研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
楠　進	GBSとガングリオシド複合体抗体 －最近の知見－	鈴木則宏	Annual Review 神 経 2011	中外医学社	東京	2011	293-299

雑誌

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Saigoh K, Izumikawa T, Koike T, Shimizu J, Kitagawa H, Kusunoki S.	*Chondroitin beta-1,4-N-acetylgalactosaminyltransferase-1 (ChGn-1)* missense mutations are associated with neuropathies.	J Hum Genet	in press		
Kaida K, Kusunoki S.	Antibodies to gangliosides and ganglioside complexes in Guillain–Barré syndrome and Fisher syndrome: Mini-review.	J Neuroimmunol	223	5-12	2010
Kusunoki S, Kaida K.	Antibodies against gangalioside complexes in Guillain-Barré syndrome and related disorders.	J Neurochem	116	828-832	2011
楠　進	CIDPと抗ガングリオシド抗体	神経内科	72	284-289	2010
海田賢一、楠　進	神経疾患と分子マーカー：Guillain-Barré症候群	Clinical Neuroscience	28	1400-1404	2010

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書籍:該当なし

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Wakao, N., Imagama, S., Tauchi, R., Muramoto, A., Zhang, H., Natori, T., Takeshita, S., Ishiguro, N., Matsuyama, Y., Kadomatsu, K.	Hyaluronan oligosaccharides promote functional recovery after spinal cord injury in rats.	**Neurosci. Lett.**	488	299-304	2010
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Kizuka Y and Oka S.	Regulation of HNK-1 (Human Natural Killer-1) Carbohydrate Expression: Multiple Control Mechanisms of Biosynthetic Enzyme Activity	*Trends Glycosci. Glycotechnol.*	22	194-199	2010
岡　昌吾	記憶学習に関与する糖鎖	臨床化学	39(4)	381-382	2010
中川直樹 森田一平 岡　昌吾	HNK-1糖鎖抗原と神経突起の形成	脳２１	14(1)	37-43	2011

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Izumikawa, T., Kanagawa, N., Watamoto, Y., Okada, M., Saeki, M., Sakano, M., Sugahara, K., Sugihara, K. Asano, M., Kitagawa, H.	Impairment of Embryonic Cell Division and Glycosaminoglycan Biosynthesis in Glucuronyltransferase-I-Deficient Mice	J. Biol. Chem.	285 (16)	12190-12196	2010
Okada, M., Nadanaka, S., Shoji, N., Tamura, J., and Kitagawa, H.	Biosynthesis of Heparan Sulfate in *EXT1*-deficient cells.	Biochem. J.	428 (3)	463-471	2010
Dejima, K., Murata, D., Mizuguchi, S., Nomura, K. H., Izumikawa, T., Kitagawa, H., Gengyo-Ando, K., Yoshina, S., Ichimiya, T., Nishihara, S., Mitani, S., and Nomura K.	Two Golgi-resident 3'-Phopho-adenosine 5'-Phosphosulfate Transporters Play Distinct Roles in Heparan Sulfate Modifications and Embryonic and Larval Development in *Caenorhabditis elegans*.	J. Biol. Chem.	285 (32)	24717-24728	2010
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Izumikawa, T., Okuura, Y., Koike, T., Sakoda, N., and Kitagawa, H.	Chondroitin 4-O-sulfotran-sfer-ase-1 regulates the chain length of chondroitin sulfate in cooperation with chondroitin N-acetylgalactosaminyltransferase-2	Biochem. J.	434 (2)	321-331	2011

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ORIGINAL ARTICLE

Chondroitin beta-1,4-*N*-acetylgalactosaminyltransferase-1 missense mutations are associated with neuropathies

Kazumasa Saigoh[1], Tomomi Izumikawa[2], Toshiyasu Koike[2], Jun Shimizu[3], Hiroshi Kitagawa[2] and Susumu Kusunoki[1]

Chondroitin sulfate proteoglycans (CSPGs) in the peripheral nervous system likely participate as regulatory molecules in the process of axonal degeneration and regeneration. We investigated the *chondroitin beta1,4-N-acetylgalactosaminyltransferase-1* (*ChGn-1*) gene in 114 patients affected with neuropathies including Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, hereditary motor and sensory neuropathy (HMSN) and unknown etiology. The controls were 196 patients with other neurological diseases. We found novel missense mutations in two patients with neuropathy (Bell's palsy, unknown HMSN) in exons 5 (H234R) and 10 (M509R), respectively. None of the patients with other neurological diseases had either of these mutations. We then synthesized the two soluble forms of ChGn-1, containing each of the above mutations. Each of the soluble mutants was expressed in COS-1 cells and the mutant proteins were purified. The purified mutant proteins were used for western blotting analysis using an anti-ChGn-1 antibody and evaluated for glycosyltransferase activities. Although the expression of the ChGn-1 mutant proteins was confirmed by western blotting, they exhibited no *N*-acetylgalactosamineT-II activities. It is possible that these mutations are associated with the pathogenetic mechanisms of the peripheral neuropathies.
Journal of Human Genetics advance online publication, 16 December 2010; doi:10.1038/jhg.2010.148

Keywords: ChGn-1; chondroitin GalNAcT-I; chondroitin GalNAcT-II; mutation; neuropathy

INTRODUCTION

Peripheral neuropathies are often caused by genetic factors. In particular, hereditary motor and sensory neuropathy (HMSN) or Charcot-Marie-Tooth disease is known to be associated with a number of causative genes.[1,2] HMSN is a heterogeneous group of degenerative peripheral nerve disorders, which altogether constitute the most common inherited neurological disease, with an incidence of 1 in 2500.[3]

Glycoconjugates, such as glycoproteins, proteoglycans and gangliosides, are important constituents of both the central and peripheral nervous systems. However, no association between human peripheral neuropathies and glycosyltransferases, which are involved in the synthesis of carbohydrate chains of glycoproteins, proteoglycans, gangliosides and so on, has been reported to date. Recently, it has been shown that beta1,4-*N*-acetylgalactosaminyltransferase (also called GM2/GD2 synthetase)-deficient mice are affected by sensory-dominant neuropathies.[4]

Chondroitin sulfate proteoglycans (CSPGs) have been shown to be present in the matrix of the nervous system. Several species of molecules are known such as neurocan, versican, phosphacan and so on. They show developmental and post-traumatic changes both spatially and temporally. CSPGs are known to act as growth inhibitory molecules.[5] On the other hand, some of the CSPGs promote neurite outgrowth.[6] CSPGs are likely to act not only as a chemical barrier but also as regulatory molecules for nerve regeneration.[7] Chondroitin beta1,4-*N*-acetylgalactosaminyltransferase-1 (ChGn-1) is involved in an important step for the synthesis of CSPGs.[8] Chondroitin sulfate chains consist of repeating disaccharide units of *N*-acetylgalactosamine (GalNAc) and glucuronic acid, which are sulfated at either the C6 or C4 position of GalNAc. The integrity of the chondroitin sulfate chain is maintained by elongation (biosynthesis) of the chain, which is catalyzed by ChGn-1, ChGn-2 and sulfotransferases.[9] Recently, it has been reported that a broad spectrum of skeletal dysplasias result from mutations causing undersulfation of chondroitin sulfate chains in humans and in mice.[10–14] In contrast, no association between CSPGs and human peripheral neuropathies has been reported to date.

In this study, we found novel missense mutations that resulted in a profound decrease of enzymatic activities in two patients with neuropathies.

MATERIALS AND METHODS

Subjects and patient populations

We recruited 310 patients with neurological disorders. We investigated 114 patients with neuropathy (40 with Guillain-Barré syndrome, 40 with chronic inflammatory demyelinating polyneuropathy, 5 with hereditary motor sensory neuropathy and 29 with unknown-etiology) and 196 disease control subjects. This study was approved by the internal review board of Kinki University

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School of Medicine. All patients provided written informed consent before participation in the study. Genomic DNA extraction and genotyping were performed using standard protocols.

Sequence analysis

Genomic DNA was extracted from whole blood using the QiaAmp Mini DNA kit (Qiagen, Tokyo, Japan). PCR amplicons generated with oligonucleotide primers were digested from *ChGn-1* gene on the basis of GenBank sequence. We sequenced all exons and their boundaries, using the ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Materials

UDP-[^{3}H]GalNAc (10 Ci mmol^{-1}) and unlabeled UDP-GalNAc were purchased from NEN Life Science Products (Waltham, MA, USA) and Sigma (Tokyo, Japan), respectively. Chondroitin was purchased from Seikagaku (Tokyo, Japan).

Construction of a Soluble Form of ChGn-1

The complementary DNA fragment of a truncated form of *ChGn-1*, lacking the first 41 N-terminal amino acids, was amplified by reverse transcription-PCR with total RNA derived from G361 human melanoma cells (ATCC CRL-1424) as a template using a 5′-primer (5′-GCTCTAGACAGCTGGCACTGCCCAGG-3′) containing an in-frame *Xba*I site and a 3′-primer (5′-CGGGATCCCATCTCT GACCCATCAGTCC-3′) containing a *Bam*HI site located 58 bp downstream of the stop codon.

Site-directed Mutagenesis

A two-stage PCR mutagenesis method was used to construct the ChGn-1 H234R or ChGn-1 M509R mutants. Two separate PCR reactions were performed to generate two overlapping gene fragments using the soluble form of *ChGn-1* complementary DNA as a template. In the first PCR, the sense 5′-primer described above and either of the antisense internal mutagenic primers listed below were used: H234R 5′-GTTTGAATTCGCGTTTGTGGTCCCC-3′ or M509R 5′-CCTGAACACCAGCCTGCCCAGCTGGCCG-3′ (the mutated nucleotides are underlined). In the second round of PCR, the respective sense internal mutagenic primers (complementary to the antisense internal mutagenic primer) and the antisense 3′-primer described above were used.

Expression of soluble forms of the ChGn-1 and ChGn-1 mutants and enzyme assays

The expression plasmids (6.0 μg each) were transfected into COS-1 cells on 100-mm plates using FuGENE 6 (Roche Molecular Biochemicals, Tokyo, Japan) according to the manufacturer's instructions. At 2 days after transfection, 1 ml of the culture medium was collected and incubated with 10 μl of HIS-Select Cobalt Affinity beads (Sigma) for 1 h at 4 °C. The beads recovered by centrifugation were washed and then resuspended in the assay buffer described below, and GalNAcT transferase activity was assessed using polymer chondroitin (167 μg) as an acceptor. Reaction mixtures were incubated at 37 °C for 1 h, and radiolabeled products were then separated from UDP-[^{3}H]GalNAc by gel filtration using a syringe column, as described previously.[9]

Western blot analysis

After 2 days of culture, the culture medium was collected and incubated with 10 μl of HIS-Select Cobalt Affinity beads (Sigma) for 1 h at 4 °C. The beads recovered by centrifugation were washed with phosphate-buffered saline, resolved on 7.5% SDS-polyacrylamide gels, and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated for 1 h with an anti-ChGn-1 mouse antibody (Transgenic, Kobe, Japan). The antibody was diluted 1:1000 with 25 mM Tris-buffered saline. The bound antibody was detected with anti-mouse IgG conjugated with horseradish peroxidase and enhanced chemiluminescence.

RESULTS

Mutation analysis of patient DNA

In mutation analysis of the *ChGn-1* gene, we found two novel heterozygous missense mutations in patients with neuropathies, H234R (1355 A >G) in exon 5 from one patient and M509R (2180 T >G) in exon 10 from the other. Those mutations were not observed in 196 unrelated disease control DNA samples.

The patient with the H234R missense mutation was a 38-year-old man. He had been affected by acquired idiopathic generalized anhidrosis since his childhood. He had no apparent family history. He was also affected by hemi-facial palsy since the age of 34. The hemi-facial palsy developed similar to an episode of acute idiopathic facial palsy, so called Bell's palsy, but was irreversible.

The patient with the M509R mutation was a 25-year-old man. He had motor and sensory neuropathy without any apparent family history. He had experienced intermittent postural tremor since he was in elementary school. Nerve conduction studies revealed an absence of sensory nerve action potentials in the median, ulnar and sural nerves. The compound muscle action potentials were reduced (left median nerve: 2.88 mV (control: >4 mV), left ulnar nerve: 0.85 mV (>3 mV) and left tibial nerve: 0.19 mV (>7 mV)). Motor nerve conduction velocities were also decreased (left median nerve: 14.7 m s^{-1} (>45 m s^{-1}), left ulnar nerve: 13.6 m s^{-1} (>45 m/s) and left tibial nerve: 9.34 m s^{-1} (>40 m s^{-1})). He had been diagnosed with HMSN of unknown type.

Sequence analysis on PMP22 and MPZ, and PMP22 duplication and deletion study using fluorescence *in situ* hybridization method showed no abnormal findings in either patient.

Expression and glycosyltransferase activities of soluble forms of the ChGn-1 H234R and M509R mutants

To clarify whether these mutations of *ChGn-1* influence glycosyltransferase activities, we constructed soluble forms of the two ChGn-1

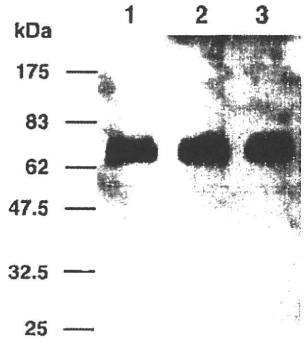

Figure 1 Western blot analysis of ChGn-1 H234R and ChGn-1 M509R. A soluble form of ChGn-1, ChGn-1 H234R or ChGn-1 M509R was expressed as a fusion protein tagged with 6× His in COS-1 cells as described in 'Materials and methods.' The recombinant proteins secreted in the medium were purified and then separated by SDS-PAGE, and the expression of each His-tagged protein was examined using an anti-ChGn-1 antibody. *Lane 1*, ChGn-1-His; *lane 2*, ChGn-1 M509R-His; *lane 3*, ChGn-1-H234R-His.

Table 1 The GalNAcT-II activity of the fusion proteins secreted into the culture medium by transfected COS-1 cells

Protein	*GalNAcT-II activity (pmol ml^{-1} medium per h)*[a]
ChGn-1	1.2
ChGn-1 mutant H234R	ND[b]
ChGn-1 mutant M509R	ND[b]

Abbreviation: ChGn-1, chondroitin beta-1,4-*N*-acetylgalactosaminyltransferase-1.
Values represent the average of three independent experiments.
[a]Polymer chondroitin was used as an acceptor substrate.
[b]ND, not detected (<0.01 pmol ml^{-1} medium per h).

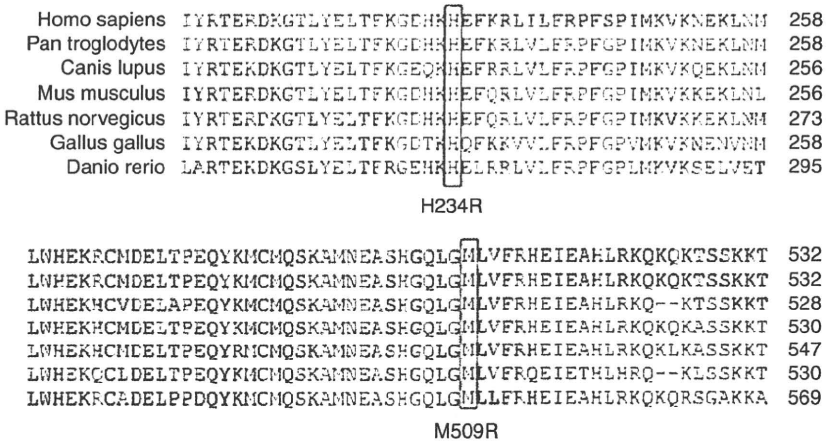

Figure 2 Amino acid sequence alignment of *ChGn-1* gene. H234R and M509R positions are highly conserved across species. Sequence include human (homo sapience; NP_060841.3), chimpanzee (Pan troglodytes; XP_519635.2), dog (Canis lupus; XP_539946.2), mouse (Mus musculus; NP_766341.3), rat (Rattus norvegicus; XP_224757.4), chicken (Gallus gallus; XP_420453.2) and zebra fish (Danio rerio; XP_001333479.2). Sequence were aligned using the NCBI homologene web site (http: //www.ncbi.nlm.nih.gov/ homologene).

H234R and M509R mutants. To examine the expression and activity of the mutant proteins, each of the soluble mutants was expressed in COS-1 cells and the culture medium was purified with HIS-Select Cobalt Affinity beads. The purified mutant proteins were used for western blotting analysis, and glycosyltransferase activities were assessed using chondroitin as an acceptor. When the soluble form of the ChGn-1 H234R and M509R mutants were expressed in COS-1 cells, proteins, ~70 kDa in size were secreted as shown by western blotting using an anti-ChGn-1 antibody (Figure 1). Although the ChGn-1 H234R and M509R mutant proteins were expressed (Figure 1, lanes 2 and 3), these mutant proteins showed no GalNAcT-II activities (Table 1).

We analyzed amino acid sequence alignment of *ChGn-1* gene. The novel coding mutations identified in this study changed the charge of the amino acid sequence. And the both mutation sites of *ChGn-1* genes are highly conserved across species from zebrafish to humans (Figure 2). The mutation of H234R and M509R would be possible to form a new salt-bridge and contribute to the formation of the protein turn around the positive-charged residue. Moreover, these two mutations were not represented in the single-nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/).

DISCUSSION

In this study, we found two novel mutations in *ChGn-1* gene, both of which were associated with a profound decrease of their enzyme activity, in two patients with neuropathies of unknown etiology.

Proteoglycans are considered to be involved in the development of the nervous system. Defects in the production of CSPGs therefore may be associated with developmental errors in both the central and peripheral nervous systems. Complete loss of chondroitin polymerization has been studied in nematodes, indicating that condroitin is required for embryonic cytokinesis and cell division.[15,16]

In addition, CSPGs are known to have an important role in nerve injury. CSPG is known to be a major component of glial scarring. It is considered to be a major obstacle for recovery of the adult nervous system after injury, especially in light of its well-known activity in limiting axonal growth. *In vitro*, many CSPG family members have been reported to inhibit neurite outgrowth. However, the inhibitory activity of CSPGs does not necessarily mean that they are simple barrier molecules that contribute to nerve regeneration. They may avoid excessive fiber regeneration and inappropriate reinnervation.[17] In addition, in some situations, CSPG has been shown to strongly promote neurite outgrowth.[18] Thus, CSPG has a pivotal role in the repair of the injured spinal cord and in the recovery of motor function during the acute phase after injury.[17]

The peripheral nervous system is not as completely protected against injuries as the central nervous system. It should therefore be more vulnerable to minor trauma, such as compressions or bruises. Defect in the glycosyltransferases of the CSPGs, such as ChGn-1, may be associated with the pathogenesis of the peripheral neuropathies by disturbing the recovery from the minor trauma.

As the mutations we identified in this study are heterozygous mutations, the amount of CSPGs may not decrease profoundly in either case. We therefore consider that those mutations may not usually cause any obvious clinical disturbances. However, these mutations may reduce the effectiveness of the reparation of the peripheral nervous system because of poor productivity of CSPGs at the time of the emergency. It may be associated with the development of the irreversible hemi-facial palsy in the patient with the H234R mutation, whereas Bell's palsy is reversible in most of the cases. Another possible mechanism is toxic or dominant-negative effect. For example, as for superoxide dismutase mutation for familial amyotrophic lateral sclerosis, the pathogenetic mechanism is not from the loss of enzymatic activities.[19] This possibility should be investigated in future.

An association between the abnormality in the CSPG genes and skeletal diseases has been reported.[12] However, to the best of our knowledge, the association with neurological disorders has never been reported. The present investigation did not prove that the heterozygous *ChGn-1* mutations cause neuropathy in two patients. However, the possible association between the mutations and pathogenesis of neuropathy may exist. Studies on larger number of patients and complete family studies are necessary.

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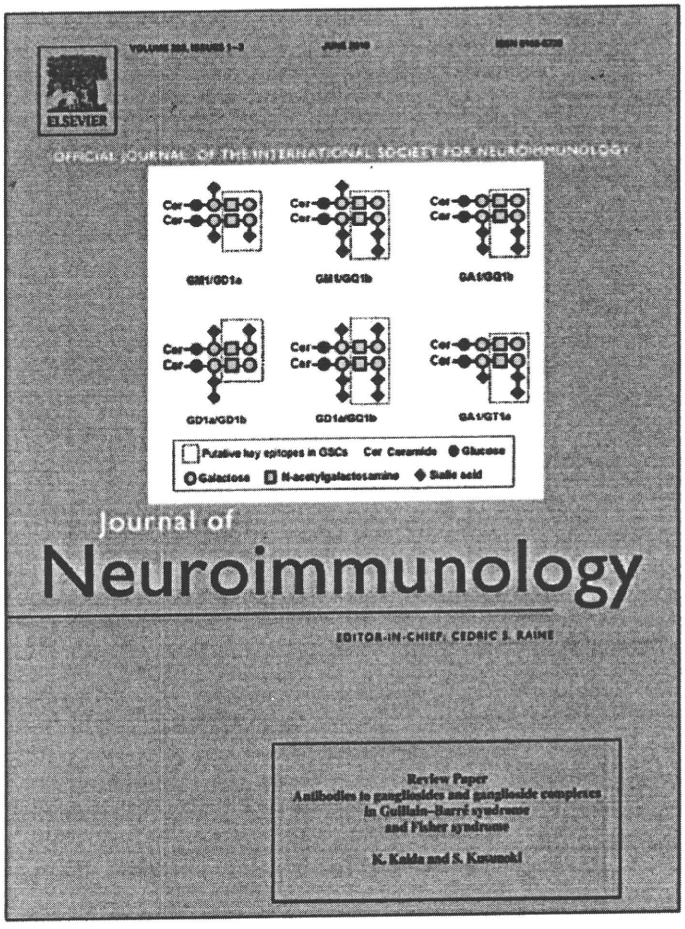

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Review article

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

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Antibody

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

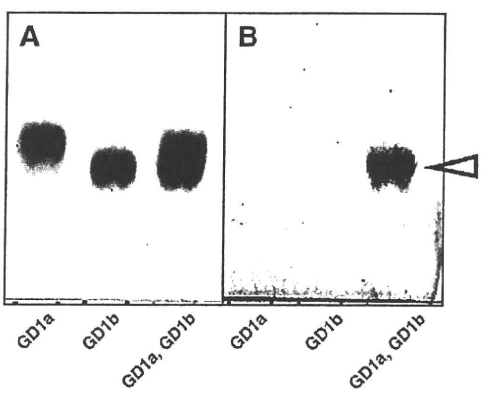

Fig. 1. Results from thin-layer chromatography (TLC). (A) TLC bands visualized with orcinol reagent. (B) TLC immunostaining using a representative anti-GD1a/GD1b-positive serum, showing that the overlapping region between GD1a and GD1b is strongly stained (arrowhead). Serum is diluted to 1:100.

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.

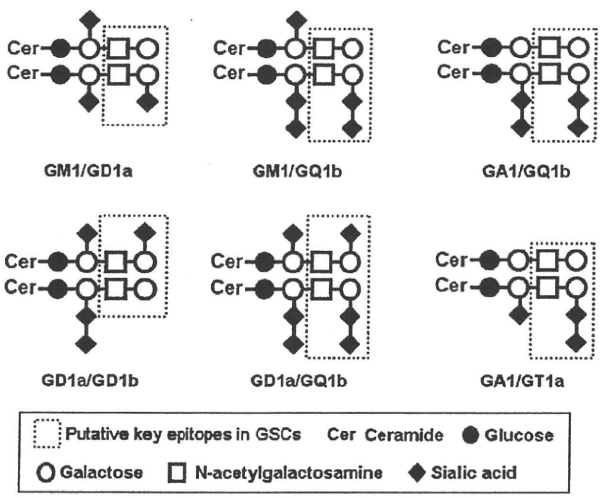

Fig. 2. Pattern diagrams of glycolipid complexes GM1/GD1a, GD1a/GD1b, GM1/GQ1b, GD1a/GQ1b, GA1/GQ1b, and GA1/GT1a. Squares with dotted lines indicate putative antigenic epitopes for antiganglioside complex antibodies.

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

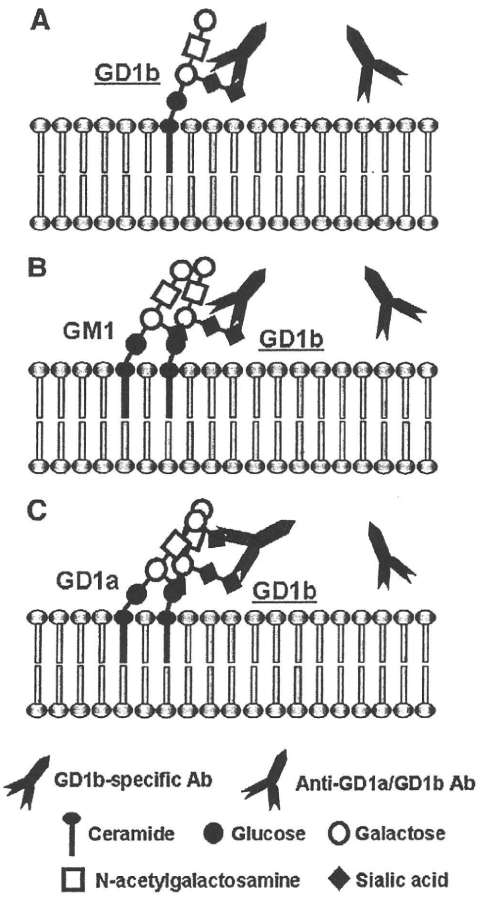

Fig. 3. Schematic diagram depicting proposed antigen–antibody interactions between GD1b-specific antibodies and GSCs containing GD1b in the nerve cell membrane. (A) The GD1b-specific antibody binds to antigenic epitopes of GD1b that are exposed and unmasked in the cell membrane. (B) GD1b and GM1 colocalize 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.

cell membrane and whether the GM1-like epitopes are unmasked. Colocalization and *cis*-interaction of gangliosides may either enable or inhibit antibody binding to the neuronal membrane or have no effect (Greenshields et al., 2009). In our recent study, the epitope targeted by monoclonal anti-GA1 antibody was masked in a glycolipid complex GA1/GQ1b, whereas that recognized by the monoclonal anti-GQ1b antibody was preserved (Ogawa et al., 2009). Therefore, even if GA1 and GQ1b actually form complexes in the biological membrane, the anti-GQ1b antibody can access GQ1b epitopes in GA1/GQ1b but the anti-GA1 antibody cannot access GA1 epitopes in the same complex. Thus, the local glycolipid environment in the plasma membrane may regulate the pathogenic effect of antiganglioside antibodies, and it should be borne in mind that the antibody–antigen interaction depends not only upon the fine specificity of individual antibodies but also upon the conformation of glycoepitopes formed in glycolipid environments in the nerve cell membrane.

7. Putative factors influencing antibody binding to target epitopes

Certain specific conditions of glycoepitopes in the cell membrane are essential for exertion of the pathogenic action of antiganglioside antibodies. First, complex glycolipid environments in the cell membrane may influence the accessibility and avidity of antiganglioside antibodies for target gangliosides, as described above (Fig. 3) (Kaida et al., 2008a; Greenshields et al., 2009). Analyses of the reactivity of antiganglioside antibodies against various GSCs are useful for evaluation of the accessibility of the antibodies. Second, the large amount of targeted gangliosides in particular loci of peripheral nerves is closely associated with antibody-mediated injury and specific clinical features. GQ1b is abundantly distributed in human oculomotor, trochlear, and abducens nerves, leading to predisposition to binding of anti-GQ1b antibodies (Chiba et al., 1993, 1997). Anti-GD1a antibody-mediated nerve injury is observed in GD3-synthase knockout mice that overexpress GD1a, but not in normal mice, probably because the abundant expression of GD1a at a particular region is critical for development of anti-GD1a-mediated nerve damage (Goodfellow et al., 2005). Third, the conformational difference of glycoepitopes between motor and sensory nerves may influence antibody binding and development of nerve injury. Ganglioside analysis of human motor and sensory nerves has shown that the amount of GM1 and GD1a is almost equal in both nerves, but that the ceramide compositions differ between the motor and sensory nerves (Ogawa-Goto et al., 1990): the gangliosides from sensory nerves are abundant in long-chain fatty acids, in contrast to those from motor nerves. In a binding assay using derivatives of GD1a bearing very long chain fatty acids, the difference in length of fatty acids in the ceramide reduced the binding ability of monoclonal anti-GD1a antibodies with GD1a derivatives, indicating that the ceramide composition can modify the steric structure of gangliosides in membranes (Tagawa et al., 2002). These findings may partly explain the preferential binding of anti-GD1a antibodies from AMAN patients to GD1a in motor nerves (Gong et al., 2002).

Finally, the conformational microstructure of sialic acids in gangliosides may regulate the binding ability of antiganglioside antibodies. In a recent immunohistochemical study using GD1a derivatives with chemically modified sialic acid residues, anti-GD1a monoclonal antibodies that preferentially stained motor axons specifically bound to GD1a-1-ethyl ester, GD1a-1-alcohol, and GD1a-1-methyl ester, in contrast to other anti-GD1a monoclonal antibodies that stained both motor and sensory axons (Lopez et al., 2008). There were no differences in binding to GD1a derivatives between anti-GD1a antibodies from AMAN patients and motor-specific anti-GD1a monoclonal antibodies. Thus, ganglioside exposure in the nerves and the fine specificity of antiganglioside antibodies is likely to regulate their accessibility to target gangliosides. The effects of phospholipids should also be considered because the presence of several kinds of phospholipids influences antibody binding to gangliosides (Hirakawa et al., 2005).

8. Perspective

Recent progress on the immunobiological mechanism in GBS has contributed to the precise understanding of antiganglioside antibody-mediated nerve dysfunction, and has encouraged development of novel therapeutic strategies for patients with GBS and its variants (Willison et al., 2008; Kaida and Kusunoki, 2009). Consideration of GSCs will provide new avenues of research on antibody–antigen interactions in GBS. Examination of anti-GSC antibodies may expand the spectrum of antiganglioside antibodies in GBS, enhancing their value as diagnostic markers and expediting understanding of the pathophysiology underlying antiganglioside antibody-mediated nerve dysfunction. New techniques such as combinatorial glycoarrays are beneficial for studies on anti-GSC antibodies (Rinaldi et al., 2009). The understanding of GSCs will also shed light on microdomain function mediated by carbohydrate–carbohydrate interactions in biological membranes. Microdomain function is controlled by carbohydrate-binding proteins such as selectins and Siglecs and is based on *cis*- or *trans*-carbohydrate–carbohydrate interactions (Hakomori, 2004; Varki, 2007). In the microdomain, complex glycoconjugates such as GSCs with clustered sialic acid epitopes may form rigid rodlike structures with multivalency and strict binding specificity, and are likely to function in cell–cell recognition or immune-mediated events in a more effective manner than a solo glycoepitope of an isolated ganglioside. This hypothesis is supported by a recent study demonstrating that a GSC, GM2/GM3, provides more efficient suppression of cell motility through blocking of cMet activation compared to GM2 or GM3 alone (Todeschini et al., 2008).

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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.

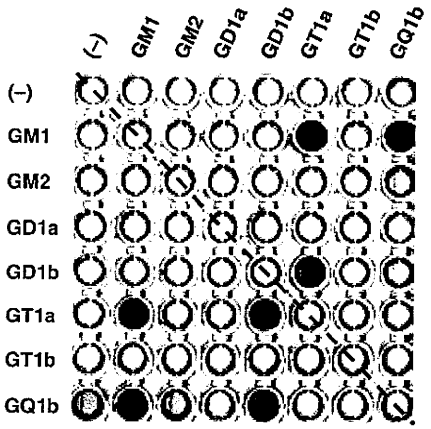

Fig. 1 An ELISA plate showing the binding activities of a serum antibody that recognizes a combination of [Galβ1-3GalNAc] and [NeuAcα2-8NeuAcα2-3 Galβ1-3GalNAc] in the terminal residues. All the wells in each line and column were coated with a respective ganglioside (e.g. the wells in the first line 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.