博士学位論文

グライコアレイを用いた免疫性ニューロパチーにおける糖脂質および糖脂質複合体に対する自己抗体の検討

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論文題目

Serological Study using glycoarray for detecting antibodies to glycolipids and glycolipid complexes in immune-mediated neuropathies.

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Doctoral Dissertation

Serological study using glycoarray for detecting antibodies to glycolipids and glycolipid complexes in immune-mediated neuropathies

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Serological study using glycoarray for detecting antibodies to glycolipids and glycolipid complexes in immune-mediated neuropathies

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Abstract

We performed a serological investigation using glycoarray in Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), and multifocal motor neuropathy (MMN). Antibodies to 10 glycolipids and 45 glycolipid complexes were tested. Anti-GM1/sulfatide and anti-GA1/sulfatide IgG antibodies were common in GBS (20.0% and 19.0%, respectively). Anti-GQ1b/sulfatide IgG antibody was detected in 14.0% of GBS patients. IgG antibodies to antigens containing GQ1b were significantly correlated with ophthalmoplegia in GBS ($p < 0.01$). IgM antibodies to antigens containing GM1 or GalNAc-GD1a were in 50% and 37.5% of MMN patients, respectively. Glycoarray is efficient for detecting antibodies against numerous glycolipid complexes in immune-mediated neuropathies.

Key words : Glycoarray, Glycolipid complex, Antibodies, Guillain-Barré syndrome, Multifocal motor neuropathy

1. Introduction

Guillain-Barré syndrome (GBS) is an acute immune-mediated neuropathy with symmetrical weakness of the limbs, and areflexia. Multifocal motor neuropathy (MMN) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) are chronic demyelinating peripheral neuropathies. MMN presents as asymmetric, distal predominant weakness of the upper limbs and is characterized by multifocal conduction blocks in electrophysiological studies. CIDP is typically a sensorimotor neuropathy with symmetric and diffused disturbance.

Anti-glycolipid antibodies are often detected in acute phase sera from patients with GBS. These antibodies are useful diagnostic markers. Some of the antibodies are associated with specific clinical features and may directly be involved in the pathogenetic mechanisms.

Not only individual glycolipid antigens, but also mixtures of two different glycolipids are sometimes targets for antibodies in the serum of GBS patients. Those antibodies may recognize new conformational epitopes, called glycolipid complexes, which are formed by the carbohydrate portions of two different gangliosides(Kaida et al., 2004).

In contrast to GBS, anti-glycolipid IgG antibodies are not so frequently detected in the sera from patients with MMN or CIDP, although a subset of patients with CIDP had anti-LM1 IgG antibodies (Kuwahara et al., 2011). Anti-GM1 IgM antibodies were found in approximately 40-60% of patients with MMN (Cats et al., 2010; Delmont et al., 2015; Nobile-Orazio et al., 2008). To date, the number of glycolipid complexes that have been investigated is limited. It is possible that antibodies to certain types of glycolipid complexes are detectable in, not only GBS patients, but also those with MMN or CIDP. Several studies using combinatorial glycoarray for detection of numerous combinations of glycolipids were recently reported (Rinaldi et al., 2012).

To search for novel target antigens for the antibodies in serum samples from patients with GBS, MMN, and CIDP, by using combinatorial glycoarray, we examined IgM and IgG antibodies against 10 glycolipids〔GM1, GM2, GD1a, GD1b, GQ1b, GalNAc-GD1a, LM1, galactocerebroside (Gal-C), asialo-GM1 (GA1), and sulfatide〕 and combinations of two different glycolipids in GBS, CIDP, and MMN patients. Furthermore, we investigated the relationships between the clinical features and presence of those antibodies.

2. Material and methods

2. 1. Patient enrollment and serum collection

Serum samples were collected from institutions throughout Japan, including our hospital. Serum were obtained from 100 patients affected with GBS, 100 patients with CIDP, and 24 patients with MMN, all in the acute or relapsing phase. All GBS sera were samples of pre-treatment. Serum was obtained from 30 healthy controls (HC). Serum was collected from disease controls in our hospital, including 39 patients with amyotrophic lateral sclerosis (ALS), 20 patients with non-immune-mediated neuropathies (five with vasculitic neuropathies, five with Charcot-Marie-Tooth disease, eight with metabolic neuropathies, and two

with toxic neuropathies), 20 patients with multiple sclerosis (MS), and 20 patients with other central nervous system (CNS) diseases [five with neuromyelitis optica (NMO), seven with encephalopathy, and eight with encephalitis].

All GBS patients were diagnosed using the criteria established by Asbury and Cornblath (Asbury and Cornblath, 1990). The results of nerve conduction studies (NCS) were obtained from 93 patients with GBS and electrophysiologically classified into acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN), and unclassified, according criteria established by Ho et al. (Ho et al., 1995).

Diagnosis of CIDP and MMN were made using the criteria established by the European Federation of Neurological Societies and the Peripheral Nerve Society (EFNS/PNS) (Joint Task Force of the EFNS and the PNS, 2010a; Joint Task Force of the EFNS and the PNS, 2010b). The CIDP phenotypes were classified into typical or atypical. Atypical cases were further categorized as multifocal demyelinating sensory and motor (MADSAM), distal acquired demyelinating symmetric (DADS), pure motor, pure sensory, and focal, based on the criteria of the EFNS/PNS.

2. 2. Combinatorial glycoarray

Stocked glycolipid solutions were diluted to concentration of 100 μg/ml with methanol. Glycolipid complexes were made by mixing equal volumes of the diluted solutions of the different glycolipids. We used an automatic thin layer chromatography (TLC) sampler (CAMAG, Switzerland) for spotting antigens (0.4 × 0.4 mm dimensions) onto Immobilon-FL polyvinylidene difluoride (PVDF) membranes (Merck Millipore) affixed to glass slides (76 × 26 mm). Because each spot should be separated at least 2mm from another spot, we could examine 11 × 11 lanes on a slide (One lane was for methanol. Remaining lanes were for 10 individual glycolipids and 45 combinations, consisting of two different glycolipids). We measured antibodies against GM4 instead of GM2 for patients with MS and other CNS diseases, because GM4 is located in the myelin and astrocytes of the human brain (Ledeen et al., 1973; Uemura et al., 2014; Ueno et al., 1978). The PDVF membranes were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature. Serum which was diluted (at 1:100) with 1% BSA in PBS, was applied to PVDF membranes on glass slides for 2 h at 4℃ . After washing three times with 0.1% BSA in PBS for a total of 1 h, Alexa 647 anti-human IgM (H+L) and Alexa 555 anti-human IgG (H+L) (Thermo Fisher Scientific), diluted (at 1:1,000) with 1% BSA in PBS, were applied for 1 h as secondary antibodies. After washing with 0.1% BSA in PBS for a total of 1 h, the glass slides were washed with distilled water for 5 min. The PVDF membranes were dried at room temperature in the dark. Reactivity to glycolipids was expressed in fluorescence intensities, and the fluorescence signals were detected by using Image Quent TL software (GE healthcare). Intensities greater than the mean plus 3 standard deviations (SD) for 30 healthy controls were considered positive. We considered that the antibody specifically recognized the glycolipid complex instead of individual glycolipids when

the exact intensity of the antibody to the glycolipid complex was higher than the sum of those to the constituent glycolipids.

2. 3. Statistical analyses

The data were analyzed using SPSS (version 20, IBM SPSS statistics). Between-group differences were tested using χ^2 tests, Fisher's exact tests, and Mann-Whitney U tests. Differences were considered statistically significant for two-tailed p-values < 0.05.

This study was approved by the Internal Review Board of Kindai University Faculty of Medicine.

3. Results

The clinical characteristics of patients with GBS, CIDP, and MMN are shown in Tables 1, 2, and 3, respectively.

Table 1 Characteristics of patients with Guillain-Barré syndrome

	Total (n = 100)	AIDP (n = 37)	AMAN (n = 23)	Unclassified (n = 33)
Age (years) : median〔range〕	54.4〔20-91〕	61.8〔29-91〕	49.3〔27-78〕	48.8〔20-85〕
Sex				
Male	44	17	13	10
Female	56	20	10	23
Cranial nerve deficit	57 (57%)	26 (70%)	10 (43%)	18 (55%)
ophthalmoplegia	18 (18%)	3 (8%)	4 (17%)	10 (30%)
facial palsy	39 (39%)	19 (51%)	8 (35%)	10 (30%)
bulbar palsy	26 (26%)	14 (38%)	6 (26%)	4 (12%)
Sensory impairment	74 (74%)	31 (84%)	15 (65%)	24 (73%)
paresthesia and dysesthesia	59 (59%)	20 (54%)	14 (61%)	21 (64%)
hypesthesia	30 (30%)	17 (46%)	8 (35%)	5 (15%)
deep sensory disturbance	24 (24%)	15 (41%)	3 (13%)	5 (15%)
Ataxia	19 (19%)	8 (22%)	4 (17%)	6 (18%)
Autonomic dysfunction	19 (19%)	14 (38%)	3 (13%)	2 (6%)
Respiratory failure	11 (11%)	8 (22%)	2 (9%)	1 (3%)

AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy.
Seven of the 100 patients with GBS could not be categorized AIDP, AMAN or unclassified because too few electrophysiological results were available.

Table 2　Characteristics of patients with chronic inflammatory demyelinating polyradiculoneuropathy

	CIDP (n = 100)
Age (years) : median〔range〕	59.2〔25-89〕
Sex	
Male	59
Female	41
Onset age (years) : median〔range〕	57.1〔18-86〕
Disease duration (months) : median〔range〕	26.4〔2-300〕
Typical (%)	63 (63%)
Atypical (%)	
MADSAM	11 (11%)
Pure motor	5 (5%)
Pure sensory	7 (7%)
Focal	3 (3%)
DADS	9 (9%)
Not defined	2 (2%)
Treatments (%)	
IVIg	83 (83%)
Steroids	48 (48%)
Plasmapheresis	10 (10%)
Immunosuppressant	9 (9%)

CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; MADSAM, multifocal demyelinating sensory and motor; DADS, distal acquired demyelinating symmetric; IVIg, intravenous immunoglobulin.

Table 3　Characteristics of patients with multifocal motor neuropathy

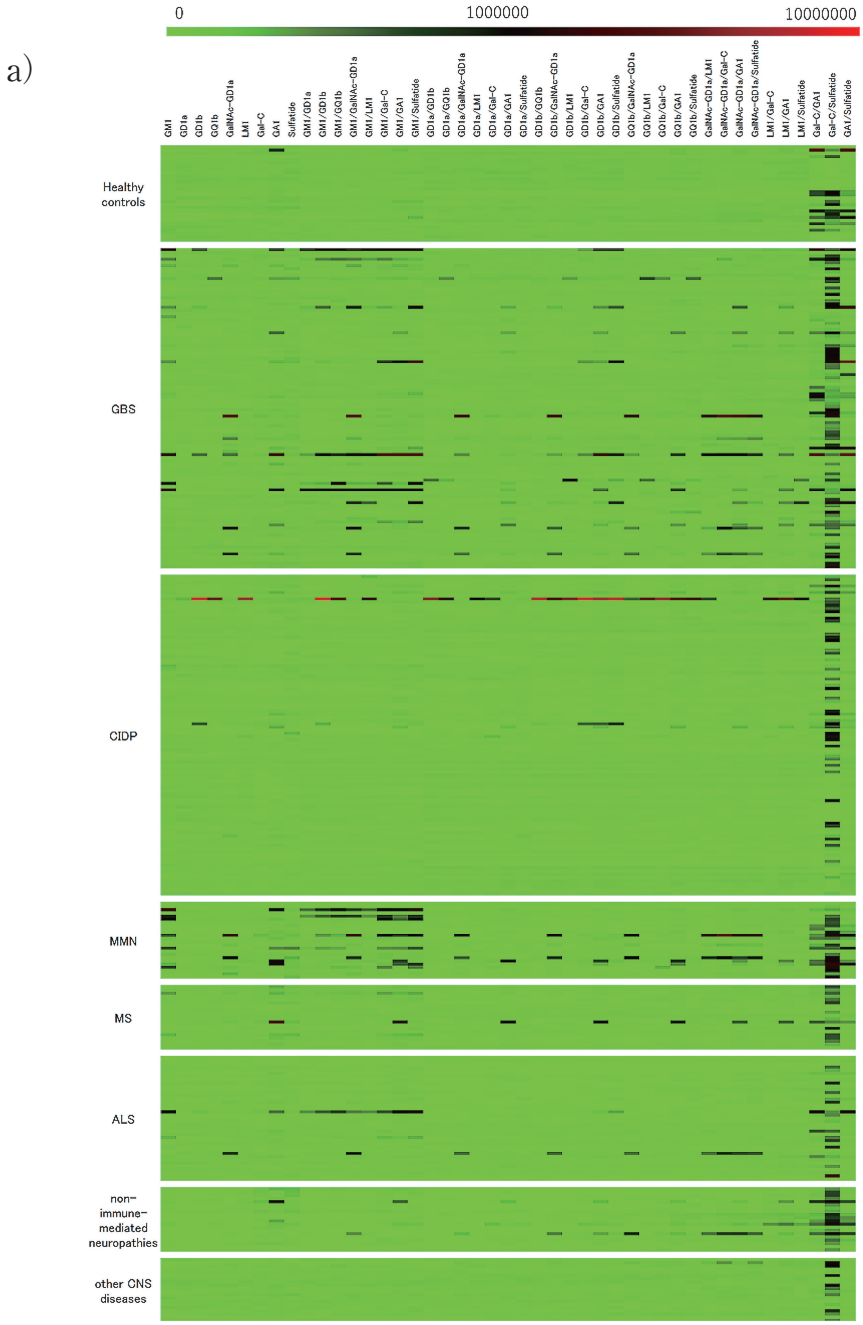
	MMN (n = 24)
Age (years) : median〔range〕	53.2〔24-79〕
Sex	
Male	14
Female	10
Onset age (years) : median〔range〕	48.4〔23-75〕
Disease duration (months) : median〔range〕	49〔2-192〕

MMN, multifocal motor neuropathy.

3. 1. Anti-glycolipid and anti-glycolipid complex antibodies

Overall IgM and IgG results for all patient groups are shown in Figure 1 as a heatmap. The positive ratios of overall anti-glycolipid and anti-glycolipid complex antibody assays using glycoarray were 56.0% in GBS, 16.0% in CIDP, 58.3% in MMN, and 19.4% in disease and normal controls.

Higher intensities of IgG antibodies were detected almost exclusively in GBS patients. In contrast, IgM antibodies were frequently detected in MMN patients and sometimes in those with GBS. Among the antibodies to individual glycolipids and glycolipid complexes, anti-GM1/sulfatide IgG antibody (20.0%), anti-GA1/sulfatide IgG antibody (19.0%) and anti-GM1/GD1a IgG antibody (17.0%) were frequently observed in patients with GBS.



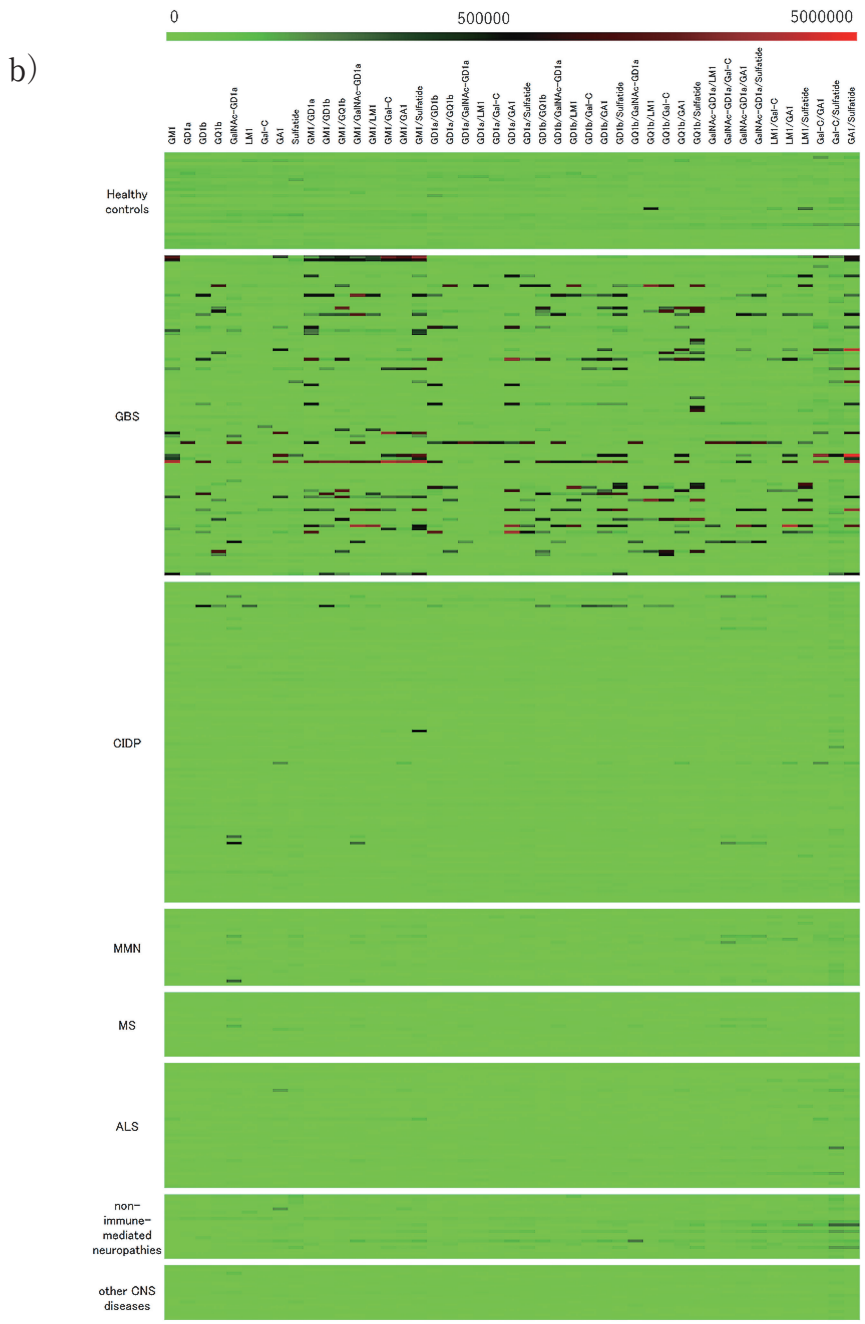


Fig. 1 a) Overall heatmap of IgM antibodies for all patient groups
b) Overall heatmap of IgG antibodies for all patient groups
The antigens which contained GM2 and GM4 are not shown on the heatmap, since the type of antigens which were examined differed in MS and other CNS diseases.
Non-immune-mediated neuropathies include five vasculitic neuropathies, five Charcot-Marie-Tooth disease, eight metabolic neuropathies, and two toxic neuropathies. Other CNS diseases include five NMO, seven encephalopathy, and eight encephalitis.
GBS, Guillain-Barré syndrome; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; MMN, multifocal motor neuropathy; HC, healthy controls; ALS, amyotrophic lateral sclerosis; MS, multiple sclerosis; CNS, central nerve system; NMO, neuromyelitis optica.

IgM antibodies were detected in 12 patients with CIDP (12.0%), and IgG antibodies were observed in six patients with CIDP (6.0%).

Anti-GM1 IgM antibody (33.3%) and anti-GM1/Gal-C IgM antibody (33.3%) were the most frequently observed antibodies in patients with MMN.

Among the disease controls, IgM antibodies were observed in four patients with ALS (10.3%), seven patients with non-immune-mediated neuropathies (three with vasculitic neuropathies, two with Charcot-Marie-Tooth disease, and two with metabolic neuropathies), three patients with MS, and one patient with NMO. IgG antibodies were observed in five patients with non-immune-mediated neuropathies (one patient with vasculitic neuropathies, one patient with Charcot-Marie-Tooth disease, and three patients with metabolic neuropathies). The patients with MS or other CNS diseases did not have IgG antibodies. In addition, none of the patients with MS or other CNS diseases had specific antibodies to GM4 or glycolipid complexes containing GM4.

3. 2. Association between clinical features and anti-glycolipid complex IgG antibodies in GBS patients

IgG antibodies to glycolipid complexes containing sulfatide were the most frequently observed antibodies (n = 41, 41.0%) in GBS patients, followed by antibodies to complexes containing GM1 (n = 33, 33.0%), GA1 (n = 31, 31.0%), or GQ1b (n = 31, 31.0%).

3. 2. 1. IgG antibodies to GM1 and glycolipid complexes containing GM1

IgG antibodies to GM1 and glycolipid complexes containing GM1 were observed in 17 of 27 GBS patients with pure motor involvement (63.0%), whereas these antibodies were detected in 16 of 73 GBS patients with both motor and sensory impairment (21.9%) ($p < 0.01$).

Seventeen GBS patients had detectable anti-GM1/GD1a IgG antibodies. Anti-GD1a/GA1 and anti-GD1a/GD1b antibodies were detected in 11 and 9 of these 17 patients, respectively. Seven GBS patients had antibodies against all three complexes (GM1/GD1a, GD1a/GA1, and GD1a/GD1b), and five of the seven had only reactivity to these complexes of gangliosides (Fig. 2).

Anti-GM1/sulfatide IgG antibodies were observed in 20 of 100 GBS patients (20.0%), and 12 of the 20 patients were pure motor GBS (60.0%). Furthermore, seven of these 20 patients had antibodies that were specific to GM1/sulfatide complex rather than to the individual GM1 and sulfatide antigens, and six of the seven patients were pure motor GBS (85.7%).

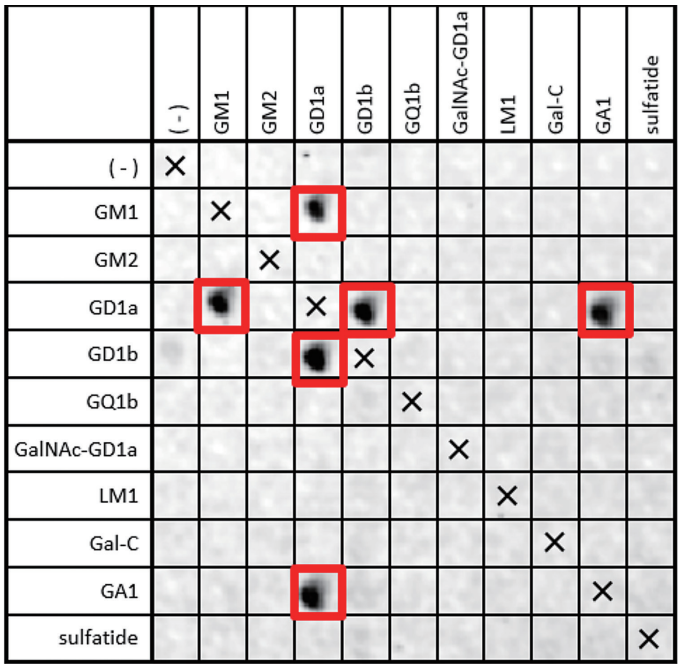


Fig. 2　IgG antibodies to GM1/GD1a, GD1a/GD1b, and GD1a/GA1 complexes
The IgG reactivities to GM1/GD1a, GD1a/GA1 and GD1a/GD1b by glycoarray are shown. In this patient with GBS, no antibodies to individual glycolipids were observed. The patient had IgG antibodies to common structures consisting of two glycolipids, such as GM1/GD1a, GD1a/GA1, and GD1a/GD1b.

3. 2. 2. IgG antibodies to GQ1b and glycolipid complexes containing GQ1b

IgG antibodies to GQ1b or glycolipid complexes containing GQ1b were observed in 14 of 18 GBS patients with ophthalmoplegia (77.8%) and 17 of 82 GBS patients without ophthalmoplegia (20.7%). Anti-GQ1b associated IgG antibodies were more frequently detected in GBS patients with ophthalmoplegia than in those without ophthalmoplegia ($p < 0.01$).

Antibodies to the GQ1b/sulfatide complex were observed in 14 of 100 GBS patients (14.0%), and 10 of the 14 patients had ophthalomoplegia (71.4%). In seven of the 14 patients, the antibodies were specific to the GQ1b/sulfatide complex rather than the individual GQ1b and suldfatide antigens. In addition, four patients did not have antibodies other than those to the anti-GQ1b/sulfatide complex. Ophthalmoplegia was observed in all four of these patients, bulbar palsy in one patient, and ataxia in two of the other three patients.

3. 3. Anti-glycolipid complex antibodies in CIDP patients

IgM antibodies were detected in 12 patients with CIDP(12.0%). Six CIDP patients had IgG antibodies(6.0%). Of these patients with CIDP, three patients had both IgM and IgG antibodies (3.0%). Among the 63 patients with typical CIDP, seven patients had IgM antibodies (11.1%). Nine patients with DADS and three patients with focal CIDP did not have any of these antibodies.

As for the IgM antibodies, antibodies to glycolipid complexes containing GM1 or sulfatide were the most frequently observed antibodies ($n = 7$, 7.0%, respectively). In addition, the IgG antibodies to glycolipid

complexes containing Gal-C were the most frequently observed antibodies (n = 4, 4.0%).

We compared the clinical features between anti-glycolipid antibodies-positive CIDP patients and antibodies-negative CIDP patients. Age (62 vs 59 years), gender rate (male/female; 2.75 vs 1.2), onset age (61 vs 56 years), disease duration (14 vs 29 months), proportion of typical CIDP (53.3% vs 65.9%), acute onset (6.7% vs 5.9%), preceding infections (6.7% vs 1.2%), cerebrospinal fluid protein levels (92 vs 107 mg/dl), and efficacy rate of immunotherapy (71.4% vs 85.7%) were not correlated with the status of the anti-glycolipid antibodies.

3. 4. Anti-glycolipid complex antibodies in MMN patients

IgM antibodies to GM1 and GM1/Gal-C were each detected in eight patients with MMN (33.3%). Overall, IgM antibodies to antigens containing GM1 were present in 12 patients (50.0%). The intensities of the antibody activity were not different between anti-GM1 and anti-GM1/Gal-C IgM antibodies. IgM antibodies to GalNAc-GD1a were detectable in five patients (20.8%). IgM antibodies to antigens containing GalNAc-GD1a were present in nine patients (37.5%). IgM antibodies to GM1/GalNAc-GD1a were detected in six of these nine patients.

IgG antibodies were observed in two patients with MMN. One had anti-GalNAc-GD1a/Gal-C antibody and another patient had anti-GalNAc-GD1a antibody.

4. Discussion

In the present study, we examined IgM and IgG antibodies to 10 individual glycolipids and 45 glycolipid complexes by combinatorial glycoarray in patients with GBS, MMN, and CIDP. IgG antibodies were frequently detected in GBS patients, and IgM antibodies were frequently observed in MMN patients and sometimes in those with GBS.

IgG antibodies to glycolipid complexes containing sulfatide were the most common antibodies in GBS patients (41.0%). Moreover, 11 patients had the specific antibodies to glycolipid complexes containing sulfatide without reactivity to the individual glycolipids and sulfatide antigens. We considered that glycolipid complexes consisting of both sulfatide and the other glycolipid often enhance the reactivity to the individual glycolipids as described in the previous report (Rinaldi et al., 2013), but sometimes possibly make a new epitope on the cell membranes. Among those antibodies, antibodies to GM1/sulfatide, GA1/sulfatide, and GQ1b/sulfatide were frequently detected antibodies in the patients with GBS (20.0%, 19.0%, and 14.0%, respectively). GBS patients with antibody to GQ1b/sulfatide had ophthalamoplegia more frequently than patients without this antibody (71.4% vs 9.3%). Furthermore, pure motor disturbance was more frequently observed in anti-GM1/sulfatide antibody-positive than in antibody–negative GBS patients (60.0% vs 38.3%). The clinical features of GBS patients with IgG antibodies to glycolipid complexes containing sulfatide may be determined by the reacitivities to the concomitant glycolipid. IgG antibody to GA1/sulfatide was detected in 17.7% of the

patients with GBS in the previous study (Rinaldi et al., 2013). Our result showed that this antibody was one of the common antibodies in GBS as well. However, the pathological role of the antibody remains unclear.

As indicated in the previous report, some antibodies to glycolipids and glycolipid complexes had relations with GBS or MMN. For example, GM1 epitope is highly expressed on axonal membranes of motor nerves and on the surface of Schwann cells. Binding of the antibodies to the axon at the nodes of Ranvier on to Schwann cells may cause complement activation and disruption of sodium channel clusters, resulting in conduction abnormalities (Cats et al., 2010; Susuki et al., 2007). IgG antibodies to GM1 and glycolipid complexes containing GM1 were associated with pure motor GBS in our present study, which is compatible with the previous report (Hafer-Macko et al., 1996).

Anti-GQ1b IgG antibody is associated with ophthalmoplegia and ataxia (Chiba et al., 1993; Kusunoki et al., 1999). To the best of our knowledge, no reports have detected anti-GQ1b/sulfatide IgG antibody in patients with GBS or Fisher syndrome whereas antibodies to GQ1b/GM1 or GQ1b/GD1a complexes have been reported (Kaida et al., 2006; Kanzaki et al., 2008). IgG antibodies to GQ1b/sulfatide complex, in addition to IgG antibodies to the individual GQ1b antigen, may play a role in GBS with ophthalmoplegia.

GA1 is a glycolipid that is the desialylated form of GM1. GD1b is a disialoganglioside with two sialic acids in its structure. Since they share a same terminal, Gal-GalNAc residue, cross-reactivity is often seen between antibodies to GM1, GD1b, and GA1. Anti-GM1/GD1a, anti-GD1a/GA1, and anti-GD1a/GD1b antibodies may be cross-reactive, because those antigens share an epitope formed by Gal-GalNAc and sialosyl-Gal-GalNAc residues (Fig. 3). All five sera with antibody activities to GM1/GD1a, GD1a/GA1, and GD1a/GD1b lost reactivity when GM2 is present instead of GM1, GA1, or GD1b in the above combinations. All five sera showed positive reactions when GT1b was used instead of GD1a but lost reactivity when GT1a was used instead of GD1a(data not shown), confirming that the antibody specifically recognized an epitope formed by Gal-GalNAc and sialosyl-Gal-GalNAc residues.

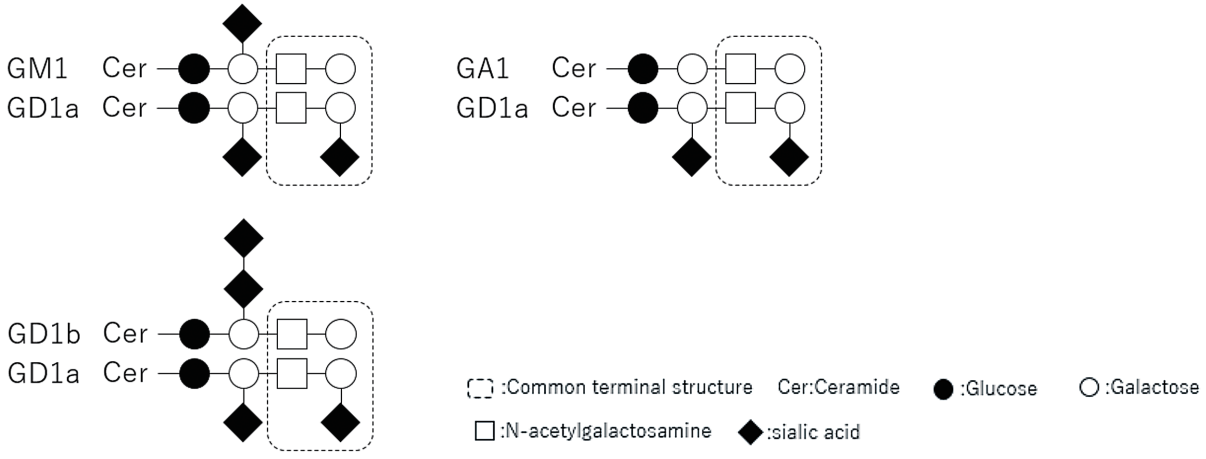


Fig. 3 Structure of GM1/GD1a, GA1/GD1a, and GD1a/GD1b complexes
The terminal residue of GM1/GD1a, GA1/GD1a, and GD1a/GD1b share a common structure.

In patients with MMN, IgM antibodies to GM1 were mentioned in diagnostic criteria of the EFNS/PNS, but the pathomechanism remains unclear. Recently, it was reported that the intensity of IgM antibodies to the GM1/Gal-C complex was greater than the intensity of antibodies to GM1 alone (Galban-Horeajo et al., 2013). In addition, the sensitivity of anti-GM1/Gal-C IgM antibody was greater than that of anti-GM1 IgM antibody in patients with MMN, when GM1 and Gal-C were mixed at a weight-to-weight ratio equivalent to 1:5 or 1:10 (Delmont et al., 2015). IgM antibodies to GM1 and GM1/Gal-C complex were the most common antibodies in patients with MMN in the present study. However, the frequency and the intensity did not differ between GM1 and the GM1/Gal-C complex. This may be the case because we mixed the antigens at a 1:1 ratio or because of ethnic differences in the samples. IgM antibodies to GalNAc-GD1a or glycolipid complexes containing GalNAc-GD1a were also frequently observed in patients with MMN. GalNAc-GD1a is assumed to exist on axonal membranes of the nodes of Ranvier and in the paranodal region in the ventral root of humans (Kaida et al., 2003). IgG antibody to the GM1/GalNAc-GD1a complex has been reported in pure motor variant GBS, especially in the form of acute motor conduction block neuropathy (AMCBN) (Kaida et al., 2008). These suggest that IgM antibodies to GalNAc-GD1a and glycolipid complexes containing GalNAc-GD1a may contribute to conduction block of the peripheral nerves in patients with MMN. Further studies to elucidate the mechanism are required.

In contrast to GBS and MMN, there were no significant findings relevant to CIDP. As CIDP consists of several subtypes, investigations of larger samples with each subtype should be performed in the future.

In conclusion, antibodies to glycolipid complexes are often observed in immune-mediated neuropathies, and some of these antibodies may have a pathogenetic role in each disease. Combinatorial glycoarray is a useful tool to detect these antibodies. We need further investigations using glycoarray in other immune-mediated diseases, including Fisher syndrome and Bickerstaff brainstem encephalitis.

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Author Contributions

Design or conceptualization of the study: MM, MK, and SK.

Acquisition of data: MM, MK, RU, MS, and YH.

Analysis or interpretation of the data: MM, MK, and SK.

Drafting or revising the manuscript for intellectual content: SK.

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