide antibodies to develop neuropathies when injected in animals (Paparounas et al. 1999). Glycolipids, and particularly gangliosides, are not randomly distributed within the membrane but rather form domains (Sonnino et al. 2006) that participate and modulate signal transduction processes. Oligosaccharides of membrane glycoconjugates, and particularly the oligosaccharides of glycolipids, are cryptic structures. Glycoproteins protrude from the plasma membrane into the extracellular environment, but neighboring proteins can prevent external interactions. The oligosaccharide chain of glycolipids protrude from the plasma membrane to a maximum of only 20–25 Å (Sonnino et al. 1994) and from many years are considered very cryptic for targeting by antibodies, lectins and enzymes (Prince 1992; Greenshields et al. 2009).

A new concept that is emerging in recent times suggests that a specific combination of oligosaccharide structure, of their position and clustering can form a distinct epitope for serum anti-oligosaccharide antibodies that not necessarily show binding properties for the individual oligosaccharides or part of them (Kaida et al. 2004; Kaida and Kusunoki 2010). This needs the availability at the same time of specific anti-oligosaccharide antibodies and of a specific plasma membrane organization, for the developing of a neuropathology.

The complexity of the natural membranes makes difficult to proof this using simple experimental models.

To overcome this, we prepared the dimeric GM1–GD1a hybrid ganglioside that contains two structural different oligosaccharide chains. We found that the serum IgG reactive with the GM1/GD1a complex actually recognize the GM1–GD1a hybrid dimer.

Results

The preparation of ganglioside dimers GM1–GM1, GD1a–GD1a and GM1–GD1a was carried out starting from lysogangliosides. Lysoganglioside preparations were described in great details (Neuenhofer et al. 1985; Sonnino et al. 1992; Mauri et al. 2004, Valiente et al. 2001) in the past and they are currently available in our laboratory.

The lysogangliosides are connected with adipic acid to form the dimer compounds. Figures 1 and 2 show schemes for the preparation of ganglioside dimers and for the hybrid dimer GM1–GD1a. The yield of preparation was good and determined to be 70% for dimers GM1–GM1 and GD1a–GD1a and ~50% for the hybrid dimer GM1–GD1a. TLC of the dimers (Figure 3) shows homogeneity over 98% for the three compounds that due to the high sugar content displayed a retention time lower than that of natural gangliosides. As expected, the chromatographic behavior of the hybrid dimer GM1–GD1a was intermediate between those of dimers GM1–GM1 and GD1a–GD1a.

The three dimers were characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR). By NMR, we also performed experiments aimed to have information on the three-dimensional structure of the hybrid dimer GM1–GD1a.

The synthesis of the three dimers started from lysogangliosides prepared from natural compounds extracted from calf brains. These gangliosides are a mixture of C18- and C20-sphingosine. Combining the lysoderivatives, each compound homogeneous in the oligosaccharide chains results heterogeneous in the lipid moiety, displaying the three combinations of sphingosine C18/C18, C18/C20 and C20/C20. MS confirmed the calculated molecular mass for the three compounds. The electro-spray ionization (ESI)-MS spectra show the main charged ions $[M-2H]^{2-}$ at 1334, 1348 and 1362, $[M-3H]^{3-}$ at 986, 995 and 1004 and $[M-4H]^{4-}$ at

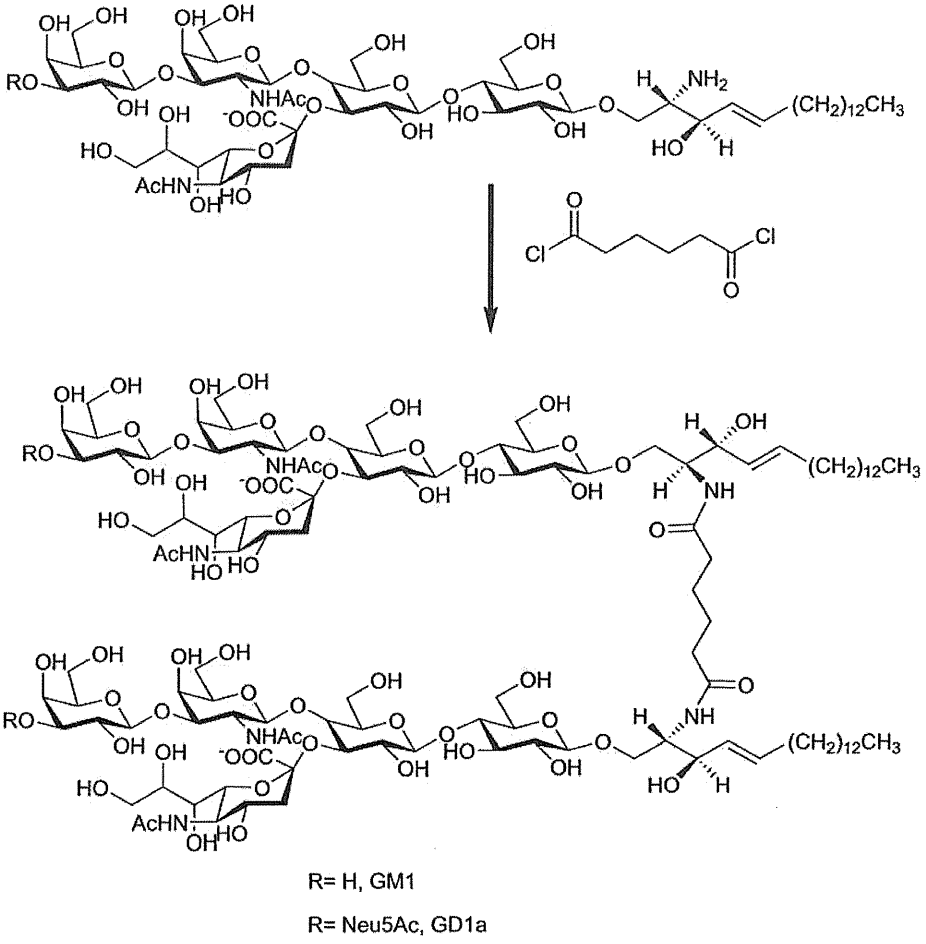

Fig. 1. Scheme of the preparation of ganglioside dimers GM1–GM1 and GD1a–GD1a.

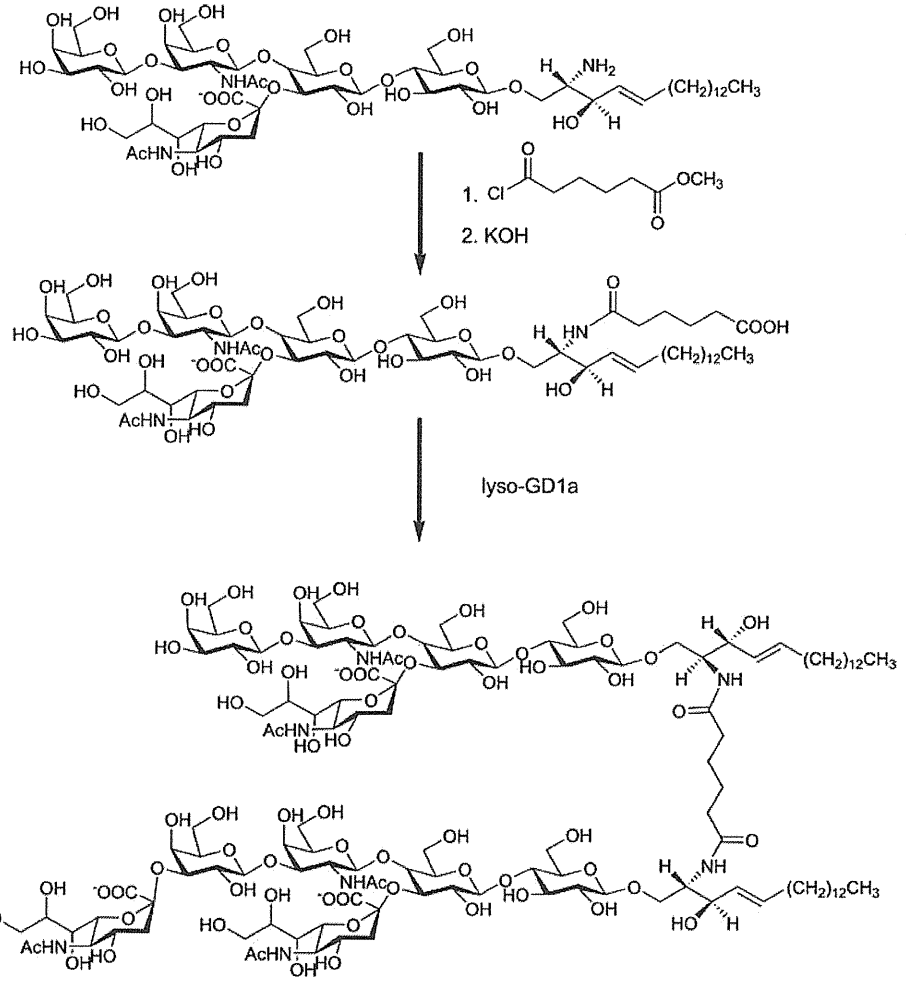

Fig. 2. Scheme of the preparation of ganglioside hybrid dimer GM1–GD1a.

812, 819 and 826 for GM1–GM1, GM1–GD1a and GD1a–GD1a, respectively. Figure 4 shows the mass spectra of the hybrid dimer GM1–GD1a. Less abundant signals corresponding to differently charged ions were also in the spectra.

The hybrid dimer GM1–GD1a was characterized by high-resolution NMR experiments. Table I reports the proton NMR assignments and Figure 5 a portion of the ^{1}H-^{13}C HSQC (heteronuclear single-quantum coherence)-NOESY (nuclear overhauser effect spectroscopy) spectrum. Amphiphilic compounds, like gangliosides and ganglioside/phospholipid mixtures, form big aggregates not suitable for NMR analyses in water solution. To mimic a membrane organization, experiments in water were carried out inserting the hybrid dimer GM1–GD1a into DPC aggregates. These aggregates have been shown to be small and to display high mobility in solution; this allowed to obtain high-resolution spectra (Poppe et al. 1994).

Figure 5 shows a correlation between the IV Gal-H1 of the monosialyl oligosaccharide chain and the IV Gal-H2 of the disialyl oligosaccharide chain. This suggests a possibility that the two oligosaccharide chains should be locked in a quite rigid conformation forming a single epitope.

GD1a has an additional sialic acid with respect to GM1 and it is very well separated by high-performance TLC (HPTLC). Figure 3 shows the results of TLC immunostaining with GBS serum. Both gangliosides were not recognized by the serum from a patient with GBS in the HPTLC-immunostaining procedure. Nevertheless, if GD1a is applied on the plate above GM1 to have a less separation from GM1, an immunostained spot with chromatographic behavior intermediate between GM1 and GD1a was observed. We know that the chromatographic resolution is not absolute and that each ganglioside is represented on the TLC plate by a large spot due to heterogeneity of the lipid moiety. Between the two spots of GM1 and GD1a, we expect to have a mixture of the more hydrophilic species of GM1 (short-chain fatty acids) and less hydrophilic species of GD1a (long-chain fatty acids). Of course, the serum antibodies recognized the GM1/GD1a mixture in a dot-spot assay.

The three dimers GM1–GM1, GD1a–GD1a and GM1–GD1a are separated each other by HPTLC. The dimeric hybrid GM1–GD1a was very well recognized by the GBS serum (Figure 3). As a control, we used dimeric GM1 and dimeric GD1a. No reactivity was observed with the patient

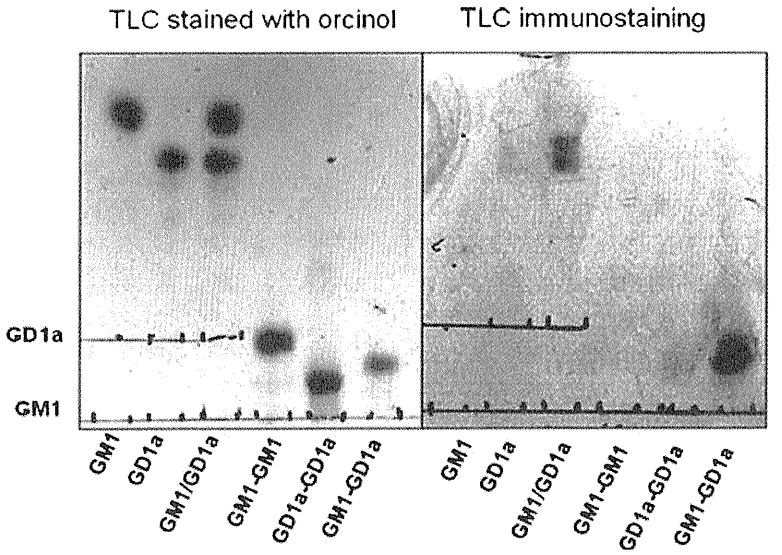

Fig. 3. HPTLC and HPTLC immunostaining of gangliosides and ganglioside dimers. Two micrograms of GM1, GD1a, a mixture of GM1 and GD1a, GM1–GM1 dimer, GD1a–GD1a dimer and GM1–GD1a hybrid dimer was applied to a TLC plate, developed with a solvent system chloroform:methanol:0.2% $CaCl_2$-$2H_2O$ (50:45:10, v/v). The left panel was stained by the orcinol reagent and the right panel was immunostained by a patient serum IgG (diluted 1:100) specifically reactive with a mixture of GM1 and GD1a. Anti-human IgG Fc, diluted to 1:200 with 1% BSA in PBS, was used as a secondary antibody. Each horizontal line indicates the start line for GM1 and GD1a. The start line for each dimer is same as that for GM1.

serum, excluding any possible artifact deriving by the presence of two general oligosaccharide chains. This strongly suggests that both the GM1 and GD1a chains are necessary for a strong interaction and to maintain the stability of the antibody–antigen complex.

Twelve sera that had been confirmed to be IgG positive for a mixture of GM1 and GD1a according to the criteria reported by Kaida et al. (2004) were used for the ELISA using the three dimers as an antigen (Table II). The 12 sera showed no reactivity against GM1 and no or minor reactivity against GD1a, suggesting that both the oligosaccharide chain must be present for antibody–antigen complex formation. Among the sera, 10 showed strong antibody activity to the GM1–GD1a hybrid dimer, whereas the two sera #3 and 11 did not. Sera #3 and 11, from patients that had ophthalmoplegia, showed a strong cross-reactivity against GD1b, GQ1b and GT1a. Within the sera that recognize the hybrid dimer GM1–GD1a, sera #4, 5 and 6 reacted with GD1b, but not with GQ1b or GT1a.

Discussion

Several data (Sonnino et al. 2006; Prinetti et al. 2009; Sonnino and Prinetti 2010; van Zanten et al. 2010) have been reported on the segregation of gangliosides in membranes and on the role of gangliosides in stabilizing lipid membrane domains known as lipid rafts. The existence of lipid rafts subdomains has been also reported (Lingwood and Simons 2010). This is probably one of the reasons that make the oligosaccharide chain cryptic reducing the possibility of an oligosaccharide–serum antibody interaction. Sometimes, this occurs and it is followed by a process of serious neuropathy. Sometimes, this interaction, in in vitro assays, requires an epitope obtained mixing two structurally different gangliosides. Anti-ganglioside complex (GSC) antibodies, in vivo, may recognize a conformational epitope formed by two different gangliosides on the lipid rafts.

Antibodies against a GM1/GD1a complex were detected in 39 of 234 GBS sera (Kaida et al. 2007). A subset of anti-GM1/GD1a-positive patients also had the antibodies against GD1a/GD1b and/or GD1b/GT1b complexes, which are significantly associated with severe GBS (Kaida et al. 2007). The antibodies against the GM1/GalNAc-GD1a complex were associated with pure motor GBS or acute motor conduction block neuropathy (Kaida, Sonoo, et al. 2008). The antibodies against GSCs containing GQ1b or GT1a are present in the Fisher syndrome and related disorders (Kanzaki et al. 2008). In contrast, the binding activities of the antibodies highly specific to GD1b, associated with sensory ataxic neuropathy, are decreased by the addition of such gangliosides as GD1a to GD1b antigen due to the interaction between the two gangliosides and resultant conformational change (Kaida, Kamakura, et al. 2008). A similar inhibitory effect of neighboring gangliosides on the antibody binding has also been reported by the other research groups (Greenshields et al. 2009; Nobile-Orazio et al. 2010). Therefore, the analyses of the clustered gangliosides on the plasma membrane are more and more important for clarifying the pathogenetic mechanisms of autoimmune neuropathies.

We successfully prepared the hybrid dimer GM1–GD1a. In addition, we also prepared the GM1–GM1 and GD1a–GD1a dimers that were very useful in biological assays as controls. The synthesis was carried out from lysogangliosides and did

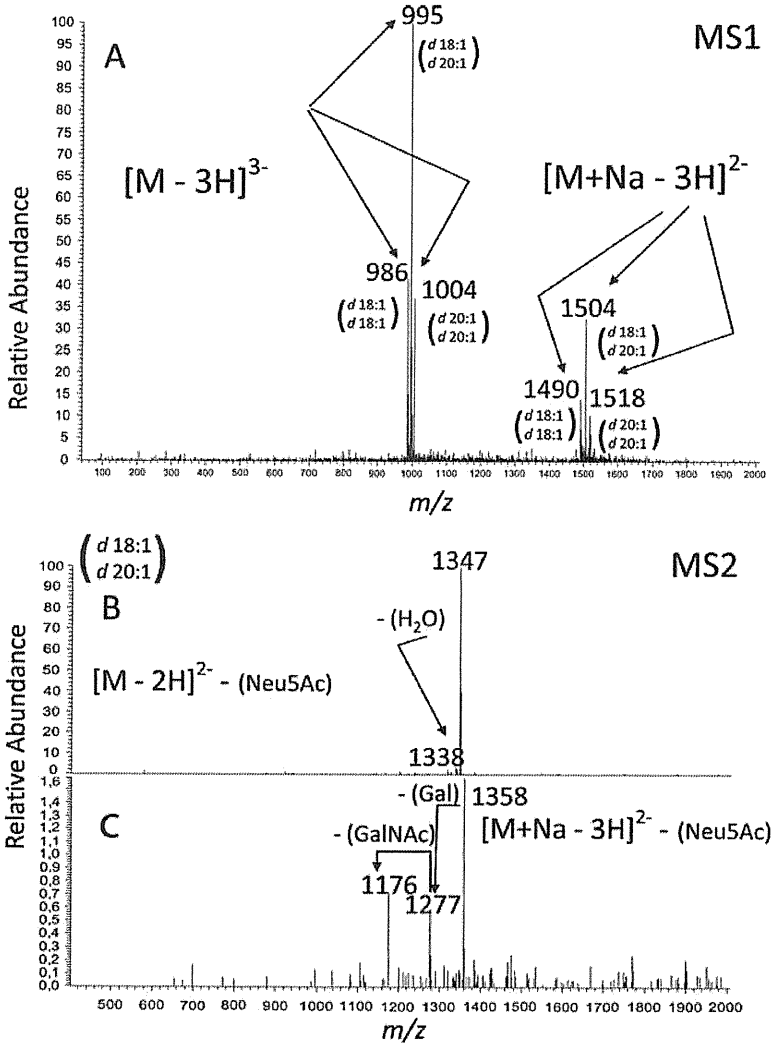

Fig. 4. Negative-ion ESI/MS spectra of ganglioside hybrid dimer GM1–GD1a. **(A)** MS1 spectrum of the double-charged ions and triple-charged ions, resulting from the ionization of the carboxylic group of sialic acid, from the three different possible combinations between lysogangliosides containing 18:1 and 20:1 sphingosine. **(B)** MS2 spectrum from ion at 995; the loss of an Neu5Ac residue originated bi-charged ions. **(C)** MS2 spectrum from ion at 1504.

not present specific difficulties and the final yields were from 50 to 70%. The availability of procedures for the preparation of several lysogangliosides, such as lyso-GM3, lyso-GM1, lyso-GD1a and lyso-GD1b, opens the possibility to prepare several ganglioside hybrid dimers.

Most of the antibodies specifically reactive with a mixture of GM1 and GD1a recognized the GM1–GD1a hybrid dimer, suggesting that, at least in some cases, the hybrid dimer actually mimics the GM1/GD1a complex, a novel antigen formed by the carbohydrate structures of GM1 and GD1a gangliosides.

However, the two sera #3 and 11 (Table I) did not recognize the GM1–GD1a dimer. Both of the patients 3 and 11 had ophthalmoplegia and anti-GQ1b and anti-GT1a IgG antibody activities in the sera. It indicates that a difference exists between the antigenic epitope of the GM1/GD1a complex and that of the GM1–GD1a dimer and that some of the IgG antibodies reactive with both GQ1b and GT1a could cross-react to the GM1/GD1a complex but not to the GM1–GD1a dimer. These results suggest that the position and the features of protein domain(s) interacting with the epitope(s) are different, also if all our sera recognized the GM1/GD1a complex.

Many further NMR experiments will be necessary to have the complete secondary structure of the two chains of the hybrid dimer GM1–GD1a. This is due to the complexity of the spectra, to some pick overlapping and to the fact that some interactions occur via water bridges (Brocca et al. 1998). Nevertheless, while chemical-shift assignments suggest that the conformation reported for the chains in the natural gangliosides should be maintained in the hybrid dimer GM1–GD1a, a defined interaction between the two external galactoses could be observed (Figure 5). Thus, the covalent linkages of the two glucoses with the lipid moiety and the hydrogen bond interactions at the end of the two chains

Table I. Chemical shifts of hybrid dimer GM1–GD1a in DPC aggregates in D_2O at 30°C

GM1 part				GD1a part				Ceramide part		
		^{1}H (ppm)	^{13}C (ppm)			^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)
Glc (I)	1	4.40	103.01	Glc (I)	1	4.40	103.01	1a	3.69	69.55
	2	3.29	72.79		2	3.29	72.79	1b	4.13	69.61
	3	3.60	74.33		3	3.60	74.33	2	3.87	53.45
	4	3.56	79.05		4	3.56	79.05	3	4.01	71.10
	5	3.54	74.92		5	3.54	74.92	4	5.37	130.23
	6	3.75, 3.93	60.31		6	3.75, 3.93	60.31	5	5.66	133.25
Gal (II)	1	4.48	102.84	Gal (II)	1	4.48	102.84	6	1.93	32.76
	2	3.32	70.09		2	3.32	70.09	7		
	3	4.10	74.47		3	4.10	74.47	8		
	4	4.08	77.36		4	4.08	77.36	9		
	5	3.71	74.32		5	3.71	74.32	10		
	6	3.7–3.8	60.5–61.3		6	3.7–3.8	60.5–61.3	11	1.2–1.3	29.0–30.3
GalNAc (III)	1	4.74	102.66	GalNAc (III)	1	4.74	102.66	12		
	2	4.00	51.17		2	4.00	51.17	13		
	3	3.76	80.65		3	3.76	80.65	14		
	4	4.12	67.98		4	4.12	67.98	15		
	5	3.68	74.50		5	3.68	74.50	16		
	6	3.7–3.8	60.5–61.3		6	3.7–3.8	60.5–61.3	17	1.20	22.50
	7				7		174.73	CH_3	0.77	13.52
	8	1.96	22.75		8	1.96	22.75			
Gal (IV)	1	4.49	104.90	Gal (IV)	1	4.56	104.66			
	2	3.48	70.80		2	3.49	69.26			
	3	3.58	72.73		3	4.04	75.63	Linker part		
	4	3.87	68.75		4	3.91	67.59		^{1}H (ppm)	^{13}C (ppm)
	5	3.64	74.93		5	3.63	74.77	1	2.11, 2.21	35.65
	6	3.7–3.8	60.5–61.3		6	3.7–3.8	60.5–61.3	2	1.49, 1.54	25.20
NeuAc (A)	1			NeuAc (A)	1		180.60			
	2				2		101.52			
	3a	1.89	37.13		3a	1.89	37.13			
	3e	2.62	37.14		3e	2.62	37.14			
	4	3.74	68.52		4	3.74	68.86			
	5	3.77	51.75		5	3.77	51.75			
	6	3.45	73.23		6	3.45	73.23			
	7	3.55	68.24		7	3.55	68.24			
	8	3.71	72.36		8	3.71	72.36			
	9a	3.59	62.97		9a	3.59	62.97			
	9b	3.83	62.97		9b	3.83	62.97			
	10				10		175.07			
	11	1.99	22.21		11	1.99	22.21			
				NeuAc (B)	1		174.17			
					2		99.86			
					3a	1.76	39.74			
					3e	2.71	39.78			
					4	3.64	68.53			
					5	3.79	51.82			
					6	3.58	72.96			
					7	3.55	68.24			
					8	3.85	71.95			
					9a	3.59	62.74			
					9b	3.83	62.73			
					10		175.07			
					11	1.99	22.21			

should arrange the eight neutral sugars in a quite fixed and rigid conformation. This suggests that the GM1–GD1a oligosaccharide system behave as a single epitope recognized by a single-antibody domain. This domain is present in 10 of the 12 sera analyzed. The remaining two sera #3 and 11 should have two distinct domains, one for the GM1 oligosaccharide and one for the GD1a oligosaccharide. Thus, these two sera are capable to recognize a mixture of GM1 and GD1a but not the single epitope formed by the two interacting chains.

The availability of hybrid gangliosides, containing two or more oligosaccharide chains, mimicking the cluster of oligosaccharide chains occurring on cell membranes, provides a new tool to analyze anti-oligosaccharide and correlated them with the clinical features of the pathology. In addition to this, their use to generate monoclonal antibodies could be useful to develop animal models. It should open new perspectives for understanding the pathogenesis and developing a novel therapy of GBS.

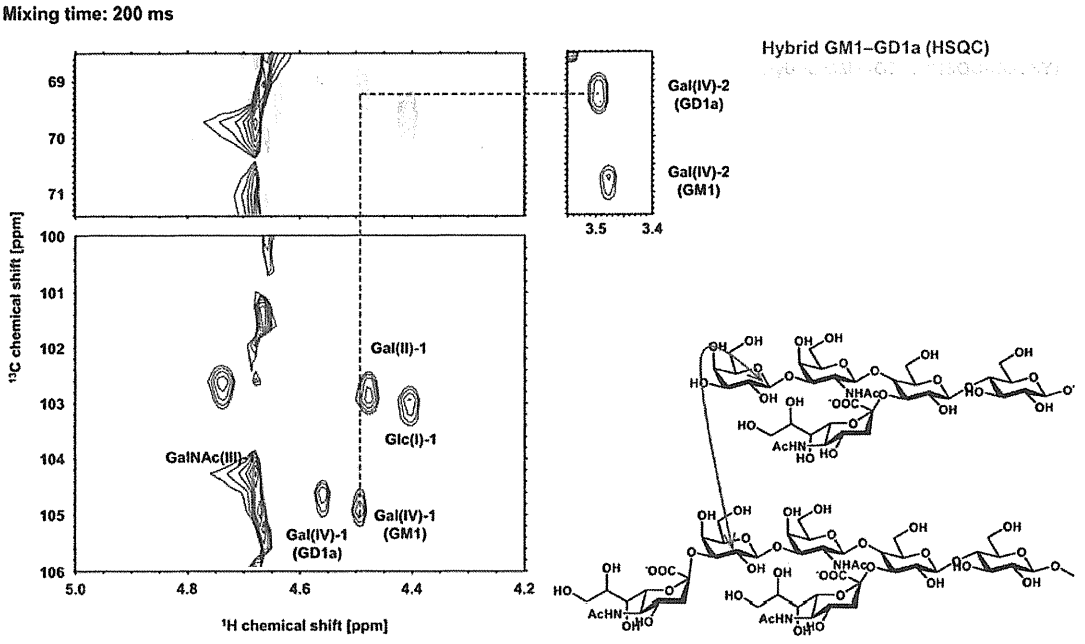

Fig. 5. Portion of the ^{1}H-^{13}C HSQC-NOESY spectrum of ganglioside hybrid dimer GM1–GD1a mixed with DPC-d_{38} in 10 mM phosphate buffer (pH 7.0) D_2O solution at 2.0 mM final concentration. Mixing time was 200 ms.

Table II. OD of the ELISA

Patient number	GM1	GD1a	GM1/ GD1a mixture	GM1–GM1 dimer	GD1a–GD1a dimer	GM1–GD1a hybrid dimer	IgG antibodies to other gangliosides
1	<0.1	<0.1	0.516	<0.1	<0.1	0.799	No
2	<0.1	0.154	0.52	0.134	0.104	0.489	No
3	<0.1	0.145	0.65	<0.1	<0.1	<0.1	GD1b, GQ1b, GT1a
4	<0.1	<0.1	0.664	<0.1	<0.1	0.583	GD1b
5	<0.1	<0.1	0.49	0.111	<0.1	0.742	GD1b
6	<0.1	0.135	0.519	<0.1	<0.1	0.548	GD1b
7	<0.1	0.199	0.497	<0.1	0.131	0.482	No
8	<0.1	0.111	0.671	<0.1	0.151	0.678	No
9	<0.1	<0.1	0.605	<0.1	<0.1	0.34	No
10	<0.1	<0.1	0.594	<0.1	0.127	0.68	No
11	<0.1	<0.1	0.382	<0.1	<0.1	<0.1	GD1b, GQ1b, GT1a
12	<0.1	0.218	0.774	<0.1	0.101	0.751	No

Antigens: GM1, GD1a, GM1 and GD1a mixture, GM1–GM1 dimer, GD1a–GD1a dimer and GM1–GD1a hybrid dimer.

Materials and methods

Chemicals

LiChroprep RP18 for column chromatography (particle size, 40–63 μm), silica gel 100 (particle size, 63–200 μm) and 60 (particle size, 15–40 μm), Amberlite® IR-120 H^+ form (particle size 300–900 μm) and high-performance silica gel-precoated thin-layer plates (HPTLC, Kieselgel 60) were obtained from Merck (Darmstadt, Germany). All the chemicals were of the highest purity available. The solvents were distilled before use and deionized water was freshly distilled in a glass apparatus. Dialysis tubes 12.000–14.000 Da were from Medicell International Ltd (London).

Gangliosides GM1 and GD1a were extracted from calf brain, purified to homogeneity and characterized by NMR and MS. Lyso-GM1 and lyso-GM1 were prepared from the corresponding natural gangliosides (Neuenhofer et al. 1985; Sonnino et al. 1992).

Methods

Preparation of GM1 and of GD1a dimers. To a solution of 0.15 M adipic acid in CH_2Cl_2/toluene 2:1 by vol., five equivalents of thionyl chloride were added drop wise at −10° C (Figure 1). After standing for 10 min at the same temperature, the reaction mixture was heated at 50°C for 3 h under reflux. The exceeding of thionyl chloride was removed by evaporation at reduced pressure and the product used without further purification. Adipoyl chloride and tributylamine were added to a solution of lyso-GM1 or lyso-GD1a in anhydrous CH_2Cl_2/CH_3OH 1:2 by vol. (50 μmol/mL) to a final molar ratio of 0.5:2:1. After vigorous stirring for 2 h at room temperature, the reaction mixture was dried and the residue purified by flash chromatography with silica gel 60 column equilibrated and eluted with $CHCl_3/CH_3OH/H_2O$ 60:35:5, by volume. The yield of reaction, after purification, was 70%.

Preparation of hybrid GM1–GD1a dimer. To a solution of 0.15 M mono-methyl adipate in CH_2Cl_2/toluene 2:1 by vol., five equivalents of thionyl chloride were added drop wise at −10°C (Figure 2). After standing for 10 min at the same

temperature, the reaction mixture was heated at 50°C for 3 h under reflux. The exceeding of thionyl chloride was removed by evaporation at reduced pressure and the product used without further purification.

Methyladipoyl chloride and tributylamine were added to a solution of lyso-GM1 in anhydrous CH_2Cl_2/CH_3OH 1/2 (50 μmol/mL) to a final molar ratio of 1:2:1. After vigorous stirring at room temperature for 2 h, the reaction mixture was dried and the residue was dissolved in 0.5 M KOH in CH_3OH. After standing at room temperature for 18 h, the solution was neutralized and the solvent was removed. The residue was dissolved in water and the salts removed by dialysis. After lyophilization, the GM1 adipate was obtained as a white powder and used without further purification. The reaction mixture was analyzed by HPTLC, using the solvent systems $CHCl_3/CH_3OH/0.2\%$ $CaCl_2$ 50:42:11 by volume.

Dicyclohexylcarbodiimide and *N*-hydroxysuccinimide, both at a final concentration of 0.02 M, were added to a 0.017 M solution of GM1 adipate, in dry dimethylformamide. After stirring for 30 min at room temperature, the lyso-GD1a was added to give a final concentration of 0.02 M. The reaction was allowed to proceed at 60°C under vigorous stirring for 1 h. The reaction mixture was dried and the residue purified by flash chromatography with silica gel 60 column equilibrated and eluted with $CHCl_3/CH_3OH/H_2O$, 60:35:5 by vol. The yield of reaction, after purification, was 70%.

High-performance TLC. Ganglioside, ganglioside derivatives, ganglioside dimers and the reaction mixtures were analyzed by HPTLC using the solvent systems $CHCl_3/CH_3OH/0.2\%$ $CaCl_2$, 50:42:11 by vol., $CHCl_3/CH_3OH/0.2\%$ $CaCl_2$/50 mM KCl, 50:50:4:8 by vol. or $CHCl_3/CH_3OH/(CH_3)_2CHOH$/50 mM KCl, 30:35:15:13 by vol., according to the polarity of compounds.

Gangliosides, ganglioside derivatives and dimers were visualized on the HPTLC plates by treatment with anisaldehyde and *p*-dimethylaminobenzaldehyde spray reagents followed by heating at 130°C; amine-containing compounds were visualized by treatment with 20% methanolic ninhydrin followed by heating at 80°C.

For HPTLC immunostaining, 2 μg of gangliosides GM1 and GD1a of dimers GM1–GM1, GD1a–GD1a and GM1–GD1a were applied to the TLC plate and developed with the solvent of chloroform/methanol/aqueous 0.2% $CaCl_2$ 50:45:10, by vol. The line of application for GD1a was 1 cm above that for GM1 and the dimers. Sera positive for the IgG anti-GM1/GD1a complex antibody were overlaid for the TLC plate at a dilution of 1:100 (Kaida et al. 2004). Peroxidase-conjugated goat anti-human IgG Fc antibody (diluted 1:200, ICN Biomedicals Inc., Aurora, OH) was the secondary antibody. Immunoreactants were made visible with phosphate-buffered saline (PBS) containing 0.01% H_2O_2 and 50 mg/dL 3,3′-diaminobenzidine tetrahydrochloride.

Enzyme-linked immunosorbent assay. Sera from 12 GBS patients that displayed the IgG antibody against a mixture of GM1 and GD1a were examined for their reactivity against the hybrid dimer GM1–GD1a. ELISA was performed using purified gangliosides GM1 and GD1a, a mixture of GM1 and GD1a, the dimers GM1–GM1, GD1a–GD1a and GM1–GD1a. Each microtiter well was coated with 0.2 μg of each antigen. Serum diluted 1:40 with 1% bovine serum albumin (BSA) in PBS was added to wells, followed by the procedure as described previously (Kaida et al. 2004). Peroxidase-conjugated goat anti-human IgG Fc antibody (diluted 1:200, ICN Biomedicals Inc.) was the secondary antibody. A color reaction was obtained by incubation with 200 μL of orthophenylenediamine dihydrochloride (40 mg/dL of phosphate–citrate buffer, pH 5.0) at room temperature for 2 min. The reaction was stopped by the addition of 8 N H_2SO_4, after which the optical density (OD) at 492 nm was read with an ELISA reader. OD values were corrected by subtracting the OD of an uncoated well that had been processed similarly (Table II). When the corrected OD was >0.1, the serum was considered positive. ELISAs were repeated twice in the same way, and the mean OD of the two experiments was calculated. Antibody activities against other gangliosides, such as GM2, GM3, GD1b, GD3, GalNAc-GD1a, GT1a, GT1b and GQ1b, also were examined as described elsewhere (Kaida et al. 2004).

Mass spectrometry. ESI-MS was carried out in a negative mode on a ThermoQuest Finnigan LCQdeca mass spectrometer equipped with an electrospray ion source and an Xcalibur™ data system. Samples were dissolved in methanol at a concentration of 20–200 ng/μL prior to direct injection into the electrospray ionization ion-trap mass spectrometer (MS). Ions were monitored as MS1 or MS2 product ions. Ionization was performed under the following conditions: spray voltage, 4 kV; sheath gas flow rate, 50 arbitrary units; capillary temperature, 260°C; capillary voltage, −42 V. The scanning range was *m/z* 200–1600, and fragmentor voltage for collision induced dissociation was 25–90%.

Nuclear magnetic resonance. Two-dimensional NMR experiments (HSQC, heteronuclear 2 bond correlation, heteronuclear multiple bond correlation, chemical-shift correlated spectroscopy, HSQC-total correlation spectroscopy, HSQC-NOESY and NOESY) were performed with JEOL ECA-920 or JEOL ECA-600 spectrometers at 30°C. The pulse delay time of 2 s was used. All NMR spectra were processed and analyzed using Delta (JEOL) and Sparky (T. D. Goddard and D. G. Kneller: SPARKY 3, University of California, San Francisco, CA). Experiments were carried out on the hybrid dimeric GM1–GD1a inserted in a micelle of dodecylphosphocholine (DPC)-d_{38} in 10 mM phosphate buffer (pH 7.0) D_2O solution. The hybrid dimer GM1–GD1a/DPC molar ratio was 1:52 and the final hybrid dimer GM1–GD1a concentration was 2.0 mM.

Quantitative determinations. Gangliosides, ganglioside derivatives and ganglioside dimers were quantified by their sialic acid content using the HCl–resorcinol method and pure *N*-acetylneuraminic acid as a reference standard (Svennerholm 1957).

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Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; DPC, dodecylphosphocholine; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; GBS, Guillain–Barré syndrome; GD1a, $IV^3NeuAc,II^3NeuAc\text{-}Gg_4Cer$; GD1b, $(II^3NeuAc)_2\text{-}Gg_4Cer$; GM1, $II^3NeuAc\text{-}Gg_4Cer$; GQ1b, $(IV^3NeuAc)_2,(II^3NeuAc)_2\text{-}Gg_4Cer$; GT1a, $(IV^3NeuAc)_2,II^3NeuAc\text{-}Gg_4Cer$; GSC, ganglioside complex; HPTLC, high-performance thin-layer chromatography; HSQC, heteronuclear single-quantum coherence; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser effect spectroscopy; OD, optical density; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

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ORIGINAL ARTICLE

Neuropathophysiological potential of Guillain–Barré syndrome anti-ganglioside-complex antibodies at mouse motor nerve terminals

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Abstract

Objectives: Anti-ganglioside antibodies are present in approximately half of Guillain–Barré syndrome (GBS) patients. Recently, it has been shown that a considerable proportion of these patients has serum antibodies against antigenic epitopes formed by a complex of two different gangliosides. However, direct experimental evidence for neuropathogenicity of this special category of antibodies is currently lacking. Here, we explored a series of GBS and GBS-variant sera with anti-ganglioside-complex antibodies for their ability to induce complement-dependent deleterious effects at the living neuronal membrane.

Methods: The neuropathophysiological potential of 31 GBS sera containing either anti-GM1/GD1a- or anti-GM1/GQ1b-ganglioside-complex antibodies was studied at motor nerve terminal presynaptic membranes in the mouse phrenic nerve/diaphragm muscle *ex vivo* experimental model. With electrophysiological measurements and confocal fluorescence microscopy, we assessed and quantified the damaging effect on neuronal membranes by anti-ganglioside-complex antibodies.

Results: We show that anti-GM1/GD1a- and anti-GM1/GQ1b-ganglioside-complex positive sera can induce complement-mediated functional and morphological injury at mouse motor nerve terminals *ex vivo*. Of the 31 investigated anti-ganglioside-complex patient sera, 17 sera induced increases in miniature end-plate potential frequency in this experimental model, mostly associated with muscle fibre twitches. Variability in potency was observed, with the anti-GM1/GD1a-complex sera inducing the most outspoken effects.

Conclusions: The present study shows the presence of ganglioside-complexes as available antigens in living neuronal membranes and supplies proof-of-principle that anti-ganglioside-complex antibodies in sera from GBS patients can induce complement-mediated damage. This strongly supports the hypothesis that autoimmune targeting of ganglioside-complexes is of pathogenic relevance in a proportion of GBS patients. (Clin. Exp. Neuroimmunol. doi: 10.1111/j.1759-1961.2011.00022.x, September 2011)

Introduction

Gangliosides form a family of sialic acid-containing amphiphilic glycosphingolipids that are enriched in neuronal membranes. Anti-ganglioside antibodies can be detected in approximately 50% of patients suffering from Guillain–Barré syndrome (GBS) or clinical variants, which are postinfectious peripheral neuropathies with diverse motor and sensory disturbances.[1–3] Anti-ganglioside antibodies are thought to exert neuropathogenic effects, either directly or through complement activation, on peripheral nerve axons including motor nerve terminals.[3–5]

It has recently been recognized that combinations of two different gangliosides can form a novel antigenic glycoepitope and that some GBS patients have antibodies against such a complex.[1,6,7] Clinical correlation and fine-specificity studies estimate that 10–20% of GBS patients has anti-ganglioside-complex antibodies.[2,8,9] Interestingly, GBS patients with anti-ganglioside-complex antibodies seem to have more severe disease symptoms than patients with antibodies against single gangliosides. In particular, GBS patients (from a Japanese population) with antibodies directed against GD1a/GD1b- and GD1b/GT1b-complexes more often require mechanical ventilation.[8] However, this was not confirmed by others in an Italian GBS patient cohort.[2] In the GBS variant, Miller Fisher syndrome (MFS), associated with anti-GQ1b ganglioside antibodies and characterized by ophthalmoplegia, ataxia and areflexia, the incidence of anti-complex antibodies seems higher than in GBS. One study showed that seven of the 12 investigated MFS sera contained antibodies against a complex of at least GQ1b or GT1a and another ganglioside.[10]

The observations that anti-ganglioside-complex antibodies disappear on clinical recovery,[9] as occurs with antibodies against single gangliosides, in combination with their association with particular disease phenotypes, suggests that they play a neuropathogenic role. However, this has not yet been directly shown in experiments on living neuronal membrane. Previously, we have shown that antibodies against single gangliosides can induce complement-mediated damage at neuronal membranes of motor axon terminals in mouse neuromuscular junctions (NMJs).[4] This effect is electrophysiologically hallmarked by a temporary extremely high frequency of miniature end-plate potentials (MEPPs; the postsynaptic responses to uniquantal acetylcholine release), causing asynchronous muscle fiber twitches and, eventually, depletion of neurotransmitter, which results in transmission block and thus paralyzes the muscle. Here, we explored a first series of 21 GBS sera with anti-GM1/GD1a-complex antibodies and 10 GBS variant sera with anti-GM1/GQ1b-complex antibodies for their ability to induce complement-dependent deleterious effects in the mouse NMJ model system.

Methods

Patient sera and mouse monoclonal antibodies

Acute-phase serum from 31 GBS (variant) patients from Japan (26 sera), the Netherlands (four sera) and Bangladesh (one serum) were obtained with informed consent and local Medical Ethical Committee approval, and stored until experimental use at −80°C. Normal human serum (NHS) from a healthy donor, stored in 0.5-mL aliquots at −80°C, was used as the complement source. Mouse monoclonal antibodies (mAbs) against GQ1b (CGM3; 50 μg/mL), GD1a (MOG35; 100 μg/mL) and GM1 (DG2; 100 μg/mL) were used as positive controls.[4,11–13] Patient sera were complement-inactivated by heating at 56°C for 30 min. Sera and mAbs were dialyzed (using a 10-kDa molecular weight cut-off dialysis membrane) overnight at 4°C against Ringer's solution (116 mmol/L NaCl, 4.5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 1 mmol/L NaH_2PO_4, 23 mmol/L $NaHCO_3$, 11 mmol/L glucose, pH 7.4), pre-gassed with 95% – O_2/5% – CO_2.

Enzyme-linked immunosorbent assay

Sera were tested in ELISA as described[9] for IgM and IgG antibody activity against individual gangliosides and complexes, as shown in the Results. Serum (1:100) scoring an optical density (OD) of >0.2 was considered positive. For anti-ganglioside-complex antibodies, positivity in sera was defined as having an OD of >0.2 higher than the highest OD for antibodies against the two individual gangliosides.[9,14] All samples were tested in duplicate. Positive sera were titrated using twofold serial dilution series starting at 1:100. The reciprocal of the highest dilution that resulted in an OD higher than the cut-off value (0.2) was taken to be the titer.

Bioassays on mouse hemidiaphragm-phrenic nerve preparations

Male and female wild-type and GD3-synthase knockout (GD3s-KO) mice[15] were used at 1–4.5 months-of-age. GD3s-KO mice lack the b- and c-series gangliosides as a result of a genetic absence

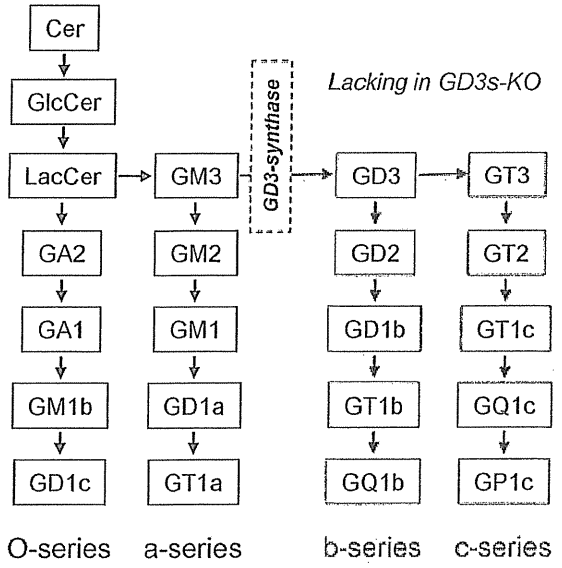

Figure 1 Ganglioside synthesis scheme. Ganglioside nomenclature is according to Svennerholm.[23] Membranes of wild-type mice contain all indicated gangliosides. GD3-synthase knockout (GD3s-KO) mice lack b- and c-series gangliosides (grey rectangle), as a result of the absence of GD3s. Cer, ceramide; GluCer, glucosylceramide; LacCer, lactosylceramide.

of GD3-synthase (Fig. 1).[16] Mice were killed with CO_2 and hemidiaphragms with phrenic nerves were dissected and kept in Ringer's medium at room temperature (20–22°C). Muscles were incubated with heat-inactivated (30 min at 56°C to destroy complement) GBS serum diluted at 33% in Ringer's medium or mouse mAbs dissolved in Ringer's medium for 3 h at 32°C, rinsed in Ringer's medium for 10 min and exposed to 33% NHS in Ringer's medium for 1 h at room temperature. Microelectrode recording of MEPPs (10–30 NMJs per session) and visual scoring of spontaneous asynchronous fiber twitching (0 for no twitching across the hemidiaphragm, 1 for twitching of <10 fibers, 2 for a small amount, 3 for a moderate amount and 4 for an extensive amount) were carried out as described.[15,17] Depending on the available volumes, sera were tested one to four times and the mean values of the parameters were calculated. Animal experiments were carried out according to Dutch law and Leiden University guidelines, including approval of the Experimental Animal Committee of the Leiden University Medical Center.

Complement immunohistochemistry

C3c deposition at NMJs was quantified in a selection of the electrophysiologically tested samples, as described previously.[11] IgG and membrane attack complex deposition at NMJs, as well as neurofilament presence at the terminal motor nerve, was qualitatively determined with confocal fluorescence microscopy, as described previously.[11,17]

Statistical analysis

The statistical significance of the correlation between the serum titer of antibodies against ganglioside-complexes, the mean MEPP frequency elevation a serum had induced at mouse NMJs and the C3c complement deposition values were tested for with the Spearman's rank correlation test using the VassarStats Website for Statistical Computation (http://faculty.vassar.edu/lowry/VassarStats.html), with P-values <0.05 considered as statistically significant.

Results

Anti-ganglioside-complex antibodies tests

On the basis of the ELISA results, sera were classified into two categories (Table 1): (i) anti-GM1/GD1a-complex positive (21 sera: 1–21); and (ii) anti-GM1/GQ1b-complex positive (10 sera: 22–31), as determined by the center of origin. In view of the possible interlaboratory variation in anti-ganglioside antibody assays,[18] all sera were retested in ELISA for IgG and IgM antibodies against gangliosides GM1, GQ1b, GD1a and GD1b, and ganglioside complexes GM1/GQ1b, GM1/GD1a, GM1/GD1b and GD1a/GD1b. Anti-ganglioside-complex positivity was confirmed in all but two sera (22 and 29), which were negative for anti-GM1/GQ1b-complex. They were still included, because they had been defined (low) positive when tested in Japan. Many sera (19/31) showed additional activity against one or both of the individual gangliosides of the complex, but generally these titers were only very low and always much lower than the titer of the anti-complex antibodies (Table 1). We reviewed the clinical neurophysiological data (mostly from the arm muscle and nerve) from the patients whose serum was tested in the present study (Table 1). Data were available from 20 of the 21 anti-GM1/GD1a-complex positive patients. The majority (15/20) had a reduced compound muscle action potential amplitude. Distal motor latency was normal in most patients (13/20), but was increased in seven of the 20 patients. From six of the 10 anti-GM1/GQ1b-complex positive patients, the neurophysiological data was available and was all normal, except for a reduced compound muscle action potential of patient 30.

Table 1 Patient neurophysiology, serum anti-ganglioside characteristics and neuropathogenicity of the investigated sera

	Patient neurophysiology		Anti-ganglioside titer			GD3s-KO mouse tissue Pathophysiology			Wild-type mouse tissue Pathophysiology		
Serum number	CMAP	DML	GM1/GD1a complex	GM1	GD1a	fMEPP (/s)	Twitching	C3c staining	fMEPP (/s)	Twitching	C3c staining
Anti-GM1/GD1a-complex sera											
1	=	=	G-25600	G-800	G-6400	58.9	+	++	1.6	–	–
2	↓	=	G-12800	G-400	G-1600	49.3	++	+	3.2	–	–
3	↓	=	G-51200	G-400	G-800	45.0	++	+	NT	NT	NT
4	↓	=	G-12800	G-400	G-400	36.8	++	+++	0.5	–	NT
5	↓	↑	G-6400	–	–	28.8	++	++	NT	NT	NT
6	=	=	G-6400	–	G-100	27.0	++	+++	0.6	–	NT
7	↓	=	G-12800	G-1600	G-100	19.8	++	+++	0.7	–	NT
8	↓	↑	G-12800	G-1600	G-100	16.2	++	++	NT	NT	NT
9	↓	↑	G-6400	G-100	G-100	14.2	++	++	NT	NT	NT
10	↓	=	G-6400	G-100	–	6.6	–	NT	1.7	–	+
11	↓	=	G-12800	–	G-200	4.9	+	NT	4.6	–	++
12	↓	↑	G-400	M-200	–	3.5	–	NT	NT	NT	NT
13	=	↑	G-6400	G-100	–	2.1	–	NT	0.5	–	NT
14	ND	ND	G-200	–	–	2.0	–	+	NT	NT	NT
15	↓	↑	G-400	–	–	1.7	–	+	NT	NT	NT
16	↓	=	G-6400	–	–	1.6	–	NT	NT	NT	NT
17	=	=	G-400	–	–	1.4	+	NT	NT	NT	NT
18	=	=	G-3200	G-200	–	1.3	–	+	NT	NT	NT
19	↓	=	G-12800	G-100	G-800	1.1	–	NT	1.5	–	+++
20	↓	↑	G-1600	G-200	G-100	1.1	–	NT	2.0	–	NT
21	↓	=	G-3200	G-800	G-400	1.1	–	–	NT	NT	NT
			GM1/GQ1b complex	GM1	GQ1b						
Anti-GM1/GQ1b-complex sera											
22	=	=	–†	–	–	1.6	–	+	7.2	–	+
23	=	=	G-1600	–	G-400	1.8	–	+	6.0	+	++
24	ND	ND	G-800	–	–	1.4	–	+	4.6	++	+
25	ND	ND	G-800	–	–	2.1	+	NT	4.3	+	NT
26	ND	ND	G-3200	–	G-400	1.5	–	NT	3.3	+	NT
27	=	=	G-1600	–	–	1.9	–	NT	2.3	+	NT
28	ND	ND	G-400	–	–	2.3	–	NT	2.3	–	NT
29	=	=	–†	M-100	–	1.3	+	NT	1.7	–	NT
30	↓	=	G-400	–	–	1.7	–	NT	1.6	+	NT
31	=	=	G-800	–	–	1.2	–	NT	1.6	+	NT

Patient neurophysiology, serum anti-ganglioside antibody activity (either IgG [G] or IgM [M]), effects on miniature end-plate potential (MEPP) frequency (fMEPP), muscle fiber twitching and C3c deposition at neuromuscular junctions of sera positive for either anti-GM1/GD1a antibodies (sera 1–21) or anti-GM1/GQ1b antibodies (sera 22–31). The sera have been ranked in descending order according to the observed MEPP frequency (for anti-GM1/GD1a-complex sera in GD3-synthase knockout [GD3s-KO] tissue and for anti-GM1/GQ1b-complex sera in wild-type tissue).
Anti-ganglioside titer: –, negative; twitching: –, <1.0; +, between 1.0 and 2.0; ++, >2.0. C3c staining was carried out in two comparable runs on 17 and seven tissue samples. Shown is the relative intensity within these series: +, low; ++, moderate; +++, high.
CMAP, compound muscle action potential; DML, distal motor latency; ND; no data available; NT, not tested.
†These sera were tested positive for anti-GM1/GQ1b-complex in the center of origin.

Pathophysiological effects at mouse NMJs

The antigenic ganglioside density on neuronal membranes is an important factor in the pathogenicity of anti-ganglioside antibodies.[12] To optimize our experimental model for anti-GM1/GD1a-complex antibodies, we used diaphragm muscles of GD3s-KO mice.[16] These mice genetically lack GD3-synthase and are therefore unable to synthesize b- and c-series gangliosides (Fig. 1). There is direct biochemical

proof that these mice upregulate the membrane density of a-series gangliosides GM1 and GD1a in the brain,[16] and we have previously shown indirectly with electrophysiological and fluorescence microscopical methods using anti-GD1a and anti-GM1 mAb that this is also the case at the motor nerve terminal membrane at the NMJ.[11,12] At GD3s-KO NMJs, 12 of the 21 anti-GM1/GD1a-complex sera induced elevations of MEPP frequency (i.e. >2.4/s, twice the control mean) during the NHS incubation (range 3.5–58.9/s; pooled control mean before incubations was 1.2/s; Figs 2a and 3a, Table 1). The elevated MEPP frequencies correlated positively and in a statistically highly significant way with the titer of the anti-GM1/GD1a-complex antibodies ($P < 0.01$, $r = 0.65$, Spearman's rank correlation test; Fig. 4). Such a positive correlation was not observed between elevated MEPP frequency and the titer of possible additionally present antibodies against single gangliosides GM1 ($P = 0.196$, $r = 0.30$) or GD1a ($P = 0.07$, $r = 0.4$). From 10 of the 21 sera, sufficient serum was available to be also studied at wild-type NMJs; only two of those sera (two and 11) induced (moderate) elevation of MEPP frequency (to 4.6 and 3.2/s, respectively; Table 1). With five of the 10 anti-GM1/GQ1b-complex sera, moderately elevated MEPP frequencies were observed (range 3.3–7.2/s) at wild-type NMJs, without correlation with anti-GM1/GQ1b-complex titer ($P = 0.74$, $r = 0.12$, Spearman's rank correlation test). No effect of these sera was observed on MEPP frequency at GD3s-KO NMJs (Fig. 2a, Table 1), which was as expected because the neuronal membranes of these mutant mice lack ganglioside GQ1b (Fig. 1).

The elevated MEPP frequency induced by anti-ganglioside-complex sera was generally accompanied by irregular twitching of individual muscle fibers throughout the preparation, (Fig. 2b, Table 1). Such twitches are most likely caused by superimposed MEPPs crossing the firing threshold of muscle fibers and have also been observed in our previous studies on the pathophysiological effects of sera positive for antibodies against single gangliosides and of mouse monoclonal antibodies against single gangliosides.[17,19] Mean twitching score was <0.5 in control (pre-incubation) sessions and was similarly low with anti-GM1/GD1a-complex sera tested in wild-type muscle (range 0.0–0.6). In GD3s-KO muscles, 11 of these 21 sera scored >1.0 (range 1.0–2.8; Fig. 2 b). Seven of the 10 investigated anti-GM1/GQ1b-complex sera scored >1.0 in wild-type muscles (range 1.2–2.3, Fig. 2b). At GD3s-KO muscles, two of these sera scored >1.0 (1.7 and 1.9). Mean

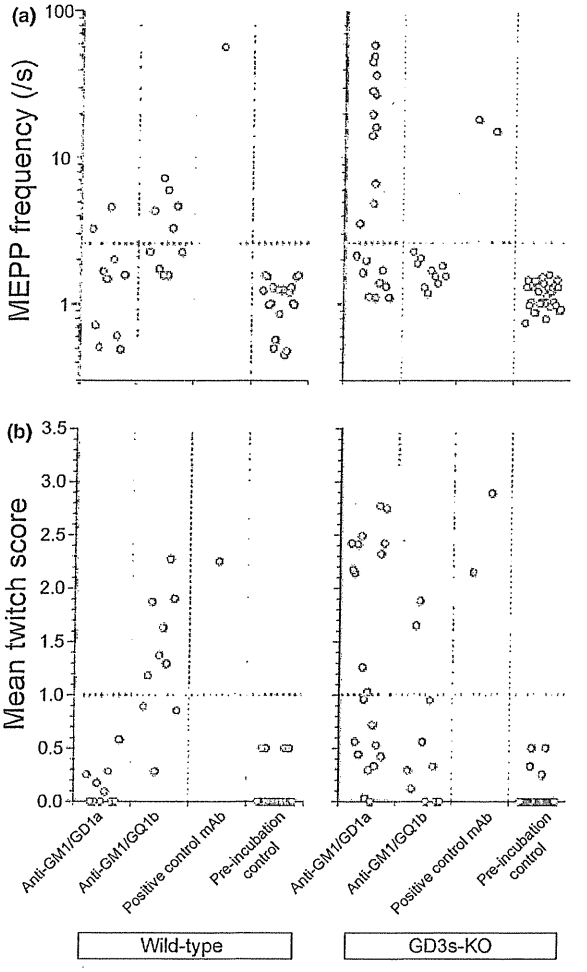

Figure 2 Overview of the neuropathophysiological effects of the investigated anti-ganglioside-complex sera. (a) Effect of anti-ganglioside-complex sera on of miniature end-plate potential (MEPP) frequency at wild-type (left panel) and GD3-synthase knockout (GD3s-KO; right panel) mouse diaphragm mouse neuromuscular junctions (NMJs). Twelve of the 21 tested anti-GM1/GD1a-complex sera induced MEPP frequency increases at GD3s-KO to a level of more than twice the pre-incubation control value (i.e. 2.4/s; dashed line). Just two of the 10 anti-GM1/GD1a-complex sera that could be tested at wild-type NMJs (modestly) increased MEPP frequency. Of the 10 investigated anti-GM1/GQ1b-complex sera, five induced (modest) MEPP frequency rise to levels of more than twice the control value, exclusively at wild-type NMJs. The anti-GQ1b mouse monoclonal antibodies (mAbs), CGM3, was used as a positive control in wild-type tissue; anti-GD1a mAb MOG35 and anti-GM1 mAb DG2 were used as positive controls in GD3s-KO tissue. (b) Effect of anti-ganglioside-complex sera on muscle fiber twitches at wild-type (left panel) and GD3s-KO (right panel) mouse NMJs. Most anti-ganglioside-complex sera that induced MEPP frequency elevation scored higher than 1 (dashed line) for muscle fiber twitching on visual inspection. Scoring: 0 = no twitching, 1 = twitching of <10 fibers, 2 = a small amount, 3 = a moderate amount and 4 = an extensive amount of fibers.

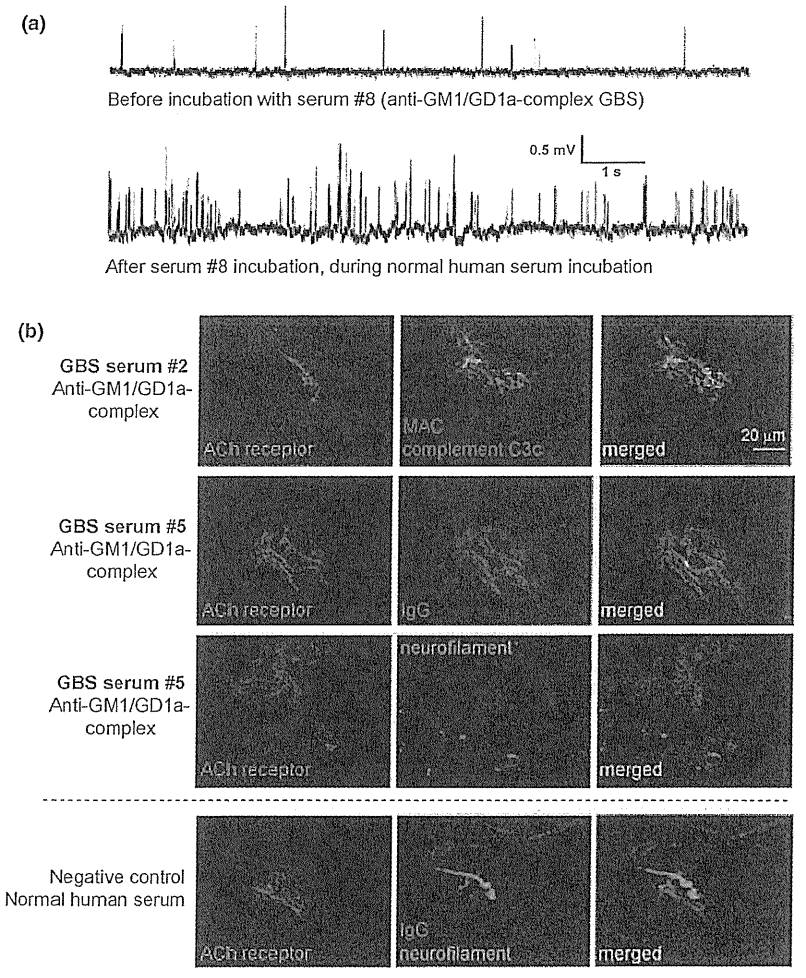

Figure 3 Examples of electrophysiological and morphological effects of anti-ganglioside-complex sera on the mouse motor nerve terminal experimental model. (a) Examples of miniature end-plate potentials (MEPPs) recorded at GD3-synthase knockout (GD3s-KO) mouse diaphragm neuromuscular junctions (NMJs) before incubation with complement-inactivated anti-GM1/GD1a-complex serum 8 (upper trace) and during subsequent incubation of the serum pre-incubated muscle with normal human serum as the complement source (lower trace). Sweep length is 10 s. (b) Examples of C3c, membrane-attack complex (MAC), IgG and neurofilament immunostaining at diaphragm NMJs (delineated by fluorescently labeled α-bungarotoxin binding to acetylcholine receptors, left column panels). Muscle preparations had been exposed to complement-inactivated anti-GM1/GD1a-complex serum 2 (first row), serum 5 (second and third row) or complement-inactivated normal human serum (as negative control, fourth row), and were all subsequently exposed to normal human serum as the complement source. IgG and C3c deposition, and associated neurofilament loss are shown at anti-GM1/GD1a-complex sera-treated NMJs.

positive control mAb score was >2.1; that is, at GD3s-KO NMJs, the anti-GM1 mAb DG2 scored 2.9 and the anti-GD1a mAb MOG35 scored 2.2; whereas at wild-type NMJs, the anti-GQ1b mAb CGM3 scored 2.3.

Complement deposition at the NMJ

In our previous studies on antibodies against single gangliosides, we have shown that these can induce complement activation, culminating in membrane attack complex formation at the presynaptic nerve terminal and that this is underlying the observed temporary dramatic increase of MEPP frequency at the NMJ as a result of the excessive influx of Ca^{2+} through membrane attack complex pores in the presynaptic neuronal membrane.[17,20,21] We investigated here with confocal fluorescence microscopy whether anti-ganglioside-complex antibody-containing sera also induced complement activation at the NMJ. For anti-GM1/GD1a-complex sera, complement C3c deposition at NMJs associated with elevated MEPP frequency ($P < 0.01$, Spearman's rank correlation test; Table 1, Fig. 3b). IgG and membrane attack complex deposition was observed at these NMJs, as well as neurofilament loss, indicating terminal motor axonal damage (Fig. 3b), as shown previously for antibodies against single gangliosides.[22] For anti-GM1/GQ1b-complex sera, C3c deposition at NMJs was sparse and not consistently associated with MEPP frequency elevation (Table 1).

Discussion

We report that sera positive for either anti-GM1/GD1a-complex or anti-GM1/GQ1b-complex antibodies clearly can produce pathophysiological effects at presynaptic neuronal membranes at NMJs