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.

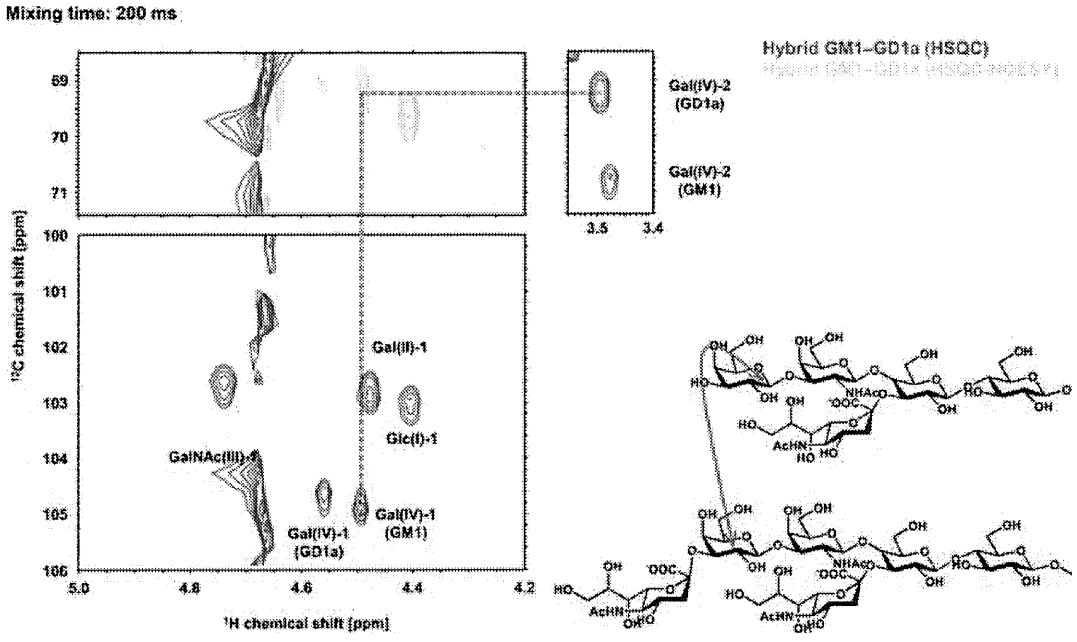

Fig. 5. Portion of the ^{1}H-^{13}C HSQC-NOESY spectrum of ganglioside hybrid dimer GM1–GD1a mixed with DPC-d_{38} in 10 mM phosphate buffer (pH 7.0) D_2O solution at 2.0 mM final concentration. Mixing time was 200 ms.

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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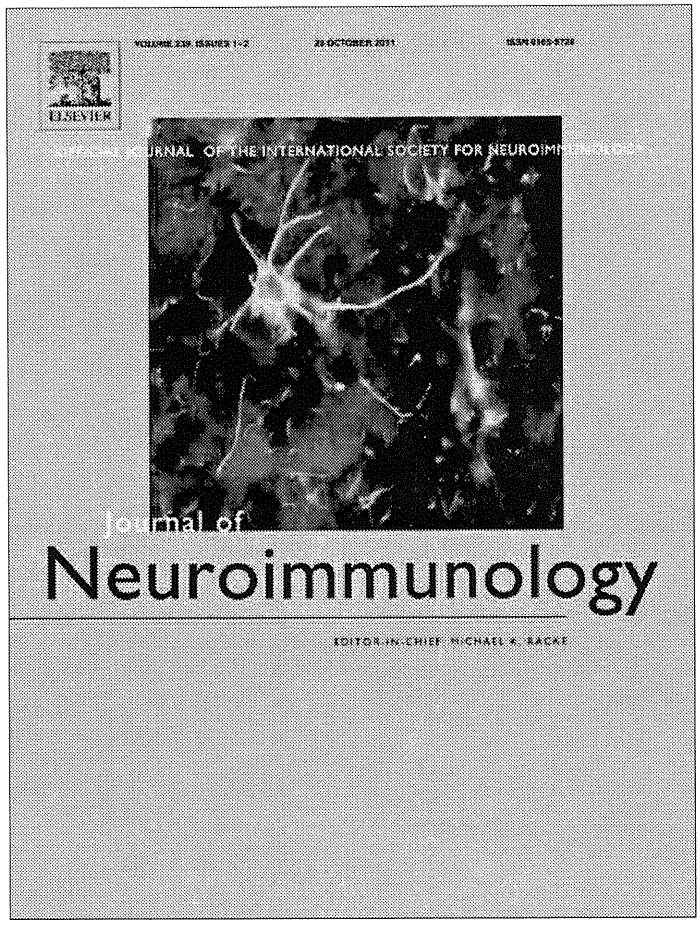

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Antibodies to LM1 and LM1-containing ganglioside complexes in Guillain–Barré syndrome and chronic inflammatory demyelinating polyneuropathy

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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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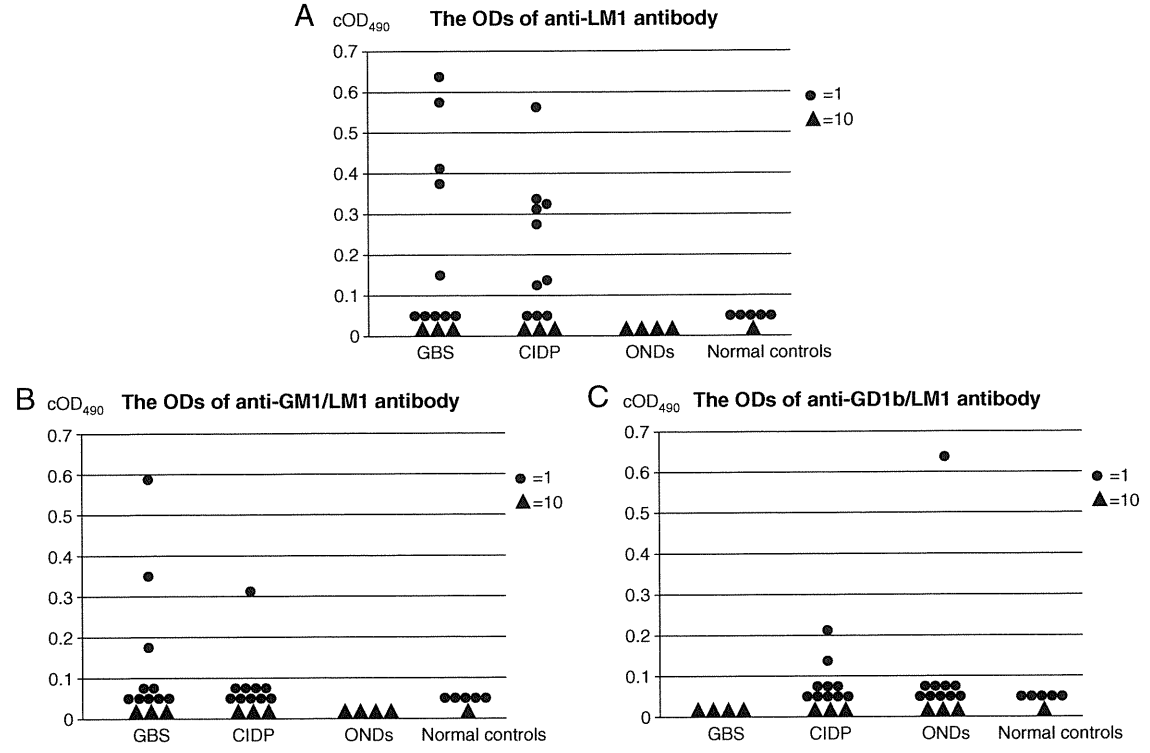

Fig. 1. The ODs of antibodies to LM1, GM1/LM1 and GD1b/LM1. Abbreviations: OD = optical density; CIDP = chronic inflammatory demyelinating polyneuropathy; GBS = Guillain–Barré syndrome; ONDs = other neurological diseases.

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.

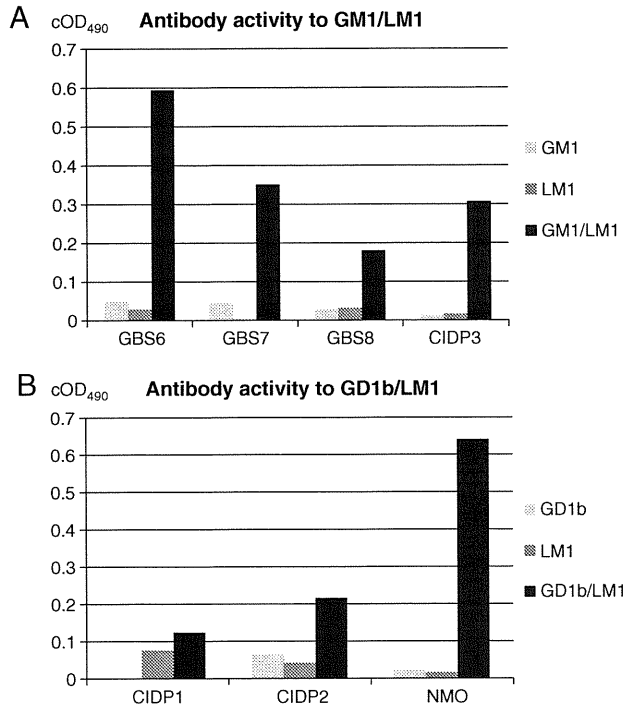

Fig. 2. The ODs of IgG antibodies to LM1-containing GSCs in antibody-positive patients. Abbreviations: OD = optical density; CIDP = chronic inflammatory demyelinating polyneuropathy; GBS = Guillain–Barré syndrome; NMO = neuromyelitis optica.

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).

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.

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;

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MINI-REVIEW

Antibodies against ganglioside complexes in Guillain–Barré syndrome and related disorders

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Abstract

Guillain-Barré syndrome (GBS) is acute autoimmune neuropathy, often subsequent to an infection. Serum anti-ganglioside antibodies are frequently elevated in titer. Those antibodies are useful diagnostic markers and possible pathogenetic factors. Recent data demonstrated that sera from some patients with GBS react with ganglioside complexes (GSCs) consisting of two different gangliosides, but not with each constituent ganglioside. Those antibodies may specifically recognize a new conformational epitope formed by two gangliosides. In particular, the antibodies against GD1a/GD1b and/or GD1b/GT1b complexes are associated with severe GBS requiring artificial ventilation. The antibodies to GM1/GalNAc–GD1a and those to GSCs containing GQ1b or GT1a are associated with pure motor GBS and Fisher syndrome, respectively. In contrast, the binding activities of the antibodies highly specific to GD1b are strongly inhibited by the addition of GD1a to GD1b. Gangliosides along with other components as cholesterol are known to form lipid rafts, in which two different gangliosides may form a new conformational epitope. Future investigation is necessary to elucidate the roles of GSCs in the plasma membrane and of the clinical relevance of the anti-GSCs antibodies.

Keywords: ganglioside, Guillain-Barré syndrome, membrane microdomain, peripheral nerve.

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Anti-ganglioside antibodies, mostly IgG type, are present in the sera from approximately 60% of patients with Guillain-Barré syndrome (GBS), acute immune-mediated polyradiculoneuropathy (Willison and Yuki 2002; Kusunoki *et al.* 2008; Van Doorn *et al.* 2008). Because the presence of anti-ganglioside antibodies in the acute-phase sera is a characteristic feature of GBS, those antibodies can be used as diagnostic markers of GBS. There are many molecular species of gangliosides, named depending on the carbohydrate sequences. Each ganglioside has unique distribution within the PNS. Considering the gangliosides are localized in the plasma membrane with their carbohydrate portions extended to the extracellular spaces, the anti-ganglioside antibodies may function in the pathogenesis of GBS through antibody-antigen interaction in PNS.

IgG anti-GQ1b antibody is one of the best studied antibodies. Ig anti-GQ1b antibodies are specifically associated with a variant of GBS, Fisher syndrome (FS) characterized by ophthalmoplegia and ataxia (Chiba *et al.* 1992). Anti-GQ1b monoclonal antibody specifically immunostains paranodal myelin of human cranial nerves innervating extraocular muscles (Chiba *et al.* 1993) and some large neurons in dorsal root ganglia (Kusunoki *et al.* 1999). It has recently been reported that the neuromuscular junctions of human extraocular muscles are richly bound by the antibodies against GQ1b and GT1a (Liu *et al.* 2009). Thus, the anti-GQ1b antibodies may cause opthalmoplegia and ataxia by binding to the regions where GQ1b is densely localized.

Measurement of anti-ganglioside antibodies has been conducted with ELISA or TLC-immunostaining by the use of purified single ganglioside antigens. Gagliosides have characteristics of forming clusters in the plasma membrane

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Abbreviations used: AMCBN, acute motor conduction block neuropathy; FS, Fisher syndrome; GBS, Guillain-Barré syndrome; GSC, ganglioside complex; LOS, lipooligosaccharides.

(Hakomori 2002). In the clusters, the carbohydrate structure of a ganglioside may interact with each other to form a novel epitope. We recently demonstrated that some GBS patients had serum antibodies that specifically recognize the novel glycoepitopes formed by two individual ganglioside molecules and named such antibodies as 'anti-ganglioside complex (GSC) antibodies' (Kaida *et al.* 2004).

Antibodies to ganglioside complexes in GBS

Antibodies to GD1a/GD1b and GD1b/GT1b complexes in severe GBS

Anti-GD1a/GD1b complex antibodies are the first identified antibodies against GSCs. We investigated a serum from a GBS patient who showed acute severe flaccid tetraparesis and needed artificial ventilation. We found an unidentified immuno-reactive band in the position just below GD1a on TLC of a crude ganglioside fraction from bovine brain. The serum was not reactive with any of such purified gangliosides as GM1, GM2, GM3, GD1a, GD1b, GD3, GalNAc–GD1a, GT1b, and GQ1b. But the serum IgG bound strongly to the well coated with the mixture of GD1a and GD1b gangliosides (GD1a/GD1b complex). When GD1a and GD1b were developed in the same lane on TLC using a developing solvent, chloroform/methanol/0.2%$CaCl_2 \cdot 2H_2O$ (50 : 45 : 10), the serum IgG strongly immunostained just the overlapping portion between GD1a and GD1b. When another developing solvent (C/M/0.2%$CaCl_2 \cdot 2H_2O$, 30/65/10) that completely separated the positions of GD1a and GD1b was used, no immunoreaction was identified. Those data indicate that mixing GD1a and GD1b may produce a new conformational glycoepitope which is different from that of GD1a or GD1b alone and the antibody in sera from the above patient may specifically recognize such a new glycoepitope.

We next investigated antibodies in sera from 234 GBS patients with ELISA using a mixture of two of the four major gangliosides (GM1, GD1a, GD1b and GT1b) (Kaida *et al.* 2007). The sera with anti-GSC antibodies often exhibited to some extent reactivity with constituent gangliosides of the GSCs. When optical density for the anti-GD1a/GD1b antibody was 0.2 higher than that corresponding to anti-GD1a or anti-GD1b antibody or it was more than the sum of those of anti-GD1a and anti-GD1b antibodies, the sera were judged to be anti-GD1a/GD1b-positive. The same criteria also were applied to the other GSCs. The cutoff value (0.2) for anti-GSC antibodies was decided arbitrarily. The results showed that 39 of 234 patients (17%) had antibodies against at least one of the mixture antigens. All the 39 patients had anti-GM1/GD1a antibodies, 27 had anti-GM1/GT1b antibodies, 16 had anti-GD1a/GD1b antibodies, and 13 had GD1b/GT1b antibodies. Most of anti-GD1a/GD1b or anti-GD1b/GT1b antibody reacted also with GM1/GT1b as well as GM1/GD1a. Immunoabsorption study suggested that anti-GSC antibodies specifically react with clustered glycoepitopes common to these GSCs, rather than individually with each GSC. An epitope formed by a combination of [Galβ1-3GalNAc] and [NeuAcα2-3Galβ1-3GalNAc] in the terminal moieties of ganglio-*N*-tetraose structures is likely to be essential for the antibody binding. Among them, antibodies against GD1a/GD1b and GD1b/GT1b complexes were significantly associated with severe GBS requiring artificial ventilation (Kaida *et al.* 2007). Those antibodies can be useful markers of severe GBS. Future study is needed to clarify why anti-GD1a/GD1b and GD1b/GT1b antibodies are associated with severe disabilities.

Antibodies to ganglioside complexes including GQ1b

Because FS is considered to be a variant of GBS, we extended an investigation of anti-GSC antibodies to FS patients. Presence of anti-ganglioside complexes antibodies in FS therefore was investigated with ELISA using seven ganglioside antigens; GM1, GM2, GD1a, GD1b, GT1a, GT1b and GQ1b (Kaida *et al.* 2006).

Acute phase serum samples were collected from 12 FS patients, 10 of whom had IgG anti-GQ1b antibodies. ELISA results showed that seven patients had antibodies to GSCs such as GQ1b/GM1, GQ1b/GD1b, GQ1b/GD1a, GQ1b/GT1b, GT1a/GM1, GT1a/GD1b, and GT1a/GD1a, but not to the complexes without GQ1b and GT1a. One patient had no anti-GQ1b or anti-GT1a antibodies, but had antibodies to GQ1b/GM1 and GT1a/GM1. Specific immunoreactivities against the overlapping portion of the two gangliosides were confirmed by TLC-immunostaining. In contrast to GBS, no FS patients had antibodies to the complexes consisting of two of the four major gangliosides, GM1, GD1a, GD1b and GT1b.

The results of anti-GSCs antibody assay on larger number of patients with FS and those with GBS with opthalmoplegia indicated that the serum antibodies could be subdivided into the three groups (Kanzaki *et al.* 2008): (i) antibodies specific to GQ1b and/or GT1a without anti-GSCs reactivity; (ii) antibodies that recognize a combination of [Galβ1-3GalNAc] and [NeuAcα2-8 NeuAcα2-3Galβ1-3GalNAc] in the terminal residues of ganglio-*N*-tetraose structures, such as antibodies to GQ1b/GM1, GQ1b/GD1b, GT1a/GM1, GT1a/GD1b (Fig. 1); and (iii) antibodies that recognize a combination of [NeuAcα2-3Galβ1-3GalNAc] and [NeuAcα2-8 NeuAcα2-3Galβ1-3GalNAc] in the terminal residues, such as antibodies to GQ1b/GD1a, GT1a/GD1a, GQ1b/GT1b, GT1a /GT1b. In addition, recent report showed that some patients have the antibodies specific to GQ1b/GA1 (Ogawa *et al.* 2009).

Sensory signs were infrequent in FS patients with antibodies to GQ1b/GM1 but were frequent in patients with other types of antibodies. However, the clinical relevance of such anti-GSC antibodies needs to be investigated in future.

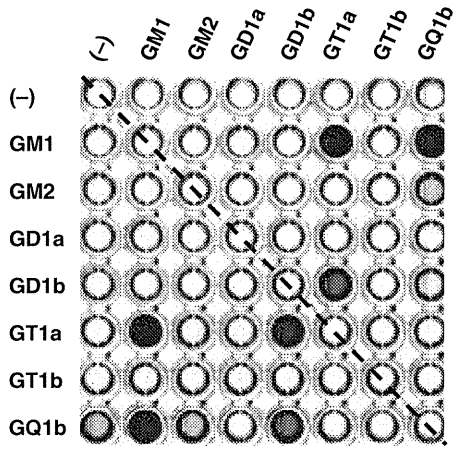

Fig. 1 An ELISA plate showing the binding activities of a serum antibody that recognizes a combination of [Galβ1-3GalNAc] and [NeuAcα2-8NeuAcα2-3 Galβ1-3GalNAc] in the terminal residues. All the wells in each line and column were coated with a respective ganglioside (e.g. the wells in the first line and column were coated only with a single ganglioside, the well in the eighth line and the second column was coated with GQ1b and GM1), except for those on the oblique dotted line that were uncoated control wells. The antibody binds strongly to GQ1b/GM1, GQ1b/GD1b, GT1a/GM1 and GT1a/GD1b but only weakly to GQ1b.

Antibodies to GM1/GalNAc–GD1a complex in pure motor GBS

IgG antibodies against GM1 or those against GalNAc–GD1a are known to closely correlate with acute motor axonal neuropathy (Kaida *et al.* 2000; Willison and Yuki 2002). We investigated antibody activities against the mixture of GM1 and GalNAc–GD1a (GM1/GalNAc–GD1a complex) in a large population of patients with GBS. The results showed that ten of 224 GBS patients had IgG antibodies to the GM1/GalNAc–GD1a complex (Kaida *et al.* 2008a).

We then analyzed the clinical and electrophysiologic findings of those 10 anti-GM1/GalNAc–GD1a-positive patients. Respiratory infections preceded the neurological onset in six cases and gastrointestinal infections in two cases. Therefore, although *Campylobacter jejuni* is an infectious agent that frequently causes the antecedent infection of GBS cases with anti-GM1 and anti-GalNAc–GD1a antibodies, *C. jejuni* may not be the major infectious agent inducing anti-GM1/GalNAc–GD1a complex antibodies. Cranial nerve involvement and sensory signs are infrequent. Early motor conduction block at intermediate nerve segments was found in five patients. Generally, the response to therapy was good. According to the criteria established by Hadden *et al.* (1998), four were categorized as demyelinative and two were axonal. When judged by other criteria (Ho *et al.* 1995), four were demyelinative and three were axonal.

Table 1 Representative anti-GSCs antibodies in GBS and FS

Antigen	Associated disease	Frequency (%)	Clinical features
GD1a/GD1b	GBS	7	Severe GBS
GD1b/GT1b	GBS	6	Severe GBS
GM1/GalNAc–GD1a	GBS	4	Pure motor GBS AMCBN
GQ1b/GM1 and related GSCs	FS	41	Infrequent sensory dysfunction
	GBS with OP	28	
GQ1b/GD1a and related GSCs	FS	6	
	GBS with OP	19	

GSC, ganglioside complex; GBS, Guillain-Barré syndrome; FS, Fisher syndrome; AMCBN, acute motor conduction block neuropathy; OP, ophthalmoplegia.
GQ1b/GM1 and related GSCs, GQ1b/GM1, GQ1b/GD1b, GT1a/GM1, GT1a/GD1b; GQ1b/GD1a and related GSCs, GQ1b/GD1a, GT1a/GD1a, GQ1b/GT1b, GT1a /GT1b.

The clinical findings of the 10 GBS patients were consistent with a pure motor variant of GBS. Clinical features of anti-GM1/GalNAc–GD1a IgG-positive GBS resemble those of acute motor conduction block neuropathy (AMCBN), in view of preserved sensory function, early conduction block at intermediate nerve segments and good recovery (Capasso *et al.* 2003). IgG anti-GM1 antibody (and sometimes anti-GalNAc–GD1a antibody) was reported in their sera. However, IgG anti-GM1 or anti-GalNAc–GD1a antibodies are frequently detected in sera of acute motor axonal neuropathy type GBS and conduction block is not common in such cases. Anti-GM1/GalNAc–GD1a antibody is likely to cause early reversible changes on the axolemma and may be more closely associated with AMCBN than the anti-GM1 or anti-GalNAc–GD1a antibody. GM1 and GalNAc–GD1a may form a complex in the axolemma at nodes of Ranvier or paranodes of the motor nerves, and may be a target antigen in pure motor GBS; especially in the form of AMCBN.

Representative anti-GSCs antibodies in GBS and FS are listed in the Table 1.

Antibodies against ganglioside complexes in chronic neuropathies

Nobile-Orazio *et al.* (2010) investigated serum IgM antibodies to GSCs in such chronic neuropathies as multifocal motor neuropathy, chronic inflammatory demyelinating polyradiculoneuropathy and IgM paraproteinemic neuropathy. As a result, one of 34 chronic inflammatory demyelinating polyradiculoneuropathy patients had IgM antibody activity to GT1b/GM1 and GT1b/GM2, and one of 23 IgM paraproteinemic neuropathy patients had IgM anti-GM2/GD1b activity.

Production of antibodies against ganglioside complexes

In GBS and related disorders subsequent to *C. jejuni* infection, anti-ganglioside antibodies are shown to be induced by the immune reaction against lipo-oligosaccharides (LOS) of pathogens causing antecedent infection (Willison and Yuki 2002; Van Doorn *et al.* 2008). A similar mechanism can be speculated in the production of anti-GSC antibodies. Kuijf *et al.* (2007) recently reported that such anti-GSC antibodies as anti-GM1/GD1a and GQ1b/GD1a cross-reacted to LOS from the autologous *C. jejuni* strain, indirectly demonstrating that the LOS contained GSC-like structures. However, carbohydrate structures expressed in the LOS may not exactly be the combination of the two carbohydrate chains expected from the reactivity of the serum anti-GSC antibodies.

Inhibition of the reactivity of the anti-ganglioside antibody by another coexistent ganglioside

If the interaction of two gangliosides creates a new epitope with conformational changes, the binding acitivity of the antibody highly specific to one ganglioside may be lessened by the addition of another ganglioside to make an antigen mixture.

We investigated sera from 17 GBS patients who had IgG antibody reactive only with GD1b in routine antibody assay. For those sera, antibody activity against a mixture of GD1b and another ganglioside was examined and compared the activity with that against GD1b alone. The results showed that the addition of GD1a, GT1a, GT1b, GQ1b and GalNAc–GD1a to GD1b caused marked decrease of the binding activity of anti-GD1b antibodies, suggesting that those gangliosides may interact with GD1b to make a novel epitope which cannot be easily recognized by the anti-GD1b antibodies (Kaida *et al.* 2008b).

In addition, the reduction rates of the binding activities caused by the addition of such gangliosides as GD1a, GT1b, GQ1b and GalNAc–GD1a were significantly more in the antibodies from ataxic patients than in those from non-ataxic patients. The addition of another ganglioside may cause conformational change. Therefore, the more specific the antibody is, the more affected its reactivity should be. It therefore suggests that the anti-GD1b IgG antibodies in ataxic patients may be more specific to GD1b than those in patients without ataxia. This may provide further evidence to the association between anti-GD1b antibody and ataxia (Kusunoki *et al.* 1996).

Thus, the antibodies specific to GD1a/GD1b complex are associated with severe GBS (Fig. 2) and those specific to GD1b itself are associated with the development of ataxia (Fig. 3).

A similar inhibitory effect of neighboring gangliosides has recently been reported in the case of anti-GM1 antibodies by

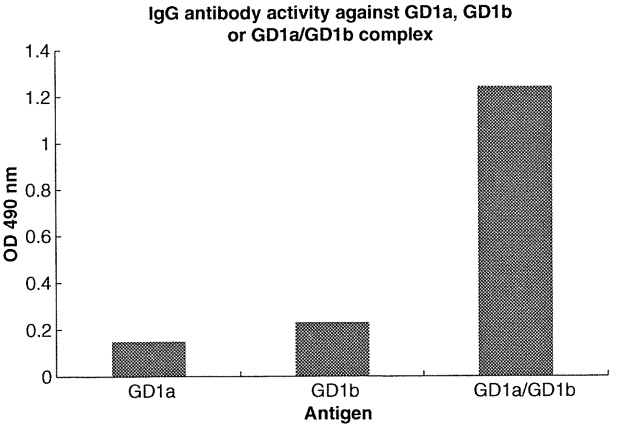

Fig. 2 ELISA result on a serum sample from a patient with severe Guillain-Barré syndrome (Kaida *et al.* 2004). This patient's serum IgG shows strong reaction with a mixture of GD1a and GD1b (GD1a/GD1b) but reacts only weakly with GD1a or GD1b alone.

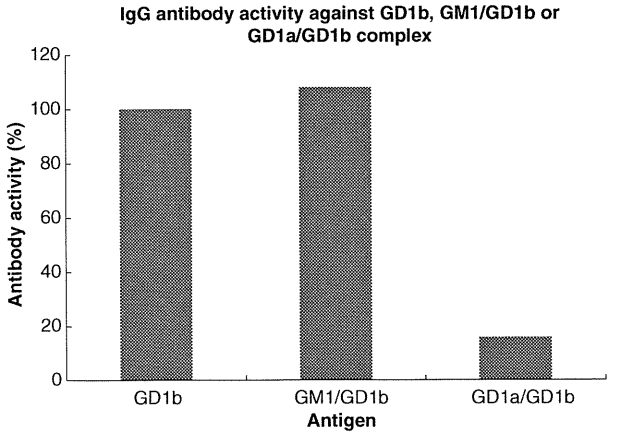

Fig. 3 The IgG antibody activities to mixture antigens in sera from nine GBS patients with ataxia who had only IgG anti-GD1b antibody in routine antibody assay (Kaida *et al.* 2008b). Bars of GM1/GD1b and GD1a/GD1b showed the average activities of the nine patients. Compared with the antibody activity to GD1b alone (100%), the activity was markedly reduced because of the addition of GD1a to GD1b antigen whereas the addition of GM1 did not affect the antibody activity.

Greenshields *et al.* (2009). Negative effects by gangalioside complexes on the binding of IgM anti-GM1 antibodies in sera from patients with chronic immune-mediated neuropathies, particularly multifocal motor neuropathy, have also been reported (Nobile-Orazio *et al.* 2010).

Future studies on the anti-GSC antibodies in the pathogenesis of autoimmune neuropathies

Gangliosides are located in the cell membranes with carbohydrate portions on the outer surfaces, and are preferentially

packaged with cholesterol, forming lipid rafts. Within rafts, gangliosides are considered to interact with important transmembrane receptors or signal transducers (Simons and Ikonen 1997; Hakomori 2002). Anti-GSC antibodies may cause dysfunction of the axon or Schwann cells through their binding to clustered epitopes of glycosphingolipids in the plasma membrane microdomains. Future study on the localization of each ganglioside complex is needed. Animal model of the autoimmune neuropathy mediated by anti-GSC antibodies should also be developed. Such investigations may lead to the understanding of the roles of GSCs in the plasma membrane and of the clinical relevance of the anti-GSCs antibodies.

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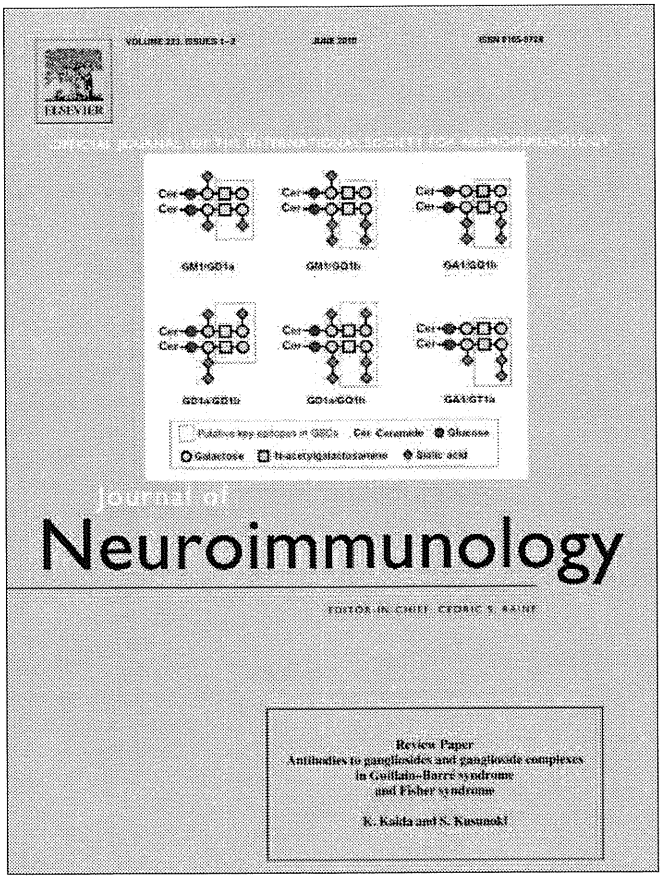

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Review article

Antibodies to gangliosides and ganglioside complexes in Guillain–Barré syndrome and Fisher syndrome: Mini-review

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ABSTRACT

Antiganglioside antibodies play a pathogenic role in the pathophysiology of Guillain–Barré syndrome (GBS) and Fisher syndrome (FS). Antiganglioside antibody-mediated nerve injury is likely to result from nerve damage through complement activation or dysfunction of molecules such as voltage-gated sodium and calcium channels. Clustered epitopes of complexes of two gangliosides in the cell membrane can be targeted by serum antibodies in GBS and FS and may regulate the accessibility and avidity of antiganglioside antibodies. The glycolipid environment or the specific distribution of target gangliosides in the peripheral nervous system may also influence the pathogenic effect of antiganglioside antibodies in GBS and FS. Structural and functional analyses of glycoepitopes of ganglioside complexes in membranes will provide new vistas on antibody–antigen interaction in GBS and shed light on microdomain function mediated by carbohydrate–carbohydrate interactions, which may lead to novel treatments for GBS and FS.

Contents

1. Introduction

Gangliosides are *N*-acetylneuraminic acid (sialic acid)-bearing glycosphingolipids that are concentrated in the outer leaflet of neuronal membranes with exposure of their oligosaccharides on the cell surface (Hakomori, 2000). Gangliosides are believed to reside in clusters within membrane microdomains that are referred to as lipid rafts or detergent-resistant membranes, together with other sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins (Simons and Toomre, 2000). Through molecular interactions with plasma membrane proteins at cell surfaces, the ganglioside glycans are involved in cell adhesion and intracellular

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signaling, myelin–axon interactions via Siglec (sialic acid-binding immunoglobulin-like lectin)-4, modulation of natural killer cell function, and inflammation through E-selectin, as addressed in a recent review (Lopez and Schnaar, 2009).

Gangliosides in the peripheral nervous system (PNS) can be targeted by serum antibodies in acute immune-mediated polyradiculoneuropathy, including Guillain–Barré syndrome (GBS) and variants such as Fisher syndrome (FS) (Chiba et al., 1992; Willison and Yuki, 2002); however, the pathogenic action of antiganglioside antibodies is not ubiquitously exerted in the PNS. Antiganglioside antibody-mediated nerve injury originates from antibody binding at specific loci in peripheral nerves, and is fundamentally regulated by antibody specificity and the specific distribution of target gangliosides (Chiba et al., 1993; Willison and Yuki, 2002; Kaida et al., 2009). Single ganglioside antigens have hitherto been utilized for conventional ELISA screening of antiganglioside antibodies. Recent studies have shown the presence of serum antibodies to ganglioside complexes (GSCs) consisting of two different gangliosides in GBS and FS (Kaida et al., 2004, Kaida et al., 2006, 2007), thereby emphasizing the significance of screening for antibodies to GSCs. Anti-GSC antibody-positive sera have no or little reactivity with constituent gangliosides, indicating that the sera react specifically with clustered glycoepitopes of GSCs. In this review we highlight the clinical and immunobiological aspects of the pathogenic action of antibodies to gangliosides and GSCs.

2. Correlation of clinical features with antiganglioside antibodies

Antiganglioside antibodies are often closely associated with clinical phenotype and specific symptoms (Willison and Yuki, 2002). This association is likely to depend upon the diverse distribution of ganglioside antigens in the peripheral nervous system.

2.1. Pure motor variant of GBS

The pure motor variant of GBS is characterized by no sensory loss, sparing of the cranial nerves, and predominant distal weakness, with frequent electrodiagnostic findings of acute motor axonal neuropathy (AMAN). This clinical phenotype is closely associated with antibodies to gangliosides such as GM1, GalNAc-GD1a, GD1a, and GM1b (Visser et al., 1995; Jacobs et al., 1996; Hao et al., 1999; Ang et al., 1999; Ho et al., 1999; Kaida et al., 2000; Yuki et al., 2000). The precise localization of GM1-like epitopes targeted by pathogenic anti-GM1 antibodies in human peripheral nerves has yet to be revealed, but recent analyses of a rabbit model of AMAN indicated that GM1 antigens are distributed at the nodes of Ranvier in motor nerves (Yuki et al., 2001; Susuki et al., 2003; Yuki et al., 2004). Using β1,4-N-acetylgalactosaminyltransferase (GalNAcT; GM2/GD2 synthase)-knockout mice, GM1 has been shown to play a role in maintaining the paranodal architecture and clusters of voltage-gated sodium channels (Susuki et al., 2007a).

GalNAc-GD1a is a minor ganglioside in the human brain and peripheral nerves (Svennerholm et al., 1973; Ilyas et al., 1988). An immunohistochemical study using rabbit anti-GalNAc-GD1a antibodies revealed that GalNAc-GD1a localizes in the vicinity of the nodes of Ranvier in human motor nerves, especially in the nodal and paranodal axolemmae (Kaida et al., 2003). An inner part of compact myelin and a periaxonal axolemma in the intramuscular nerves are also candidates for the target region of the anti-GalNAc-GD1a antibody (Kaida et al., 2003). Human motor and sensory nerves both contain GD1a, but the precise location of GD1a is unknown. As described below, structural differences of glycoepitopes of GD1a between motor and sensory nerves may explain the predisposition of the motor nerves for selective breakdown. The anti-GD1a antibody inhibits regeneration of damaged peripheral nerves, inducing delayed or poor recovery in patients with AMAN (Lehmann et al., 2007).

The tissue localization of GM1b in human PNS also remains to be determined. One study showed that among GBS patients with IgG anti-GM1b antibodies, 36% had IgG anti-GalNAc-GD1a antibodies and 32% had anti-GM1 antibodies, but the anti-GM1b antibodies were not associated with development of AMAN (Kusunoki et al., 1996a). In a collaborative study performed in Japan and the Netherlands, 56% of anti-GM1b-positive GBS patients had anti-GM1 antibodies and suffered from pure motor neuropathy, but there was no correlation between the presence of anti-GM1b antibodies and electrodiagnostic findings indicative of axonal neuropathy (Yuki et al., 2000).

2.2. Other phenotypes of GBS

IgG anti-GQ1b antibody has been identified as a diagnostic marker and a pathogenic factor in FS, and is often cross-reactive with GT1a (Chiba et al., 1992, 1993; Kusunoki et al., 1999b). An immunohistochemical investigation showed that GQ1b is densely localized in the paranodal regions of cranial nerves innervating the extraocular muscles and in a subpopulation of large neurons in dorsal root ganglia. Nerve terminals inside muscle spindles and in touch with intrafusal fibers can also be targeted by antibodies to GQ1b, GT1a, and GD1b (Liu et al., 2009). Therefore, GQ1b is likely to be a prime antigen in FS and the IgG anti-GQ1b antibody may cause ophthalmoplegia and ataxia through specific binding to these regions.

Acute neuropathy characterized by pharyngeal–cervical–brachial (PCB) weakness has been recognized as a variant of GBS, and a recent clinical study showed that PCB, GBS, FS, and Bickerstaff brainstem encephalitis form a continuous spectrum (Nagashima et al., 2007). A monospecific anti-GT1a antibody without GQ1b reactivity is essential for the development of bulbar palsy in patients with GBS (Nagashima et al., 2004). Human glossopharyngeal and vagal nerves contain both GQ1b and GT1a (Koga et al., 2002), but the localization of GT1a in human peripheral nerves has not been determined.

Monospecific anti-GD1b antibodies are likely to induce ataxia in GBS (Kusunoki et al., 1996b, 1999a; Kaida et al., 2008a). A recent analysis in a rabbit model of anti-GD1b-positive ataxic neuropathy indicated that an apoptotic mechanism in dorsal root ganglion cells is associated with development of ataxia (Takada et al., 2008), suggesting that activation of an apoptotic cascade plays a key role in development of ataxia in anti-GD1b-positive GBS.

3. Antiganglioside antibody-mediated pathophysiology in GBS and FS

3.1. Nerve injury through complement activation

Pathological studies on human specimens and recent experiments have shown that inappropriate activation of the complement cascade triggered by antiganglioside antibodies may induce nerve injury in GBS (Hafer-Macko et al., 1996a, 1996b; Lu et al., 2000; Putzu et al., 2000; Wanschitz et al., 2003; Willison et al., 2008). Especially, complement activation through the classical pathway is considered to be a key process in the development of GBS and FS (Willison et al., 2008). *Ex vivo* and *in vitro* experiments using mouse hemi-diaphragm preparations have shown that GQ1b-reactive monoclonal IgM antibodies and anti-GQ1b-positive sera impair neurotransmission at neuromuscular junctions (NMJs) through complement activation (Plomp et al., 1999; Goodyear et al., 1999). Among the classical, lectin, and alternative pathways of the complement activation system, activation of the classical pathway accompanied by MAC formation seems to play a central pathophysiologic role in experimental models of GBS and FS (Halstead et al., 2004; Halstead et al., 2005). In C6-deficient mice, monoclonal anti-GQ1b IgM antibodies do not provoke formation of MAC or increase MEPP frequency at NMJs. CD59-deficient ($CD59^{-/-}$) mice are unable to inhibit formation of MAC and are characterized by deposits of MAC and damage to perisynaptic

Schwann cells and neurofilament at nerve terminals (Halstead et al., 2004). Furthermore, this study demonstrated strong inhibition of MAC formation and loss of neurofilament under Ca^{2+}-free conditions, suggesting that activation of the classical pathway is essential for nerve injury since this pathway is Ca^{2+} dependent, whereas the alternative pathway is Ca^{2+} independent (Halstead et al., 2004). These observations indicate that nerve damage in GBS and FS occurs principally through antiganglioside antibody-mediated activation of the classical pathway.

3.2. Antibody-mediated dysfunction of ion channels in peripheral nerves

Recent *in vitro*, *in vivo*, and *ex vivo* studies suggest involvement of ion channels in the pathophysiology of GBS. The most potent molecules are ion channels associated with generation of muscle action potentials such as voltage-gated sodium channels (Navs). Dysfunction of Navs located and clustered at high density on the axonal membrane at the nodes of Ranvier may play an important role in the development of muscle weakness in GBS (Arasaki et al., 1993; Takigawa et al., 1995; Weber et al., 2000). GBS patients show marked refractoriness to axonal excitability in AMAN with IgG antibodies to GM1, GM1b, or GalNAc-GD1a (an increase in threshold current during the relative refractory period) followed by rapid normalization and a recovery of compound muscle action potentials (Kuwabara et al., 2002), suggesting that Nav dysfunction at the nodes of Ranvier is a primary cause of reversible conduction failure in GBS. AIDP patients without antiganglioside antibodies do not show similar refractoriness (Kuwabara et al., 2002). In view of localization of GM1-like epitopes and GalNAc-GD1a at high density at the nodes of Ranvier (Corbo et al., 1993; Sheikh et al., 1999; Kaida et al., 2003), anti-GM1 and anti-GalNAc-GD1a antibodies may directly or indirectly alter the regulatory function of Navs via antibody binding to antigens on the axonal membrane at the nodes.

Several studies have shown that anti-GM1 antibodies can exert a blocking effect on Navs at the nodes of Ranvier through complement activation (Arasaki et al., 1993; Takigawa et al., 1995; Weber et al., 2000; Santoro et al., 1992), but others have not found this blocking effect (Hirota et al., 1997; Dilley et al., 2003). It is intriguing that reversible disruption of Nav clusters with structural changes of the nodes was observed in ventral roots in a rabbit AMAN model immunized with a bovine brain ganglioside mixture including GM1 (Susuki et al., 2007b). Lengthened nodes and complement-mediated impairment of paranodal and nodal structures were also observed in the anti-GM1-positive rabbit model, with gradual recovery of these changes (Susuki et al., 2007b). Taken together, these findings suggest that antiganglioside antibody-mediated dysfunction of Navs is a principal pathogenesis in the AMAN variant of GBS. The prompt recovery (within one day) after immunomodulatory therapy that is often seen in clinical practice may be explained by functional blockage of Navs with little or no structural destruction of nodes.

Calcium (Ca) channels have been shown to be involved in the pathophysiology of GBS. In a co-culture of rat muscle-spinal cord cells, human and rabbit IgG anti-GalNAc-GD1a antibodies exerted a complement-independent inhibitory effect on acetylcholine (Ach) release at NMJs (Taguchi et al., 2004). Similarly, rabbit anti-GalNAc-GD1a-positive sera reversibly inhibits voltage-gated Ca channel currents of PC12 pheochromocytoma cells (Nakatani et al., 2007), and the Cav2.1 voltage-gated Ca channel current in cerebellar Purkinje cells is inhibited by sera containing IgG antibodies to GM1, GalNAc-GD1a, or GD1a (Nakatani et al., 2009). Such complement-independent inhibition of voltage-gated Ca channel current has also been observed in other *ex vivo* and *in vitro* studies using anti-GM1 or anti-GD1a monoclonal antibodies (Buchwald et al., 2007). Antibody–antigen interaction in the presynaptic membrane may cause inhibition of depolarization-induced calcium influx. The presynaptic membranes are likely to be susceptible to antiganglioside antibody attack because the blood–nerve barrier is absent and gangliosides are abundant in these membranes (Martin, 2003), but how target gangliosides interact with Ca channels in the presynaptic membrane remains to be elucidated. Taken together, the results showing complement-independent inhibition of voltage-gated Ca channel current at the presynaptic membrane may reflect an alternative pathophysiology in GBS, although clinical and electrophysiological examinations in GBS patients with antibodies to GM1, GD1a, or GalNAc-GD1a have not shown neuromuscular transmission failure.

4. Antibodies to ganglioside complexes in GBS

4.1. Clinical correlates of anti-GSC antibodies in GBS

Conventional measurement of antiganglioside antibodies has been done for purified single ganglioside antigens using enzyme-linked immunosorbent assays (ELISAs) or thin-layer chromatogram (TLC)-immunostaining. However, a mixture of two gangliosides can generate new epitopes that differ from those of the constituents and may be targeted by serum autoantibodies from GBS patients (Kaida et al., 2004). Such a mixture of gangliosides is referred to as a ganglioside complex (GSC). Antibodies to the GD1a-GD1b complex (GD1a/GD1b) were first found in GBS sera by ELISA and TLC immunostaining (Kaida et al., 2004). When GD1a and GD1b were developed such that they overlapped in the same lane on the TLC plate, the serum IgG reacted strongly with the overlapping portion (Fig. 1). With another developing solvent that produced completely separate positions of GD1a and GD1b, the reaction disappeared. In ELISA with GD1a, GD1b, and a mixture of the two, the serum IgG had a positive reaction only in a well coated with the mixture, with an optimal reaction at a GD1a to GD1b ratio of approximately 1 to 1. These findings indicate that a mixture of GD1a and GD1b induces formation of a GD1a/GD1b complex with a novel glycoepitope that differs from that of GD1a or GD1b.

We next investigated IgG antibodies to GSCs consisting of two of the four major gangliosides (GM1, GD1a, GD1b, and GT1b) using 234 GBS sera, and demonstrated that 39 sera (17%) had IgG antibodies to at least one GSC, including GD1a/GD1b, GM1/GD1a, GD1b/GT1b, GM1/GT1b, or GM1/GD1b (Table 1) (Kaida et al., 2007). All 39 anti-GSC-positive sera reacted with GM1/GD1a, 27 reacted with GM1/GT1b, 16 with GD1a/GD1b, 13 with GD1b/GT1b, and 6 with GM1/GD1b. Anti-GD1a/GT1b antibodies were not found in the sera. Since a particular combination of gangliosides is recognized by serum

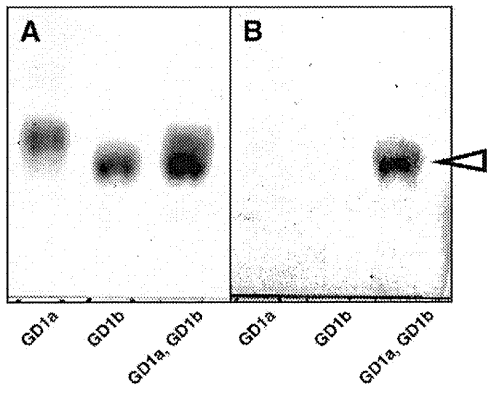

Fig. 1. Results from thin-layer chromatography (TLC). (A) TLC bands visualized with orcinol reagent. (B) TLC immunostaining using a representative anti-GD1a/GD1b-positive serum, showing that the overlapping region between GD1a and GD1b is strongly stained (arrowhead). Serum is diluted to 1:100.

Table 1
Antiganglioside complex IgG antibodies and the associated clinical features.

GSC antigens	Disorders (frequency)[a]	Clinical features
GM1/GD1a	GBS (17%)	
GM1/GT1b	GBS (12%)	
GD1a/GD1b	GBS (7%)	Severe disability, need for artificial ventilation, impairment of lower cranial nerves
GD1b/GT1b	GBS (6%)	Severe disability, need for artificial ventilation, impairment of lower cranial nerves
GM1/GalNAc-GD1a	GBS (3–11%)	Pure motor, AMCBN
GM1/GQ1b, GM1/GT1a, GD1b/GQ1b, GD1b/GT1a	FS (41%), GBS with OP (28%)	Infrequent sensory dysfunction
GD1a/GQ1b, GD1a/GT1a, GT1b/GQ1b, GT1b/GT1a	FS (6%), GBS with OP (19%)	
GA1/GQ1b, GA1/GT1a	FS, GBS, BBE	

GSC = ganglioside complex, GBS = Guillain–Barre syndrome, FS = Fisher syndrome, AMCBN = acute motor conduction block neuropathy, OP = ophthalmoplegia, BBE = Bickerstaff brainstem encephalitis.
[a] "Frequency" indicates frequency of anti-GSC antibodies in the disorder.

antibodies, an epitope formed by a combination of [Galβ1-3GalNAc] and [NeuAcα2-3Galβ1-3GalNAc] in the terminal residues of gangliotetraose structures is essential for antibody binding (Fig. 2). Most anti-GD1a/GD1b- or anti-GD1b/GT1b-positive sera also reacted with GM1/GD1a and GM1/GT1b, suggesting that they are more multivalent than the antibodies reacting only with GM1/GD1a or GM1/GT1b, or with a single ganglioside antigen. Predisposition to severe disability in patients with anti-GD1a/GD1b or anti-GD1b/GT1b antibodies may be associated with this multivalency. Whether GSCs consisting of three or more different gangliosides can be target antigens in GBS and its variants remains unclear. When mixtures of three or four gangliosides were used as antigens in ELISA, antibodies to GSCs consisting of two different gangliosides often decreased the antibody activities (Kaida et al., 2007). These results suggest that combinations of two gangliosides appear to form target epitopes in biological membranes.

Anti-GM1 and anti-GalNAc-GD1a antibodies are associated with a pure motor variant of GBS (Visser et al., 1995; Rees et al., 1995; Jacobs et al., 1996; Hao et al., 1999; Ang et al., 1999; Kaida et al., 2000, 2001). Pathological studies using peripheral nerve specimens from patients with AMAN suggest that AMAN-associated antigens are likely to be expressed in the axolemma of motor nerves, especially at the nodes of Ranvier (Hafer-Macko et al., 1996b). GM1-like epitopes are present in the axolemma at the nodes of Ranvier (Sheikh et al., 1999), although immunohistochemical studies of normal human peripheral nerves have not provided conclusive evidence for the distribution of the GM1 antigen (Kusunoki et al., 1993). GalNAc-GD1a is found in the vicinity of the nodes of Ranvier in human motor nerves; at nodal and paranodal axolemmae in the ventral roots and in a periaxonal axolemma-related region in intramuscular nerves (Kaida et al., 2003). From these findings, it can be speculated that GM1 and GalNAc-GD1a colocalize in the motor axolemma, and that antibodies to GSCs containing GM1 or GalNAc-GD1a may be associated with pure motor GBS. Actually, we found an antibody to a GSC consisting of GM1 and GalNAc-GD1a (GM1/GalNAc-GD1a) in 10 of 224 GBS sera (Kaida et al., 2008b), and the anti-GM1/GalNAc-GD1a-positive patients suffered from a pure motor variant of GBS, as expected. However, their electrophysiological findings featured early conduction block at intermediate nerve segments of motor nerves. In serial nerve conduction studies, the conduction block promptly improved and there were no findings indicative of remyelination or axonal degeneration. From these observations, we inferred that the conduction block results from reversible conduction failure on the axolemma at the nodes of Ranvier (Kuwabara et al., 1998; Kaida et al., 2008b). In view of the dense cluster of Navs at the nodes, antibody binding to GM1/GalNAc-GD1a at these nodes can cause reversible conduction block through alteration of the regulatory function of Nav. The prompt recovery after immune-mediated treatment such as IVIG may result from functional block with little or no pathological changes of the nodes. It remains to be determined whether the antibody–antigen interaction causes Nav dysfunction through complement activation or direct breakdown of Nav function, or both. Regardless, GM1, GalNAc-GD1a, and Nav may assemble in microdomains at the nodes of Ranvier.

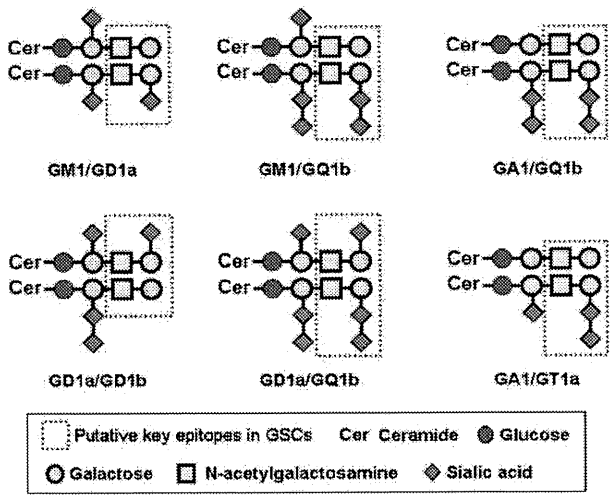

Fig. 2. Pattern diagrams of glycolipid complexes GM1/GD1a, GD1a/GD1b, GM1/GQ1b, GD1a/GQ1b, GA1/GQ1b, and GA1/GT1a. Squares with dotted lines indicate putative antigenic epitopes for antiganglioside complex antibodies.

4.2. Induction of anti-GSC antibodies

Analyses of the molecular structure of *C. jejuni* lipooligosaccharide (LOS) showed molecular mimicry between the LOS and GSCs targeted by serum antibodies from GBS patients (Kuijf et al., 2007). Inhibition ELISA using GBS sera with antibodies to such GSCs as GM1/GD1a, GD1a/GD1b, GD1a/GQ1b, and GD3/GQ1b revealed that each anti-GSC antibody cross-reacted with the LOS from the autologous *C. jejuni* strains, indicating that the LOS contained GSC-like structures. Interestingly, ganglioside-like structures expressed in some LOS of *C. jejuni* strains were not in accord with those expected from anti-GSC antibodies. Strains isolated from GBS patients with anti-GD1a/ GQ1b antibodies expressed a homogeneous LOS with only a GD1c-like structure (Kuijf et al., 2007). Further studies on the structures of GSCs may explain the unexpected antibody–antigen interactions, such as the cross-reaction between the anti-GD1a/GQ1b antibodies and GD1c-like moieties.

5. Antibodies to ganglioside complexes in FS and GBS with ophthalmoplegia

Analysis of FS sera for antibodies to GSCs containing GQ1b or GT1a revealed that a half of FS patients had antibodies to GSCs such as GM1/

GQ1b and GD1a/GQ1b (Table 1) (Kaida et al., 2006; Kanzaki et al., 2008). Based on antibody specificity, the FS-associated antibodies were subdivided into three types: GQ1b-specific, GM1/GQ1b-reactive, and GD1a/GQ1b-reactive (Kaida et al., 2006). Given the combination of GQ1b and other gangliosides in the targeted GSCs, the conformation of terminal residues containing sialic acids is likely to regulate the antibody binding. A combination of [Galβ1-3GalNAc] and [NeuAcα2-8 NeuAcα2-3Galβ1-3GalNAc] in the terminal residues of ganglio-N-tetraose structures is essential for binding of the anti- GM1/GQ1b-antibody, whereas a combination of [NeuAcα2-3Galβ1-3GalNAc] and [NeuAcα2-8 NeuAcα2-3Galβ1-3GalNAc] in the terminal residues is targeted by the anti-GD1a/GQ1b antibody (Fig. 2) (Kaida et al., 2006; Kanzaki et al., 2008). Such diversity of antibody specificity may produce clinical difference among FS patients, and sensory function was preserved in FS patients who had anti-GM1/GQ1b-reactive sera (Kanzaki et al., 2008). However, patients with FS displayed the clinical triad regardless of the presence of such anti-GSC antibodies, suggesting that molecules targeted by GQ1b-specific, GM1/GQ1b-reactive, or GD1a/GQ1b-reactive antibody are in the vicinity of the nerve membrane. GQ1b must be a key molecule in the immunobiology of FS, and GSCs containing GQ1b appear to be preferential antigens in most FS patients. Anti-GQ1b IgG antibody remains as an excellent diagnostic marker of FS.

IgG anti-GQ1b antibody is also associated with development of ophthalmoplegia in GBS, acute ophthalmoplegia without ataxia, and Bickerstaff brainstem encephalitis, as well as FS (Chiba et al., 1993; Odaka et al., 2001). A recent study of anti-GSC antibodies in GBS revealed that IgG antibodies to GSCs containing GQ1b or GT1a were present in 47% of GBS patients with ophthalmoplegia, whereas no such anti-GSC antibodies were found in those with GBS without ophthalmoplegia (Kanzaki et al., 2008). This indicates that the antibodies to GSCs containing GQ1b or GT1a are closely associated with development of ophthalmoplegia in GBS. Our recent study on antibodies to glycolipid complexes consisting of asialo-GM1 (GA1) and GQ1b have made us reconsider the conformational structure of the glycoepitopes targeted by the FS-associated anti-GSC antibodies (Ogawa et al., 2009). Some anti-GM1 antibodies in GBS sera are cross-reactive with GA1 and probably bind to the terminal N-acetylgalactosamine-galactose moiety (Koga et al., 2001). Because terminal residues with a gangliotetraose structure in GA1 are shared with GM1 or GD1b, the terminal residues of a glycolipid complex, GA1/GQ1b should be analogous to those of GM1/GQ1b or GD1b/GQ1b. However, approximately 70% of anti-GA1/GQ1b or anti-GA1/GT1a positive sera did not react with GM1/GQ1b and GD1b/GQ1b (Ogawa et al., 2009). In view of the terminal residues of such glycolipid complexes, the specificity of antibodies to GSC containing GQ1b or GT1a may be regulated not only by sialic acids in the terminal residues but also by those attached to an internal galactose. Conformational analyses of glycoepitopes in the GSCs are required for identification of the exact target antigens and understanding of the antibody-mediated pathophysiology in GBS and its variants.

6. Glycolipid environment and avidity of antiganglioside antibodies

Ataxia is a well-known symptom in GBS that is thought to be closely associated with IgG anti-GD1b antibodies. This is supported by studies showing that IgG GD1b-specific antibodies induce experimental ataxic neuropathy (Kusunoki et al., 1996b, 1999a). GD1b has been shown to be localized in large neurons in dorsal root ganglia (Kusunoki et al., 1993), indicating that anti-GD1b antibodies cause ataxia by binding to large primary sensory neurons that mediate deep sensation. However, only half of GBS patients with IgG anti-GD1b antibody present with ataxia (Miyazaki et al., 2001). To unveil the reason for this discrepancy, we examined the specificity of IgG anti-GD1b antibodies using GSC antigens containing GD1b and analyzed the association of the antibody specificity with ataxia (Kaida et al., 2008a). We found that anti-GD1b activities were strongly inhibited by the addition of gangliosides with two or more sialic acids to GD1b in patients with GBS with ataxia, compared to those with GBS without ataxia (Kaida et al., 2008a). These results suggest that target epitopes of GD1b can be masked or modified by colocalization of gangliosides with two or more sialic acids, such as GD1a. Thus, IgG antibodies with high specificity for GD1b may play a critical role in development of ataxia in GBS and colocalization of another ganglioside with GD1b may influence the accessibility of the anti-GD1b antibodies (Fig. 3).

Cis-interaction of the sugar chain of gangliosides in membrane microdomains may modify the conformation of the glycoepitopes. Such complex glycolipid environments in the cell membrane may govern the accessibility and avidity of antiganglioside antibodies for target gangliosides. A recent intriguing study using GalNAc transferase-deficient ($GalNAcT^{-/-}$) and GD3 synthase-deficient ($GD3s^{-/-}$) mice supports this hypothesis (Greenshields et al., 2009). The binding ability of the pathogenic anti-GM1 antibody to GM1-like epitopes is dependent upon which gangliosides are in the vicinity of GM1 on the

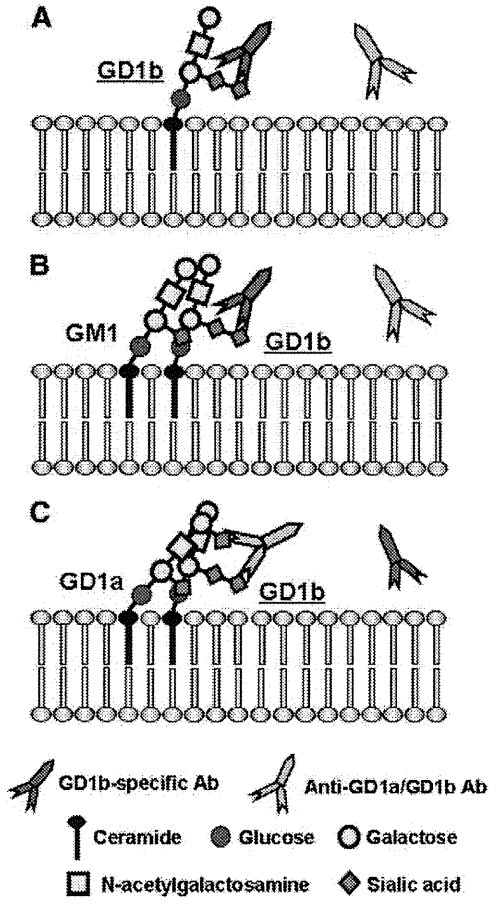

Fig. 3. Schematic diagram depicting proposed antigen–antibody interactions between GD1b-specific antibodies and GSCs containing GD1b in the nerve cell membrane. (A) The GD1b-specific antibody binds to antigenic epitopes of GD1b that are exposed and unmasked in the cell membrane. (B) GD1b and GM1 colocalize and *cis*-interact together in the membrane. The GD1b-specific antibody can access antigenic epitopes of GD1b in the GM1–GD1b complex. Colocalization and *cis*-interaction between GD1b and monosialogangliosides do not interrupt the binding of the GD1b-specific antibody to GD1b in the membrane (for details, see text). (C) GD1b and GD1a colocalize and *cis*-interact in the membrane. The GD1b-specific antibody cannot access antigenic epitopes of GD1b in the GD1a/GD1b complex, while the anti-GD1a/GD1b antibody can bind to glycoepitopes formed in the GD1a/GD1b.