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Short Communication

## Apoptosis of primary sensory neurons in GD1b-induced sensory ataxic neuropathy

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### Abstract

Experimental autoimmune sensory ataxic neuropathy was induced in three of six rabbits sensitized with GD1b ganglioside (GD1b-SAN). TUNEL assay was performed on sections of dorsal root ganglia in the cauda equina. The results showed the presence of TUNEL-positive neurons in all three rabbits affected with GD1b-SAN. In contrast, no such neurons were observed in any of the sections from the unaffected rabbits that had been inoculated with GD1b, rabbits inoculated with adjuvant alone or those without inoculation. These data support that an apoptotic mechanism is involved in the pathogenesis of GD1b-SAN.

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**Keywords:** Ganglioside; Neuropathy; Guillain-Barré syndrome; Autoimmunity; Antibody; Apoptosis; Sensory neuron; Dorsal root ganglion

### Introduction

Antiganglioside antibodies are frequently present in sera from patients with autoimmune neuropathies, such as Guillain-Barré syndrome and IgM paraproteinemic neuropathy (Kusunoki, 2000; Willison and Yuki, 2002). Immunohistochemical studies have demonstrated that some gangliosides exhibit a unique localization in the nervous system (Kusunoki et al., 1993; Chiba et al., 1993; Kaida et al., 2003). The antibodies to these gangliosides may therefore be associated with unique clinical features by binding to regions in which the target antigen gangliosides are localized.

Immunohistochemistry using a monoclonal antibody against GD1b ganglioside has shown that GD1b is densely localized in the large neurons in the dorsal root ganglia (DRGs) of rabbits as well as humans (Kusunoki et al., 1993, 1996). These large neurons are known to mediate proprioception. IgM M-proteins that recognize a disialosyl residue of GD1b are specifically present in sera from patients with sensory ataxic neuropathy

(Willison et al., 2001). We have previously reported that sensitization of rabbits with GD1b induces sensory ataxic neuropathy (GD1b-SAN) (Kusunoki et al., 1996), in which IgG antibodies monospecific to GD1b is essential to the pathogenesis of the disease (Kusunoki et al., 1999a). GD1b-SAN is the first established animal model of autoimmune neuropathy mediated by antiganglioside antibodies. Passive transfer of anti-GD1b antisera from rabbits affected with GD1b-SAN-induced degeneration of rabbit sensory neurons, indicating that anti-GD1b antibody is directly involved in the pathogenesis of GD1b-SAN (Kusunoki et al., 1999b). The precise mechanism by which the anti-GD1b antibody causes the disease remains to be elucidated.

Pathological investigation of GD1b-SAN demonstrated that there was no lymphocytic infiltration in the affected regions. Degeneration of the axons was evident in the dorsal root and dorsal column with macrophage infiltration (Kusunoki et al., 1996). In contrast, in spite of the looseness of the blood-nerve barrier in the DRG, there was no significant finding in the DRG except for few Nageotte nodules (Kusunoki et al., 1996). This prompted us to investigate the possibility that the anti-GD1b antibody induces apoptosis of the large sensory neurons.

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Through the use of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay, we found evidence of apoptosis in the DRGs from GD1b-SAN-affected rabbits.

#### Materials and methods

Immunization with GD1b was performed as described previously (Kusunoki et al., 1996). Six rabbits were immunized

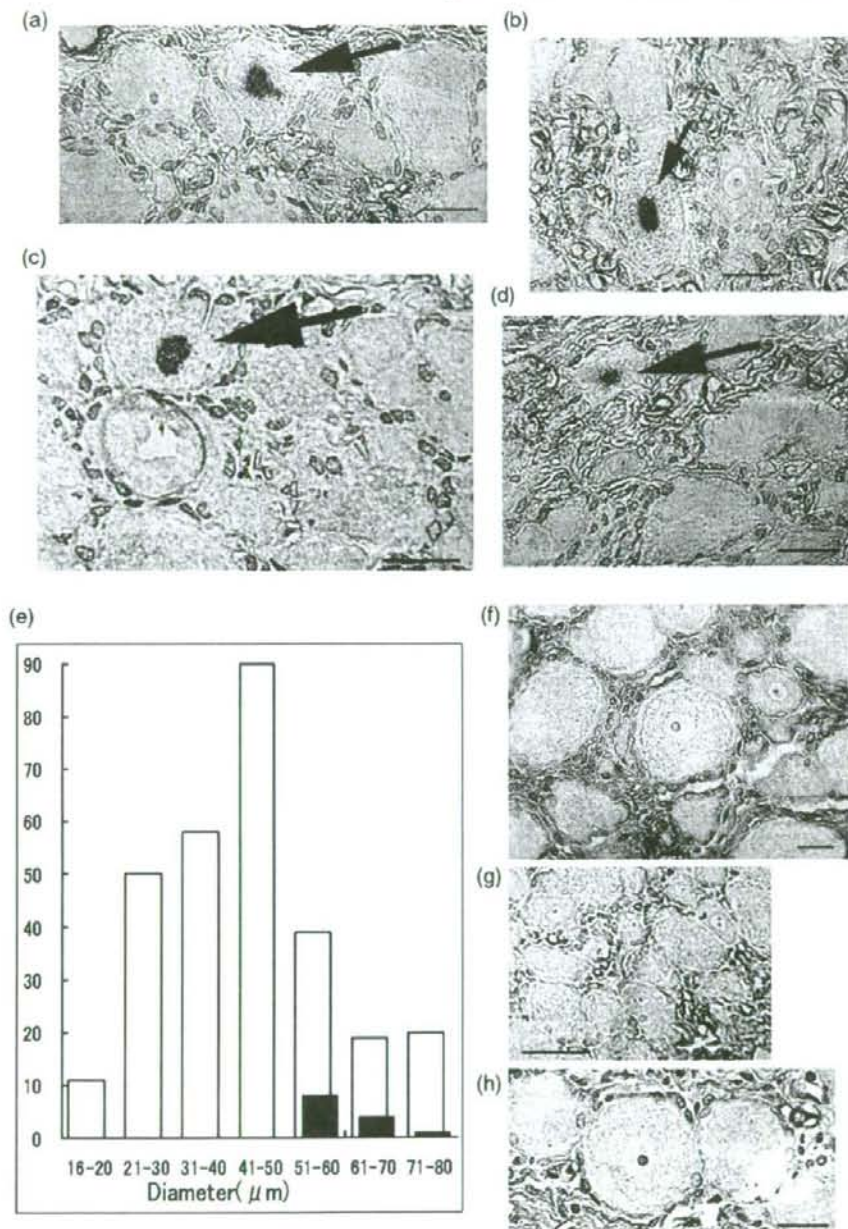


Fig. 1. (a-d) TUNEL assay of sections from rabbits affected with GD1b-SAN. Primary sensory neurons containing nucleotides that react positively (arrows). Scale bar = 50 μm. (e) The histogram of the diameters of TUNEL-positive and TUNEL-negative neurons. The TUNEL-positive neurons were of large ones. (f-h) TUNEL assay of sections from unimmunized rabbits inoculated with GD1b (f), inoculated with adjuvant alone (g) and without any inoculation (h). No TUNEL-positive cells were observed. Scale bar = 50 μm.

with GD1b, and two were given the same inoculum without GD1b. Two rabbits without inoculation were also kept in the same room of the animal center. Serum samples were taken by ear vein puncture at 1- or 2-week intervals. The rabbits were checked daily for clinical signs and weighed twice per week.

Serum anti-GD1b antibodies were investigated by the use of enzyme-linked immunosorbent assay, as described previously (Kusunoki et al., 1996). Microtiter wells were coated with 200 ng of GD1b, with an uncoated well serving as the control. Peroxidase-conjugated antibody to rabbit IgM (mu-chain specific; Cappel, West Chester, PA; diluted 1:400) or rabbit IgG (gamma-chain specific; Southern Biotechnology Associates Inc., Birmingham, USA; diluted 1:2000) was used as the secondary antibody. The optical density (OD; 492 nm) was corrected by subtracting the OD of the control well that had been processed in the same manner. A reaction with a corrected OD of more than 0.1 was considered positive.

The rabbits affected with GD1b-SAN were sacrificed 2 or 3 days after the neurological onset (namely, 35, 42 and 90 days after the first inoculation, respectively). The rabbits that had been immunized with GD1b but did not exhibit any neurological problems were sacrificed 121, 136 and 156 days after the first inoculation. The rabbits inoculated with adjuvant alone were sacrificed on day 35 and 42 after the first inoculation and those without any inoculation were sacrificed after 14 and 21 days of observation.

The lumbar spinal cord and the DRGs in the cauda equina were removed from all of the rabbits except for the two unaffected GD1b-sensitized rabbits (sacrificed on 121 and

136 days after first inoculation). Specimens were fixed either in 4% formaldehyde in phosphate-buffered saline for 48 h or in 2.5% glutaraldehyde and 2% paraformaldehyde buffered with 0.1 M sodium cacodylate (half Karnovsky solution) for 12 h.

The formaldehyde-fixed specimens were embedded in paraffin and serial sections, 10  $\mu$ m in thickness, were prepared. TUNEL assay was performed using a kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. Briefly, the sections underwent protein digestion at 37 °C for 5 min, after deparaffinization and hydration. After washing, they were incubated with 50  $\mu$ l of TdT reaction solution for 10 min in a moist chamber at 37 °C. After inactivation of intrinsic peroxidase, sections were incubated with 100  $\mu$ l of peroxidase-conjugated antibody solution for 10 min in a moist chamber at 37 °C. After removing the antibody solutions, they were incubated with 100  $\mu$ l of diaminobenzidine solution for 5 min at room temperature. After washing, sections were dehydrated and covered with a cover glass. The sections of DRGs in the cauda equina obtained from each of the three rabbits affected with GD1b-SAN, each of the two adjuvant controls, one unaffected rabbit sensitized with GD1b, and two rabbits without inoculation were examined with TUNEL assay. Seventy sections from each rabbit were examined. In the DRGs from the affected rabbits, the diameters of the TUNEL-positive and TUNEL-negative neurons containing a nucleus were measured. Immunohistochemistry with anti-caspase 3 antibody also was performed on deparaffinized sections as described by Gown and Willingham (Gown and Willingham, 2002). Mouse monoclonal anti-caspase 3 (3G2, 1:40 diluted, Abcam, Cambridge, UK) was used as the primary antibody, and peroxidase-conjugated goat

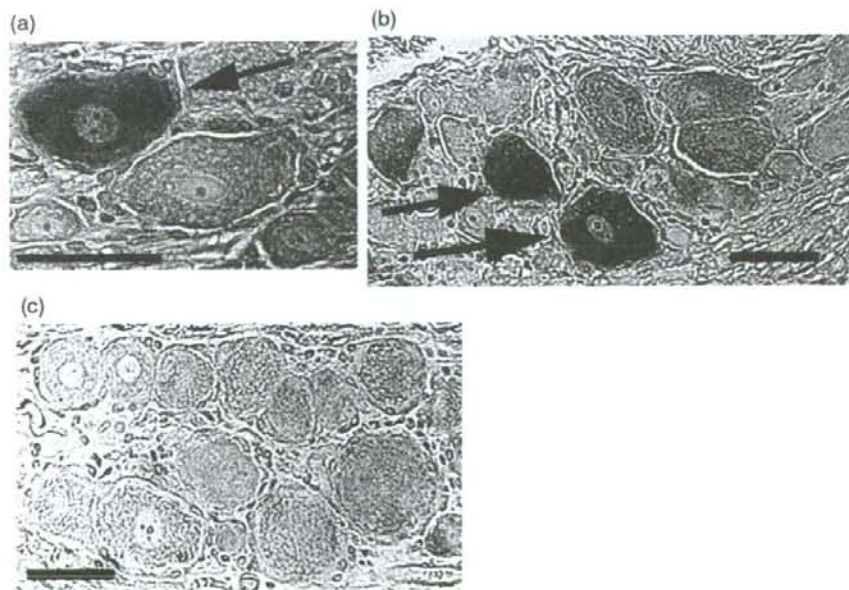


Fig. 2. Immunohistochemistry using anti-caspase 3 antibody. (a and b) Some DRG neurons from rabbits affected with GD1b-SAN were immunostained with anti-caspase 3 antibody (arrows). (c) No such staining was observed in DRG from a rabbit inoculated with adjuvant alone. Scale bar = 50  $\mu$ m.

anti-mouse IgG (1:250 diluted, MP Biomedicals, Ohio, USA) was the secondary antibody.

The Karnovsky-fixed specimens were postfixed in 2% osmium tetroxide for 2 h, dehydrated with ethanol and embedded in epoxy resin. Semi-thin sections, 1.0  $\mu$ m in thickness, were cut from the Epon block and were stained with toluidine blue.

The experiments involving animals were approved by the local ethics committee in Kinki University.

## Results

Anti-GD1b IgM antibody was detected 2 weeks after the first inoculation and reached a maximum at 4 weeks. IgG anti-GD1b antibody was elevated later, after the elevation of IgM.

Three of the six rabbits immunized with GD1b developed SAN 33, 40 and 88 days after the first inoculation, respectively.

In the sections stained with toluidine blue, axonal degeneration of the dorsal column of the spinal cord was evident, as described previously.

A few TUNEL-positive cells were observed in the formaldehyde-fixed sections from all three rabbits affected with GD1b-induced SAN (Figs. 1a–d). Two sections were mounted on each glass slide, in which approximately one TUNEL-positive cell was observed. In contrast, no TUNEL-positive cells were observed in any section from the unaffected rabbits that had been immunized with GD1b (Fig. 1f), the rabbits inoculated with adjuvant alone (Fig. 1g) or the rabbits without inoculation (Fig. 1h).

Of the 300 neurons of DRGs from the affected rabbits, 13 were TUNEL-positive and 287 were TUNEL-negative. The histogram was shown in Fig. 1e.

A few neurons per section of DRG from the affected rabbits were immunostained with anti-caspase 3 antibody, whereas no such staining was seen in DRG from control rabbits (Fig. 2).

## Discussion

Anti-GD1b antibodies were detected in all six rabbits sensitized with GD1b. Three of those six rabbits developed SAN. This finding is compatible with the previous results that about half of the rabbits sensitized with GD1b developed SAN (Kusunoki et al., 1996, 1999a).

We previously reported that downregulation of trkC occurs in the dorsal root ganglia from rabbits in the acute phase of GD1b-SAN (Hitoshi et al., 1999). TrkC serves as a receptor for neurotrophin-3 (NT3). It is known that the large primary sensory neurons in the dorsal root ganglia, which mediate proprioception, depend mainly on neurotrophin-3-mediated trkC signaling. It has been reported that mice defective for trkC exhibit abnormal movement due to lack of proprioception (Klein et al., 1994). Our above result of trkC downregulation therefore suggests that anti-GD1b antibody-mediated trkC downregulation and subsequent apoptosis of the large neurons of DRG contribute to the pathogenesis of GD1b-SAN.

The present investigation provides clear evidence that an apoptotic mechanism is involved in the pathogenesis of GD1b-SAN. Positive immunostaining of some DRG neurons from

affected rabbits with anti-caspase 3 antibody also indicates the involvement of an apoptotic mechanism in GD1b-SAN. Although the number of neurons with apoptotic changes appears to be small, approximately 4% of neurons were TUNEL-positive, indicating that quite a few sensory neurons could be affected in each animal. An apoptotic neuron should not remain *in situ* for a long time but would soon disappear. Therefore, we were not able to identify many apoptotic neurons at the same time in the pathological specimens.

Gangliosides are known to form microdomains called lipid rafts (Simons and Toomre, 2000). Within the rafts, gangliosides are believed to interact with important transmembrane receptors or signal transducers (Kasahara et al., 2000). Treatment of rat cerebellar cultures with a monoclonal anti-ganglioside GD3 antibody induced the activation of the Src family kinase Lyn and rapid tyrosine phosphorylation of some proteins (Kasahara et al., 1997). Thus, antiganglioside antibodies may alter the function of neurons through binding to target gangliosides in the raft. The mechanism(s) by which antibody binding causes apoptosis and whether the downregulation of trkC is involved in the process need to be clarified in future investigations.

Neuropathy with IgM M-protein binding to a disialosyl residue of several gangliosides, including GD1b, GT1b and GQ1b, is a human counterpart of GD1b-SAN (Willison et al., 2001). There has been one reported autopsy case of this kind of neuropathy, demonstrating a reduction in the number of sensory neurons in the DRG and pallor of the dorsal column in the spinal cord (Obi et al., 1999). In addition to the IgM paraproteinemic neuropathy, GBS with a monospecific anti-GD1b IgG antibody has been associated with ataxia due to disturbance in deep sensation (Wicklein et al., 1997). It has been reported that human IgM monoclonal antibody recognizing GD2, GD1b, GT1b and GQ1b resulted in death of rat dorsal root ganglion neurons (Ohsawa et al., 1993). However, the precise mechanism was not elucidated. Our present findings strongly suggest that apoptosis of the large primary sensory neurons contributes to the pathogenesis of human neuropathies with antibodies recognizing gangliosides with disialosyl residue, in particular GD1b.

## Acknowledgments

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## Roles of complex gangliosides in the development of experimental autoimmune encephalomyelitis

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We induced experimental autoimmune encephalomyelitis (EAE) in GM2/GD2 synthase knockout mice (GM2/GD2<sup>-/-</sup>), which cannot synthesize complex gangliosides, such as GM1, GD1a, GD1b, GT1b, and GQ1b, to investigate the roles of complex gangliosides in the pathogenesis of this disease. We used myelin-oligodendrocyte glycoprotein (MOG) as an immunogen. In active immunization EAE, the severity of clinical score was not different but the disease onset was significantly delayed in GM2/GD2<sup>-/-</sup> compared with those in wild-type mice. When we transferred MOG-reactive T cells from GM2/GD2<sup>-/-</sup> or wild-type mice to wild-type mice, no significant differences were observed between the two groups. In contrast, when we transferred MOG-reactive T cells from wild-type mice to GM2/GD2<sup>-/-</sup> or to wild-type mice, the onset of EAE in GM2/GD2<sup>-/-</sup> mice was delayed. The recall response of MOG-specific T cells, the function of antigen presenting cells, or the expression of several adhesion molecules in the endothelium were not significantly different between GM2/GD2<sup>-/-</sup> and wild-type mice. On the other hand, quantitative analysis of cellular infiltration in the central nervous system (CNS) on day 9 of active immunization EAE showed that the CD4<sup>+</sup> cell number in the CNS isolated from GM2/GD2<sup>-/-</sup> mice was significantly less than that from wild-type mice. It indicated that the delayed onset of EAE in GM2/GD2<sup>-/-</sup> mice was due to the delay of the migration of pathogenic T cells into the CNS. Thus, the complex gangliosides may be involved in the T cell–endothelial cell interaction in the pathogenetic process of EAE.

**Keywords:** EAE/MS/neuroimmunology/rodent/cell surface molecules

### Introduction

Gangliosides are sialic acid-containing glycosphingolipids that are highly enriched in the mammalian nervous system. They consist of an oligosaccharide core structure with an attached sialic acid and ceramide and are found primarily in the outer

leaflet of the cell membrane. Although they are the major sialo-glycoconjugates in the brain, their biological functions remain to be elucidated.

In human autoimmune neuropathies, such as Guillain-Barré syndrome and IgM paraproteinemic neuropathy, antibodies against gangliosides are present in the patients' sera (Chiba et al. 1992). They are considered as useful diagnostic markers and essential factors involved in the pathogenetic mechanisms (Obi et al. 1992). Gangliosides may therefore act as targets for autoimmune mechanisms in autoimmune neuropathies (Kusunoki et al. 1996). In contrast, the role(s) of the gangliosides in the pathogenesis of the immune-mediated diseases of the central nervous system (CNS) have not yet been elucidated.

Experimental autoimmune encephalomyelitis (EAE) is an immune-mediated encephalomyelitis that can be generated in experimental animals using myelin proteins as an immunogen. The roles of gangliosides in the pathogenesis of EAE remain controversial. It has previously been reported that sensitization with gangliosides does not induce EAE, whereas the administration of gangliosides with myelin proteins either enhances or suppresses the disease activities or has no effect (Kusunoki et al. 1988; Shimada et al. 1994; Saez-Torres et al. 1998). The presence of definite immune responses against gangliosides has not been shown previously. On the other hand, gangliosides on the neural, glial, or immune cell membranes might be required in the pathogenetic mechanisms of EAE, in particular in the process of lymphocyte activation, migration, and their cytotoxic activities against the CNS.

Mice engineered to lack a key enzyme in complex ganglioside biosynthesis (GM2/GD2 synthase), which consequently do not synthesize complex gangliosides, such as GM1, GD1a, GD1b, GT1b, and GQ1b, have been reported previously (Takamiya et al. 1996; Furukawa et al. 2002). These animals express only the simple ganglioside molecular species, such as GM3 and GD3. We induced EAE in this strain of mice, between 8 and 12 weeks of age, to investigate the roles of complex gangliosides in the pathogenetic mechanisms of EAE by examining any possible difference in the clinical phenotype, disease course, and immunopathological findings. Investigations of EAE in GM2/GD2<sup>-/-</sup> mice should give us a clue to elucidate the roles of complex gangliosides in the nervous system, as well as in the immune system.

### Results

#### *Disease onset of EAE was delayed in GM2/GD2<sup>-/-</sup> mice*

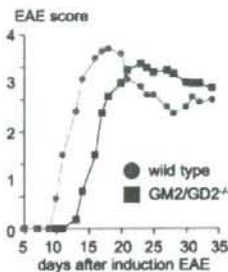
To examine the role of complex gangliosides in the development of EAE, we first performed active immunization to establish EAE. As for the severity of EAE score, including maximum EAE score and cumulative score, there was no significant

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**Table I.** Statistical analysis of clinical EAE scores

	Incidence	Day of onset	Max. score	Cumulative score
A				
Mouse				
GM2/GD2 <sup>-/-</sup>	18/21 (85.7%)	17.6 ± 0.9*	3.10 ± 0.31	47.9 ± 5.4
Wild-type	15/17 (88.2%)	12.8 ± 0.9	3.03 ± 0.34	50.4 ± 6.5
B				
Donor mouse				
GM2/GD2 <sup>-/-</sup>	10/15 (66.7%)	11.7 ± 0.9	0.97 ± 0.26	6.80 ± 1.84
Wild-type	12/18 (66.7%)	12.9 ± 1.1	0.97 ± 0.22	6.72 ± 1.76
C				
Recipient mouse				
GM2/GD2 <sup>-/-</sup>	18/24 (75.0%)	15.4 ± 0.4*	1.29 ± 0.24	5.23 ± 1.01
Wild-type	17/20 (85.0%)	12.5 ± 1.1	1.45 ± 0.21	8.10 ± 1.64

(A) Active immunization EAE. Each mouse was immunized with MOG<sub>35-55</sub> peptide for the induction of EAE. Data were the same as Figure 1. (B) Adoptive transfer EAE. One million encephalitogenic CD4<sup>+</sup> T cells from wild-type mice or GM2/GD2<sup>-/-</sup> mice (donor) were injected into wild-type mice (i.v.). Data were the same as Figure 3A. (C) Another type of adoptive transfer EAE. One million encephalitogenic CD4<sup>+</sup> T cells from wild-type mice were injected intravenously into wild-type mice or GM2/GD2<sup>-/-</sup> mice (recipient). Data were the same as Figure 3B. Mean ± SEM of the following parameters are shown: maximum score of EAE (Max. score), the days of EAE onset, incidence of paralyzed mice among sensitized rats (Incidence), and summation of the clinical scores from days 0 to 30 (Cumulative score). The statistical significance of the difference was determined using ANOVA; \**P* < 0.05 versus wild-type mice.



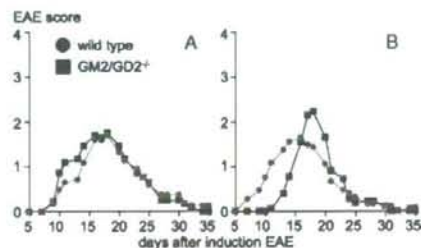
**Fig. 1.** Disease onset of EAE is delayed in GM2/GD2<sup>-/-</sup> mice. EAE was induced in GM2/GD2<sup>-/-</sup> or wild-type mice by immunization with MOG<sub>35-55</sub> in the CFA, as described in *Materials and methods*. Statistical analysis is shown in Table IA. One representative experiment of two independent experiments was expressed as the mean EAE score.

difference between GM2/GD2 synthetase-deficient mice (GM2/GD2<sup>-/-</sup>) and wild-type mice. However, the disease onset was significantly delayed in GM2/GD2<sup>-/-</sup> compared with wild-type mice (Figure 1, Table IA).

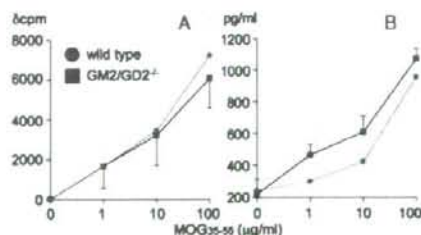
We next performed the adoptive transfer EAE. When we transferred myelin-oligodendrocyte glycoprotein (MOG)-reactive T cells from GM2/GD2<sup>-/-</sup> or wild-type mice to wild-type mice, the onset of EAE was not significantly different between the two groups (Figure 2A, Table IB). On the other hand, when we transferred MOG-reactive T cells from wild-type mice to GM2/GD2<sup>-/-</sup> mice or wild-type mice, the onset of EAE in the GM2/GD2<sup>-/-</sup> mice was delayed compared with wild-type mice (Figure 2B, Table IC). These results indicate that a lack of complex gangliosides does not have any effect during the induction phase of EAE, but exhibits effects during the effector phase of EAE.

#### Complex gangliosides do not have an effect on the recall response of MOG-specific T cells

To determine the mechanisms of complex gangliosides in the T-cell activation, we examined the proliferative response



**Fig. 2.** Complex gangliosides play a role in the effector and in the induction phases of EAE. (A) Encephalitogenic T cells were prepared by immunizing wild-type mice or GM2/GD2<sup>-/-</sup> mice and culturing their lymph node cells in the presence of MOG and IL-12 for 4 days. One million CD4<sup>+</sup> cells were injected into the tail vein of wild-type mice. EAE clinical scores were assessed as described in Table IB. (B) As described above, MOG-specific CD4<sup>+</sup> cells from wild-type mice were transferred into wild-type mice or GM2/GD2<sup>-/-</sup> mice. EAE clinical scores were assessed as described in Table IC. These data are shown as a mean clinical score ± SEM. Data are representative of three independent experiments.



**Fig. 3.** Comparison of MOG<sub>35-55</sub>-specific T-cell response in GM2/GD2<sup>-/-</sup> and wild-type mice. Popliteal and inguinal lymph nodes cells from GM2/GD2<sup>-/-</sup> mice or wild-type mice were incubated in the presence of MOG<sub>35-55</sub> for 48 h. The proliferative response was determined by the uptake of [<sup>3</sup>H] thymidine (A), and IFN- $\gamma$  was detected by ELISA (B). Representative data of two independent experiments are shown (*n* = 5 for each group). Error bars represent SEM.



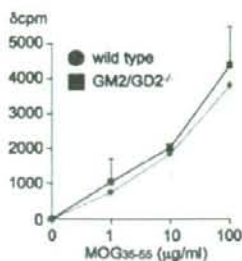


Fig. 4. Evaluation of MOG<sub>35-55</sub>-specific T-cell proliferation stimulated with APC from GM2/GD2<sup>-/-</sup> mice. A MOG-specific T cell line was established as described in *Material and methods*. One million T cells were cultured with MOG<sub>35-55</sub> and APCs for 72 h. Irradiated splenocytes from GM2/GD2<sup>-/-</sup> mice or wild-type mice were used as APCs. The proliferative response was evaluated by the uptake of [<sup>3</sup>H] thymidine. Representative data of two independent experiments are shown ( $n = 5$  for each group). Error bars represent SEM.

and cytokine production of draining lymph node (LN) cells in vitro GM2/GD2<sup>-/-</sup>, or wild-type mice were immunized with MOG<sub>35-55</sub>. Ten days after immunization, draining LN cells were collected and cultured with MOG<sub>35-55</sub> peptide. As shown in Figure 3A, there was no significant difference in the proliferative response of MOG-reactive T cells between GM2/GD2<sup>-/-</sup> and wild-type mice. We next examined the levels of cytokines in the culture supernatant by ELISA. The levels of IFN- $\gamma$ , IL-4, and IL-10 were similar in the culture supernatants of LN cells obtained from GM2/GD2<sup>-/-</sup> compared to wild-type mice (Figure 3B).

We next examined the function of complex gangliosides in antigen-presenting cells (APCs) using a MOG-specific T cell line. APCs and T cells engage in a series of complex and interconnecting signals to trigger a cellular immune response. Antigen processing and presentation by APCs allow T cells to recognize antigens. In order to proliferate the T cells, APCs are necessary to present the antigen. A MOG-specific T cell line was established, as described in *Materials and methods*. We used irradiated splenocytes from GM2/GD2<sup>-/-</sup> or wild-type mice as APCs. There was no significant difference in APCs between GM2/GD2<sup>-/-</sup> and wild-type mice (Figure 4). This suggested that complex gangliosides do not exhibit any effect on antigen presentation.

#### Lack of complex gangliosides has no effect on the presentation of adhesion molecules of the BBB

To analyze the mechanism of complex gangliosides on the infiltration of the inflammatory cells into the CNS, we focused on the blood-brain barrier (BBB). We performed histopathological staining of the brain and spinal cord from immunized mice. The degree of cell infiltration and demyelination in the brain and spinal cord was not different between wild-type and GM2/GD2<sup>-/-</sup> mice. For the recruitment of autoreactive T cells into the brain through the BBB, some adhesion molecules and chemokines are required. We performed immunohistochemical staining of sliced brain sections from EAE mice, with antibodies for adhesion molecules. Immunohistochemical staining revealed that the staining pattern of vascular endothelial cells and choroid plexus in the brain with ICAM-1, VCAM-1, E-

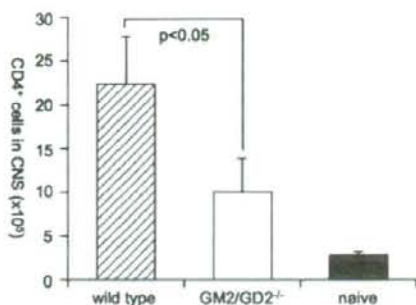


Fig. 5. Analysis of infiltrating cells isolated from the CNS. Infiltrating cells in the CNS from each mouse were collected as described in *Material and methods*. CD4<sup>+</sup> cell number in the infiltrating cells was calculated by flow cytometry. Data were presented as mean  $\pm$  SEM ( $\times 10^5$ ), wild-type ( $22.3 \pm 5.4$ ,  $n = 7$ ), GM2/GD2<sup>-/-</sup> ( $10.0 \pm 3.9$ ,  $n = 7$ ), and naive mice ( $2.7 \pm 0.4$ ,  $n = 5$ ). Data are representative of two independent experiments. The CD4<sup>+</sup> cell number in the CNS isolated from GM2/GD2<sup>-/-</sup> mice was significantly less than the cell number in the CNS from wild-type mice ( $P < 0.05$  by the Mann-Whitney  $U$ -test).

selectin, and P-selectin was not significantly different between GM2/GD2<sup>-/-</sup> and wild-type mice. These findings suggested that complex gangliosides do not have an effect on the presentation of adhesion molecules via the BBB. In addition, we examined MCP-1, which is also an important chemokine for immigration of autoreactive cells into the brain, using ELISA. MCP-1 was detected in the serum from EAE mice at 7 and 10 days after induction of EAE. The amount of MCP-1 was almost the same between GM2/GD2<sup>-/-</sup> and wild-type mice (data not shown).

#### Delayed infiltration of pathogenic T cells into the CNS in GM2/GD2<sup>-/-</sup> mice

To confirm the delayed infiltration of pathogenic T cells into the CNS in GM2/GD2<sup>-/-</sup> mice, we isolated mononuclear cells in the CNS obtained from each mouse on day 9 of active immunization EAE. It is the time just before the onset of EAE for wild-type mice. No mice actually showed EAE symptoms at that time. As shown in Figure 5, the CD4<sup>+</sup> cell number in the CNS isolated from GM2/GD2<sup>-/-</sup> mice was significantly less than the cell number from wild-type mice ( $10.0 \pm 3.9$  versus  $22.3 \pm 5.4$ , as mean  $\pm$  SEM,  $\times 10^5$ ). This result suggests that the delayed onset of EAE in GM2/GD2<sup>-/-</sup> mice was due to the delay of the migration of pathogenic T cells into the CNS.

#### Discussion

By the active immunization and the adoptive transfer experiment, we demonstrated that the lack of complex gangliosides delayed the disease onset of EAE.

There have been several reports describing the association of gangliosides and lymphocyte activation or differentiation (Rouquette-Jazdani et al. 2005; Shen et al. 2005). Actually, the administration of gangliosides has been reported to have an effect on the immune system in animals affected with EAE. Suppression of EAE in Lewis rats by the administration of gangliosides has been reported. It was shown that gangliosides

significantly suppressed myelin basic protein-induced proliferation in a dose-dependent manner (Rouquette-Jazdani et al. 2005). Chronic relapsing-remitting EAE, induced with MOG<sub>35-55</sub> in NOD mice, was successfully treated with brain-derived gangliosides. Splenocytes from the ganglioside-treated mice displayed markedly attenuated levels of MOG<sub>35-55</sub>-specific proliferation and IFN- $\gamma$  production (Sekiguchi et al. 2001). These reports raise the possibility that gangliosides are incorporated into the cell membrane of the lymphocytes to suppress their activity and ameliorate EAE. However, in the present study, adoptive transfer of MOG-specific T cells from GM2/GD2<sup>-/-</sup> or wild-type mice showed no significant difference, indicating that the presence of complex gangliosides does not influence the activation of the T cells.

As for the immune system, the spleen and thymus are slightly smaller in GM2/GD2<sup>-/-</sup> mice compared with those in wild-type (Zhao et al. 1999). GM1, asialo-GM1, and GD1b were representative gangliosides expressed on T cells of the wild-type mice and were completely abrogated on those of the GM2/GD2<sup>-/-</sup> mice. Splenocytes from the GM2/GD2<sup>-/-</sup> mice showed clearly attenuated proliferation compared with the wild-type mice when stimulated by IL-2 but not when they were treated with concanavalin A or anti-CD3 cross-linking. Expression levels of IL-2 receptor  $\alpha$ ,  $\beta$ , and  $\gamma$  were almost equivalent, and upregulation of  $\alpha$  chain after T-cell activation was also similar between the GM2/GD2<sup>-/-</sup> and wild-type mice. Activation of JAK1, JAK3, and SAT5 after IL-2 treatment was reduced, and c-fos expression was delayed and reduced in the mutant splenocytes, suggesting that the IL-2 signal was attenuated in the mutant mice, likely due to the modulation of the IL-2 receptors by the lack of complex gangliosides (Zhao et al. 1999). In our study, the proliferative response of MOG<sub>35-55</sub>-specific T cells in GM2/GD2<sup>-/-</sup> mice did not decrease in comparison with that in wild-type mice. Thus, the attenuation of IL-2 signal due to the lack of complex gangliosides was not involved in the induction phase of EAE.

Gangliosides are known to activate microglia via mitogen-activated protein kinase and NF- $\kappa$ B (Pyo et al. 1999; Marconi et al. 2005). Microglia are the brain-resident macrophages and play a crucial role in EAE. GT1b can induce production of nitric oxide (NO), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and expression of cyclooxygenase-2 (COX-2). GM1 and GD1a are also able to induce expression of COX-2 (Pyo et al. 1999). These molecules are important for an inflammatory response in EAE. COX-2 is inducibly expressed in inflammatory cells following an exposure to proinflammatory and mitogenic stimuli, and is primarily responsible for the synthesis of prostanoids involved in acute and chronic inflammation (Xie et al. 1991). We have reported that COX-2 inhibition is effective for the treatment of the autoimmune diseases, EAE and EAN (Miyamoto et al. 1998, 1999, 2002, 2006). Therefore, the lack of complex gangliosides may reduce an inflammatory response in the CNS due to inadequate activation of microglia.

Myelin-associated glycoprotein (MAG) and complex gangliosides contribute to axon stability in both the CNS and peripheral nervous system (PNS). Similar neuropathological and behavioral deficits in Galgt1-, Mag-, and double-null mice support the hypothesis that MAG binding to gangliosides contributes to a long-term axon-myelin stability (Pan et al. 2005). However, these neuropathological findings were not reported

in the GM2/GD2<sup>-/-</sup> mice aged between 8 and 12 weeks. In addition, even if there was any instability in the nervous system of GM2/GD2<sup>-/-</sup> mice of this age range, it would likely have an enhancing effect on the development of EAE. It is therefore suggested that the latent instability of the nervous system is not the cause of the delay of the EAE onset observed in GM2/GD2<sup>-/-</sup> mice.

Cell surface gangliosides inhibit cellular immune responses, including APC development and function, which is critical for Th1 and Th2 cell development (Caldwell et al. 2003). APC activity is an important step in the cellular immune response. Gangliosides have the ability to interfere with a number of APC functions, including Ag processing and presentation, cytokine production, and induction of lymphocyte proliferation. For example, when human monocytes were preincubated with purified ganglioside GD1a, the expected Ag-induced proliferative response of autologous normal T cells added to these monocytes was inhibited. Upregulation of the monocyte costimulatory molecule, CD80, was almost completely inhibited (Caldwell et al. 2003). In a study using human dendritic cells (DCs) and naive CD4<sup>+</sup> T cells, preincubation with purified GD1a reduced the upregulation of costimulatory molecules and pertussis toxin-induced IL-12 production, whereas cholera toxin-induced IL-10 production was increased. Thus, ganglioside exposure of DCs could adversely affect the development of an effective cellular immune response (Shen et al. 2005). Therefore, the immune ability of APCs in GM2/GD2<sup>-/-</sup> mice could be more active than that in wild-type mice. However in the present study, the clinical feature of EAE was the opposite; i.e., the EAE response was delayed in GM2/GD2<sup>-/-</sup> mice. Furthermore, there was no significant difference in APC function between GM2/GD2<sup>-/-</sup> and wild-type mice in an *in vitro* study. This suggested that complex gangliosides do not affect the APC function in the development of EAE.

It is also possible that the migration of the activated lymphocytes is affected by the lack of complex gangliosides. The presentation of several adhesion molecules in the BBB and the serum levels of MCP-1 were not significantly different between GM2/GD2<sup>-/-</sup> and wild-type mice. However, several complex gangliosides have been shown to be present in a human cerebrovascular endothelial cell line (Duvar et al. 2000). Complex gangliosides themselves may be involved as adhesion molecules in the process of infiltration of the activated lymphocytes into the CNS. In the present study, we demonstrated that the number of the pathogenic T cells within the CNS on day 9 of active immunization EAE was significantly less in GM2/GD2<sup>-/-</sup> mice in comparison with that in wild-type mice. This indicates that the lack of complex gangliosides in the BBB may delay the immigration of pathogenic T cells into the CNS. Thus, the complex gangliosides may be involved in the T cell-endothelial cell interaction in the pathogenic process of EAE.

GM2/GD2<sup>-/-</sup> mice not only lack complex gangliosides, but are rich in simple gangliosides, such as GM3 and GD3 (Takamiya et al. 1996). GD3 strongly affected IL-15-dependent immune responses of murine microglia (Gomez-Nicola et al. 2006). GD3 has also been reported as a natural ligand for NKT cells (Wu et al. 2003). The delay of EAE onset in this type of knockout mouse may therefore be due to the increased content of GM3 and GD3. This possibility should be examined in future investigations.

## Materials and methods

### Mouse

Wild-type C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). GM2/GD2<sup>-/-</sup> were originally obtained from Dr. K. Furukawa and have been described previously. The mice have been backcrossed to the C57BL/6 background for more than five generations. Genotyping of GM2/GD2<sup>-/-</sup> mice was performed by polymerase chain reaction as described elsewhere. These mice were maintained under specific pathogen-free conditions. All mice for experiments were 8–12 weeks old.

### Peptides

MOG<sub>35–55</sub> (single-letter amino acid code; MEVGYRSPFS-RVYHLYRNGK) was synthesized by Tore Research Institute (Tokyo, Japan). The peptides were >90% pure, as determined by HPLC.

### Induction and assessment of EAE

Mice were injected subcutaneously in the flank, bilaterally, with 200 µL of inoculum containing 100 mg of MOG<sub>35–55</sub> and 0.5 mg of mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI) in incomplete Freund's adjuvant. Pertussis toxin (List Biological Laboratories Inc., Campbell, CA, 200 ng) was injected intravenously on day 0 and day 2 after immunization. For EAE induction in the adoptive transfer model, recipient mice were injected intravenously with encephalitogenic cells, prepared as described below, and 200 ng of pertussis toxin. Immunized mice were examined daily and scored as follows: 0, no clinical signs; 1, limp tail; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund or dead. Mice were examined daily for signs of EAE in a blind fashion.

### Preparation of cells for EAE induction in the adoptive transfer model

To prepare MOG-specific cells that were able to induce EAE in the adoptive transfer model, mice were immunized with MOG/CFA in the same fashion as when inducing EAE, but no pertussis toxin was administered. Draining LNs were collected 10 days later, and a single-cell suspension was prepared. The cells were stimulated with 30 µg/mL MOG<sub>35–55</sub> in 24-well flat-bottomed plates (5 × 10<sup>6</sup> cells/well) in the T-cell medium (RPMI media enriched with 10% FBS, 2 mM L-glutamine, 5 × 10<sup>-5</sup> M2-ME, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin). Recombinant mouse IL-12 was added at 20 ng/mL. Four days after initiation of the cultures, cells were harvested and CD4<sup>+</sup> cells were selected using a column (R&D). One million CD4<sup>+</sup> cells were injected into recipient mice as described above for EAE induction.

### Establishment of a MOG-specific T cell line

A MOG-specific T cell line was established using cells for adoptive transfer EAE as described above. The cells were cultured in the T-cell medium with 10 ng/mL of IL-2. Half of the medium was replaced every few days. Stimulation with 30 µg/mL MOG<sub>35–55</sub> was performed every 10 days with irradiated (30 Gray) splenocytes as APC.

### T-cell proliferation assay

For proliferation assays, mice were immunized with peptide/CFA as described above, but the mice were not treated with pertussis toxin. A single-cell suspension was prepared from the draining LNs on day 10 after immunization. Cells were cultured in DMEM medium (Gibco, Grand Islands, NY) supplemented with 5 × 10<sup>-5</sup> M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, and 1% autologous mouse serum, and seeded onto 96-well flat-bottomed plates (1 × 10<sup>6</sup> cells/well). The cells were restimulated with peptide for 72 h at 37°C under a humidified air condition with 5% CO<sub>2</sub>. To measure cellular proliferation, [<sup>3</sup>H]-thymidine was added (1 mCi/well) and uptake of the radioisotope during the final 18 h of culture was counted with a beta-1205 counter (Pharmacia, Uppsala, Sweden). To evaluate proliferative responses of LN cells to the peptide, we determined the Δc.p.m. value for cells in each well by subtracting the background c.p.m. and used the mean of these values to represent each mouse.

### Cytokine ELISA

In parallel, the LN cells from immunized mice were cultured with peptide concentrations of 0, 1, 10, and 100 µg/mL. Supernatants from the cultures were harvested 48 h postactivation and tested for the presence of various cytokines. The concentrations of IFN-γ, IL-2, IL-4, and IL-10 in the supernatants were measured by a sandwich ELISA according to the manufacturer's guideline (BD Biosciences, San Jose, CA). Limits of detection for IFN-γ, IL-2, IL-4, and IL-10 were 195 pg/mL, 25 pg/mL, 12.5 pg/mL, and 50 pg/mL, respectively.

### Pathological analysis

On day 35 after immunization, mice were sacrificed. Brains and spinal cords were harvested and fixed in 10% neutral buffered formalin. Paraffin sections were stained with hematoxylin and eosin (HE)–Luxol fast blue stain to assess inflammation and demyelination. The numbers of inflammatory foci in the meninges and parenchyma were counted for each sample by a blinded observer, as described previously (Miyamoto et al. 2005). For immunopathological analysis, the brain and spinal cord were obtained same as described above on day 7, 10, 14 after immunization of EAE. Thin-sliced (10 µm) frozen sections were fixed with acetone, and stained with HE, Luxol fast blue, and antibodies for adhesion molecules. ICAM-1 (CD54), VCAM-1 (CD106), E-selectin (CD62E), and P-selectin (CD62P) (BD Biosciences) were stained following the protocols provided by BD Bioscience.

The studies have been reviewed and approved by the local ethics committee in Kinki University.

### Analysis of infiltrating cells isolated from CNS

Wild-type and GM2/GD2<sup>-/-</sup> mice were anesthetized with diethyl ether on day 9 after induction of EAE. After perfusion with PBS, brain and spinal cord were removed and homogenized. After washing with PBS, mononuclear cells were isolated using Percoll gradient (Amersham Biosciences, Piscataway, NJ) and were counted (Miyamoto et al. 2006). The cells were stained with a PE-labeled anti-CD4 antibody (BD Biosciences), and were analyzed by a flow-cytometer (BD FACS Calibur). Naive mice were also analyzed the infiltrated cell in CNS as the same method.

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## Conflict of interest statement

None declared.

## Abbreviations

APC, antigen-presenting cell; BBB, blood-brain barrier; CNS, central nervous system; COX-2, cyclooxygenase-2; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; GM2/GD2<sup>-/-</sup>, GM2/GD2 synthase knockout mice; HE, hematoxylin and eosin; LN, lymph node; MOG, myelin-oligodendrocyte glycoprotein; MAG, myelin-associated glycoprotein; NO, nitric oxide; PNS, peripheral nerve system; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

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# Ganglioside complexes containing GQ1b as targets in Miller Fisher and Guillain-Barré syndromes

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## ABSTRACT

**Background:** Serum antibodies to GQ1b are associated with Miller Fisher syndrome (MFS) and Guillain-Barré syndrome (GBS) with ophthalmoplegia. Antibodies to ganglioside complexes (GSCs) have not yet been examined in a large population of patients with MFS or GBS. This study aimed to determine the clinical significance of antibodies to GSCs in MFS and GBS.

**Methods:** The study investigated serum anti-GSC antibodies and the clinical features in 64 MFS patients, 53 GBS patients with ophthalmoplegia (GBS-OP(+)) and 53 GBS patients without ophthalmoplegia (GBS-OP(-)).

**Results:** Thirty patients with MFS (47%), 25 with GBS-OP(+), 47% and none with GBS-OP(-) had antibodies to GSCs containing GQ1b or GT1a. Patients with MFS and GBS-OP(+) were subdivided according to the antibody reactivities; patients with antibodies specific to GQ1b and/or GT1a (without anti-GSC antibodies) were placed in Group 1, those with antibodies against GSCs with a total of two sialic acids in the terminal residues, such as GQ1b/GM1, were placed in Group 2, and those with antibodies against GSCs with a total of three sialic acids in the terminal residue, such as GQ1b/GD1a, were placed in Group 3. In MFS, sensory disturbances were infrequent in Group 2 compared with the other groups ( $p < 0.0001$ ). Antibodies specific to GQ1b were observed more often in MFS than in GBS-OP(+) ( $p = 0.0002$ ).

**Conclusions:** IgG antibodies to GSCs containing GQ1b or GT1a were closely associated with the development of ophthalmoplegia in GBS, as well as MFS. Both GQ1b and clustered epitopes of GSCs containing GQ1b or GT1a may be prime target antigens for MFS and GBS-OP(+).

Miller Fisher syndrome (MFS) is characterised by ophthalmoplegia, ataxia and areflexia,<sup>1</sup> and is thought to be a variant of Guillain-Barré syndrome (GBS). Anti-GQ1b immunoglobulin G (IgG) antibody, an excellent diagnostic marker in MFS, is found in the acute-phase sera of more than 90% of MFS patients.<sup>2</sup> Moreover, anti-GQ1b IgG antibody is also associated with ophthalmoplegia in GBS and Bickerstaff's brain-stem encephalitis.<sup>3,4</sup>

Glycosphingolipids are known to form microdomains called lipid rafts, together with cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins.<sup>5</sup> Within the microdomains, gangliosides may play an important role in membrane-mediated functions.<sup>6-8</sup> We previously reported antibodies to ganglioside complexes (GSCs) as new target antigens in GBS,<sup>9</sup> and that 58% of MFS patients exhibited serum antibodies to GSCs containing GQ1b.<sup>10</sup> These studies suggest that the clustered glycoepitopes of GSCs in peripheral nerves may be targets for serum antibodies in acute

immune-mediated polyradiculoneuropathy, such as GBS and MFS. We also demonstrated that the specificity of anti-GSC antibodies might be associated with the clinical features of GBS and MFS.

To clarify the clinical significance of antibodies to GSCs containing GQ1b in MFS and GBS, we retrospectively analysed the clinical features of anti-GSC-positive patients with GBS or MFS in a larger population.

## METHODS

### Patients

We collected data from 64 consecutive patients with MFS and 53 GBS patients presenting with ophthalmoplegia (GBS-OP(+)) who were recruited between January 2003 and June 2005. The serum samples were submitted to us from various teaching and general hospitals for screening of anti-ganglioside antibodies. They were acute-phase sera obtained before specific treatment and within 2 weeks after the onset of the disease. The clinical records were collected with the sera. If the record was insufficient, we sent a questionnaire about clinical findings to the attendant physicians to analyse the clinical features. GBS patients were diagnosed according to the diagnostic criteria of Asbury and Cornblath.<sup>11</sup> The diagnosis of MFS was based on acute self-limited ophthalmoplegia, ataxia and areflexia without significant limb weakness, central nervous system (CNS) involvement or other neurological diseases. Patients who developed limb weakness after appearance of ophthalmoplegia, ataxia or areflexia were categorised into GBS-OP(+) (score of 4 or less on the Medical Research Council scale).

We analysed MFS patients who exhibited at least two symptoms in the triad of ophthalmoplegia, ataxia and areflexia. Concerning the ophthalmoplegia, we excluded patients who only exhibited internal ophthalmoplegia. Ataxia was defined as instability in gait and standing, including sensory and cerebellar ataxia. Anti-GSC antibodies were also determined in 53 consecutive GBS patients without ophthalmoplegia (GBS-OP(-)). 20 normal subjects (normal control), and from 88 patients with neurological disorders other than GBS (disease control) multiple sclerosis, 8; myasthenia gravis, 11; amyotrophic lateral sclerosis, 10; spinocerebellar degeneration, 3; Parkinson's disease, 4; cerebrovascular disease, 8; frontotemporal dementia, 3; brain tumour, 3; myelopathy, 8; chronic inflammatory demyelinating polyradiculoneuropathy, 5; multifocal motor neuropathy, 3; acute cerebellitis, 4; mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes, 1; Creutzfeldt-Jakob Disease, 1; and other neuropathy, 16.

### The enzyme-linked immunosorbent assay for anti-ganglioside and anti-GSC antibodies

Serum samples were investigated for antibodies to GM1, GM2, GM5, GD1a, GD1b, GD3, GT1a, GT1b, GQ1b and GalNAc-GD1a using the enzyme-linked immunosorbent assay (ELISA). GalNAc-GD1a was isolated in our laboratory from bovine brain,<sup>17</sup> and the other gangliosides were purchased from Sigma-Aldrich Co. (St Louis, MO). The patients' sera (diluted 1:40 with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)) were applied to wells coated with 200 ng of single antigens and uncoated wells as the control. Peroxidase-conjugated anti-human IgG antibodies were purchased from MP Biomedicals (Solon, OH). Anti-human IgG was diluted 1:500 with 1% BSA in PBS, and were applied to each well as the secondary antibodies. We corrected the optical density (OD) values by subtracting those of control wells. The serum was considered to be positive when the corrected OD was more than 0.1. The serum samples were also investigated for anti-GSC antibodies as described elsewhere.<sup>9, 10</sup> The GSCs used in this study consisted of a mixture of 100 ng of each of two of the seven gangliosides—GM1, GM2, GD1a, GD1b, GT1a, GT1b and GQ1b. The presence of anti-GSC antibodies was established according to previously described criteria.<sup>10</sup>

### Clinical analysis of anti-GSC antibody-positive patients with MFS and GBS with ophthalmoplegia

According to our recent study results,<sup>10</sup> we divided MFS patients and patients with GBS-OP(+) into three groups based on the anti-GSC antibodies and anti-GQ1b IgG antibody, as described below in the Results section. The clinical and electrophysiological data of the subjects were obtained from the attending physicians in each hospital. Patients with no information on neurological signs and symptoms were excluded from the analysis. GBS patients' disabilities were assessed using the Hughes Functional Grading Scale.<sup>15</sup>

### Antecedent *Campylobacter jejuni* enteritis

IgM and IgG anti-*Campylobacter jejuni* (*C. jejuni*) antibodies were investigated in serum samples from MFS and GBS-OP(+) patients using an ELISA kit for *C. jejuni* (SERION ELISA classic, *C. jejuni* IgG/IgM; Virion/Serion, Würzburg, Germany). The ELISA was performed on *C. jejuni* antigen-coated plates according to the manufacturer's instructions, and the results were evaluated. When the patient exhibited antecedent gastrointestinal episodes, such as diarrhoea and abdominal pain, and exhibited positive ELISA results for IgM or IgG antibodies to *C. jejuni*, they were judged to have antecedent gastrointestinal infection associated with *C. jejuni*.

### Antibody specificity against GQ1b/GM1 or GQ1b/GD1a complex in MFS

To investigate the specificity of antibodies to GSCs including GQ1b, we assessed reactivity against GQ1b/GM1 or GQ1b/GD1a with ELISA, using representative sera from MFS patients with no anti-GSC antibody, anti-GQ1b/GM1 antibody or anti-GQ1b/GD1a antibody. In addition to GQ1b (200 ng), we added another ganglioside, GM1 or GD1a (0–600 ng)—ie, GQ1b/GM1 or GQ1b/GD1a = 200/0, 200/100, 200/200, 200/400 and 200/600 ng. The ELISA was performed as described above. A monoclonal anti-mouse GQ1b antibody (Seikagaku Corporation, Tokyo, Japan; diluted 1:40 with PBS) was used as a control, and peroxidase-labelled anti-mouse IgA+IgG+IgM antibody (KPL, Inc., Gaithersburg, MD; diluted 1:500 with PBS) was used as the secondary antibody. The

corrected OD for each of the values was used to analyse their activities according to the mixture of these antigens.

### Statistical analyses

Statistical analyses were performed using SPSS version 12.0J for Windows (SPSS Inc., Chicago). Two-tailed p-values of <0.05 were considered significant. One-way factorial analysis of variance (ANOVA) was used to compare ages in multiple group comparisons, the Bonferroni test was used as a post-hoc test. The Kruskal-Wallis test was used for nonparametric comparisons of the Hughes functional grading scale. Differences in proportions were examined using contingency tables and the Chi square test or the Fisher's exact test. When significance was demonstrated, all groups were compared by calculating the odds ratio (OR) with 95% confidence intervals (CIs).

## RESULTS

### Antibodies to GSCs or single ganglioside antigens

ELISA demonstrated that approximately half of MFS and GBS-OP(+) patients (47%) had one of the IgG antibodies to GSCs containing GQ1b or GT1a (table 1). Patients with antibodies specific to GQ1b (without anti-GSCs antibodies) were categorised into Group 1 (table 2). There were significantly more MFS patients compared with the GBS-OP(+) in Group 1 (table 1). More than one-third of GBS-OP(+) patients did not have either antibodies to GQ1b or GSCs containing GQ1b or GT1a. MFS patients who had at least one of the antibodies to GQ1b/GD1b, GT1a/GM1, GT1a/GD1b or GQ1b/GM1 were categorised into Group 2, because antibodies to GQ1b/GD1b, GT1a/GM1 or GT1a/GD1b were elevated concomitant with anti-GQ1b/GM1 antibody, but not with anti-GQ1b/GD1a antibody,<sup>10</sup> and MFS patients who had at least one of the antibodies to GQ1b/GD1a, GQ1b/GT1b, GT1a/GD1a or GT1a/GT1b were categorised into Group 3 (table 2). In short, MFS patients who had antibodies reactive to GSCs with a total of two sialic acids in the terminal residues in GSCs, such as GQ1b/GM1, were classified into Group 2, and MFS patients who had antibodies to GSCs with a total of three sialic acids in the terminal residue, such as GQ1b/GD1a, were classified into Group 3. None of the MFS patients had any different types of anti-GSC antibodies simultaneously. In the same manner, GBS-OP(+) patients could be subdivided into three groups: anti-GQ1b-positive patients without anti-GSC antibodies (Group 1); patients who had antibodies reactive to GSCs with a total of two sialic acids in the terminal residues in GSCs, such as GQ1b/GM1 (Group 2); and who had antibodies reactive to GSCs with a total of three sialic acids in the terminal residue, such as GQ1b/GD1a (Group 3). However, five patients in the GBS Group 2 had IgG antibodies to GQ1b/GD1a, GQ1b/GT1b, GT1a/GD1a or GT1a/GT1b (table 2). These five patients were excluded from clinical analysis between Groups 1, 2 and 3. Three patients in the MFS Group 2, two in the GBS Group 2 and one in the GBS Group 3 had no anti-GQ1b IgG antibodies but had antibodies to GSCs containing GQ1b or GT1a. None of the MFS patients had antibodies to GSCs, consisting of two of the four major gangliosides (GM1, GD1a, GD1b and GT1b), which differed from the GBS patients. No antibodies to GSC were detected in the normal and disease control groups.

As for IgG antibodies to single ganglioside antigens other than GQ1b and GT1a, anti-GT1b IgG antibodies were found in seven MFS patients. In GBS-OP(+) patients, anti-GT1b antibodies were found in 16 patients, anti-GD1a antibodies in 6, anti-GalNAc-GD1a in 5, anti-GD1b in 3, anti-GM1 in 2 and anti-GM2 in 1 patient.

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**Table 1** Anti-GQ1b IgG antibodies and antibodies to ganglioside complexes containing GQ1b or GT1a in MFS, GBS-OP(+) and GBS-OP(-) groups

Anti-GQ1b antibody	Antibodies to GSCs containing GQ1b or GT1a	MFS (n = 64)	GBS-OP(+) (n = 53)	GBS-OP(-) (n = 53)
+	-	32 (50%)*	9 (17%)*	5 (9%)*
<+ or ->	+	30 (47%)†	25 (47%)†	0 (0%)†
-	-	2 (3%)‡	19 (36%)‡	48 (91%)‡

\*MFS vs GBS-OP(+), odds ratio (OR) 4.89 (95% confidence intervals (CI) 2.05 to 11.65); MFS vs. GBS-OP(-), OR 9.60 (95% CI 3.38 to 27.25).

†MFS vs GBS-OP(-), p<0.0001; MFS vs. GBS-OP(+), p<0.0001.

‡MFS vs GBS-OP(+), OR 0.058 (95% CI 0.013 to 0.263); MFS vs. GBS-OP(-), OR 0.003 (95% CI 0.001 to 0.018); GBS-OP(+) vs. GBS-OP(-), OR 0.058 (95% CI 0.020 to 0.171).

GBS-OP(+), Guillain-Barré syndrome with ophthalmoplegia; GBS-OP(-), Guillain-Barré syndrome without ophthalmoplegia; GSCs, ganglioside complexes; IgG, immunoglobulin G; MFS, Miller Fisher syndrome.

**Clinical features in MFS and GBS with ophthalmoplegia**

The clinical features of patients with MFS or GBS-OP(+) are shown in tables 3 and 4. Patients who did not have antibodies to GQ1b or GSCs containing GQ1b or GT1a were excluded from clinical analysis. Group 2 MFS patients were characterised by infrequent sensory disturbances (table 3) and tended to exhibit preserved bulbar function. Ataxia was common in the MFS and GBS-OP(+) groups except for GBS Group 3. Antibodies specific to GQ1b were highly associated with disturbances of deep sensation in GBS-OP(+). In GBS-OP(+), the number of patients requiring artificial ventilation was greatest in Group 1; however, there were no significant differences between groups. There were no statistical differences in functional score at the nadir of the disease between each group in GBS-OP(+) (data not shown). MFS and GBS-OP(+) patients who had antibodies to GSCs with a total of two sialic acids in the terminal residues in GSCs (Group 2) tended to suffer from antecedent respiratory infection more frequently than gastrointestinal infection. Antecedent *C. jejuni* enteritis was evident in seven MFS Group 1, one MFS Group 3, three GBS Group 1, two GBS Group 2 and five GBS Group 3 patients. There were no significant differences in the frequency of *C. jejuni* enteritis among these groups. Of 41 MFS patients with available electrophysiological results, 18 patients did not exhibit any abnormalities.

**Antibody specificities to GQ1b/GM1 or GQ1b/GD1a in sera from MFS patients**

In anti-GSC antibody-negative sera, anti-GQ1b activity decreased with increasing concentrations of GM1 and GD1a (fig 1A), similar to the results with mouse monoclonal anti-GQ1b antibodies (fig 1D). In anti-GQ1b/GM1 antibody-positive sera, antibody activity increased proportionally with GM1 concentrations and decreased with increasing concentrations of GD1a, which differed from the results with anti-GQ1b/GD1a-positive sera (fig 1B, C).

**DISCUSSION**

The present study showed that antibodies to GSCs, including GQ1b or GT1a, as well as anti-GQ1b antibodies, are significantly associated with MFS and ophthalmoplegia in GBS. A survey of anti-GSC antibodies made it apparent that MFS-associated antibodies were subdivided into three types based on antibody specificity: ie, antibody specific to GQ1b, antibody reactive to GSCs containing GQ1b or GT1a with a total of two sialic acids in the terminal residues,<sup>16</sup> and antibody reactive to GSCs with a total of three sialic acids in the terminal residues. The findings that none of the MFS patients had two or more types of these antibodies supported the validity of the

**Table 2** IgG antibodies to GSCs and single ganglioside antigens in MFS, GBS-OP(+) and GBS-OP(-)

	MFS			GBS-OP(+)		
	Group 1 (n = 32)	Group 2 (n = 26)	Group 3 (n = 4)	Group 1 (n = 9)	Group 2 (n = 15)	Group 3 (n = 10)
GQ1b	32 (100%)	23 (89%)	4 (100%)	9 (100%)	12 (80%)	9 (90%)
GT1a	27 (84%)	13 (50%)	3 (75%)	6 (67%)	12 (80%)	9 (90%)
<b>To GSCs containing GQ1b or GT1a:</b>						
GQ1b/GM1	0	13 (50%)	0	0	6 (40%)	0
GQ1b/GD1b	0	4 (15%)	0	0	8 (53%)	0
GT1a/GM1	0	21 (81%)	0	0	13 (87%)	0
GT1a/GD1b	0	20 (77%)	0	0	14 (93%)	0
GT1a/GM2	0	5 (19%)	0	0	1 (6.7%)	0
GQ1b/GM2	0	1 (4.0%)	0	0	1 (6.7%)	0
GQ1b/GD1a	0	0	2 (50%)	0	2 (13%)	4 (40%)
GQ1b/GT1b	0	0	3 (75%)	0	3 (20%)	7 (70%)
GT1a/GD1a	0	0	2 (50%)	0	4 (27%)	6 (60%)
GT1a/GT1b	0	0	3 (75%)	0	1 (6.7%)	6 (60%)
GQ1b/GT1a	0	0	1 (25%)	0	0	3 (30%)
Others	None	None	None	None	None	None

Thirty MFS patients and 25 GBS-OP(+) patients had anti-GSC antibodies. Of these, 26 MFS patients were categorised in group 2 and only four in group 3. For 25 GBS-OP(+) patients, these numbers were 15 (in group 2) and 10 (in group 3).

GBS Group 2 and GBS-OP(-) patients had IgG antibodies to other GSCs: two (13%) patients in GBS Group 2 had antibodies to GM1/GD1a, 5 (33%) to GM1/GT1b, 1 (7%) to GD1a/GD1b and 2 (13%) to GD1b/GT1b. Two (4%) patients in GBS-OP(-) had antibodies to GM1/GD1a, 2 (4%) to GM1/GT1b and 1 (2%) to GD1a/GD1b.

GBS-OP(+), Guillain-Barré syndrome with ophthalmoplegia; GBS-OP(-), Guillain-Barré syndrome without ophthalmoplegia; GSCs, ganglioside complexes; IgG, immunoglobulin G; MFS, Miller Fisher syndrome.

**Table 3** Comparison of the clinical features in each MFS group

	MFS (n = 62)			p Value
	Group 1 (n = 32)	Group 2 (n = 26)	Group 3 (n = 4)	
Age; mean (95% CI), years	45.3 (39.8–50.8)	43.7 (37.4–50.0)	46.5 (33.4–59.7)	0.898
Gender (male/female); n	24/8	15/11	2/2	0.304
Antecedent infection; n (%)				
Respiratory	22 (69)	24 (92)	3 (75)	0.071
Gastrointestinal	7 (22)	1 (4)	1 (25)	0.086
Ataxia; n (%)	28 (88)	22 (85)	4 (100)	1
Areflexia; n (%)	29 (91)	26 (100)	4 (100)	0.384
Cranial nerve deficits; n (%)				
VII	9 (28)	3 (12)	0 (0)	0.209
IX, X	7 (22)	2 (8)	2 (50)	0.057
Sensory disturbances; n (%)	26 (81)	7 (27)	4 (100)	<0.0001*
Superficial	23 (72)	7 (27)	4 (100)	0.0003†
Deep	10 (31)	1 (4)	1 (25)	0.020‡

\*Group 2 vs Group 1, odds ratio (OR) 0.085 (95% confidence intervals (CI) 0.025 to 0.294); Group 2 vs Group 3, p = 0.012.

†Group 2 vs Group 1, OR 0.144 (95% CI 0.045 to 0.460); Group 2 vs Group 3, p = 0.012.

‡Group 2 vs Group 1, OR 0.088 (95% CI 0.010 to 0.743); Group 2 vs Group 3, OR 0.120 (95% CI 0.006 to 2.458).

MFS; Miller Fisher syndrome.

classification. The reason for the diversity of antibody specificity remains to be determined.

IgG antibodies specific to GQ1b were more frequently in MFS than in GBS-OP(+) (Group 1), suggesting a strong association between the anti-GQ1b antibody and the development of MFS (table 1). Our study confirmed a close association between the GQ1b-specific antibody and ataxia or impaired deep sensation in GBS-OP(+), as shown in a previous study.<sup>11</sup> Moreover, impairment of deep sensation was infrequent in patients with MFS despite the profound degree of ataxia, as pointed out by Mori *et al.*<sup>10</sup> It is unclear why the loss of deep sensation is less common in MFS than in GBS in Group 1. Impairment of muscle spindle afferents might explain ataxia in MFS without loss of deep sensation.<sup>18,17</sup> In GBS-OP(+), antiganglioside antibodies other than those to GSCs containing GQ1b or GT1a, might influence neurological dysfunction such as impairment of superficial sensation.

IgG antibodies, which are reactive to GSCs with a total of two sialic acids in the terminal residues, such as GQ1b/GM1, appear to

be associated with ophthalmoplegia in GBS as well as in the development of MFS. The present study confirmed that these types of antibodies led to the preservation of sensory function in MFS.

IgG antibodies, which are reactive to GSCs with a total of three sialic acids in the terminal residues, such as GQ1b/GD1a, were infrequent in MFS and we were not able to adequately identify the clinical characteristics in Group 3 patients. In GBS-OP(+) patients, on the other hand, ataxia and loss of deep sensation were attenuated in Group 3 patients. Such clinical differences might result from specific localisation of clustered epitopes consisting of a combination of [NeuAc $\alpha$ 2-3Gal $\beta$ 1-3GalNAc] and [NeuAc $\alpha$ 2-8NeuAc $\alpha$ 2-3Gal $\beta$ 1-3GalNAc]. Thus, each of the three types of the antibodies is likely to be associated with some clinical features. However, clinical prospective studies and experimental studies are needed to confirm such associations.

It is interesting to note whether anti-GSC and anti-GQ1b antibodies bind to identical sites in neuronal membranes. As shown in figure 1, epitopes targeted by each of three types of the MFS-associated antibodies appear to be different.

**Table 4** Clinical features of GBS-OP(+) patients

	GBS-OP(+) (n = 29)			p Value
	Group 1 (n = 9)	Group 2 (n = 10)	Group 3 (n = 10)	
Age; mean (95% CI), years	45.1 (29.4 to 60.8)	41.0 (29.9 to 52.2)	36.5 (24.0 to 49.0)	0.582
Gender (male/female); n	4/5	5/5	6/4	0.897
Antecedent infection; n (%)				
Respiratory	5 (56)	8 (80)	5 (50)	0.400
Gastrointestinal	4 (44)	2 (20)	5 (50)	0.400
Ataxia; n (%)	9 (100)	8 (80)	3 (30)	0.003*
Cranial nerve deficits; n (%)				
VII	5 (56)	6 (60)	6 (60)	1
IX, X	7 (78)	4 (40)	8 (80)	0.181
Sensory disturbances; n (%)	9 (100)	9 (90)	9 (90)	1
Superficial	8 (89)	8 (80)	9 (90)	1
Deep	8 (89)	2 (20)	3 (30)	0.005†
Mechanical ventilation; n (%)	3 (33)	2 (20)	2 (20)	0.751

\*Group 3 vs Group 1, p = 0.003; Group 3 vs Group 2, p = 0.070.

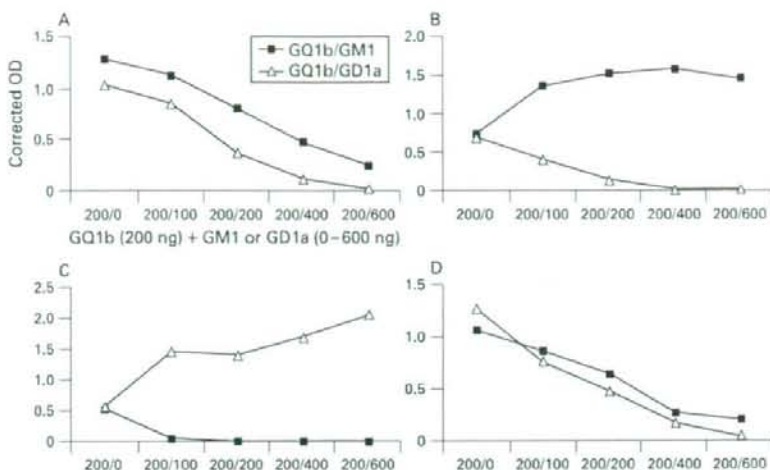
†Group 2 vs Group 1, odds ratio (OR) 0.031 (95% confidence intervals (CI) 0.002 to 0.418); Group 2 vs Group 3, OR 0.583 (95% CI 0.075 to 4.562); Group 1 vs Group 3, OR 18.67 (95% CI 1.56 to 222.93).

GBS-OP(+), Guillain-Barré syndrome with ophthalmoplegia.



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**Figure 1** Antibody specificities to GQ1b/GM1 or GQ1b/GD1a in sera from MFS patients. GM1 or GD1a (0–600 ng) was added to GQ1b (200 ng) in a well of an ELISA plate; ie, GQ1b:GM1 or GQ1b:GD1a = 200:0, 200:100, 200:200, 200:400 and 200:600 ng. Anti-GSC-negative serum (Group 1) (A), anti-GQ1b/GM1 antibody-positive serum (Group 2) (B), anti-GQ1b/GD1a antibody-positive serum (Group 3) (C) and monoclonal mouse anti-GQ1b IgG antibody (D) were used. Black squares show the antibody activity to a mixture of GQ1b and GM1. Triangles show the antibody activity to a mixture of GQ1b and GD1a. Each line shows a mean of two measurements.



It is notable that 19 GBS-OP(+) patients (36%) had neither anti-GQ1b IgG antibodies nor antibodies to GSCs containing GQ1b. In these 19 patients, GQ1b antigen may be uninvolved in the immune response leading to the development of ophthalmoplegia. As described recently,<sup>16</sup> anti-GalNAc-GD1a antibodies might be associated with ophthalmoplegia in four of the 19 GBS-OP(+) patients with IgG anti-GalNAc-GD1a antibodies. Anti-ganglioside antibodies associated with ophthalmoplegia in GBS may be more diverse than in MFS.

MFS patients had no IgG antibodies to GSCs consisting of two of the four major gangliosides (GM1, GD1a, GD1b and GT1b), and no IgG antibodies to single ganglioside antigens except for GQ1b, GT1a and GT1b. This differs from the situation in GBS patients. The IgG antibodies to GSCs consisting of the four major gangliosides might correlate with development of limb weakness.<sup>19</sup>

Specific immunoadsorption therapy to remove antibodies could ameliorate the course of GBS and MFS.<sup>20, 21</sup> The synthetic disialylgalactose immunoaffinity columns with the minimal epitopes of GQ1b and GT1a were useful for eliminating anti-GQ1b antibodies from sera.<sup>22</sup> The present and the previous immunoadsorption studies,<sup>20</sup> however, suggest that an immunoadsorption column with the epitopes of GSCs, such as GQ1b/GM1 and GQ1b/GD1a, is required in a half of MFS patients to achieve optimal effects.

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**Competing interests:** None.

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# GD1b-specific antibody induces ataxia in Guillain-Barré syndrome

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## ABSTRACT

**Background:** Rabbit ataxic neuropathy and several case reports have suggested a close association of IgG anti-GD1b antibodies with ataxia in Guillain-Barré syndrome (GBS). However, about half of the patients with GBS having IgG anti-GD1b antibodies with no reactivities against other gangliosides (GD1b-mono IgG) do not exhibit ataxia. Antibodies specific to ganglioside complexes (GSCs) containing GD1b have been found in sera from some patients with GBS. Objective: To investigate whether the reactivities of anti-GD1b IgG to such complexes are different between ataxic and nonataxic patients.

**Methods:** The authors examined sera from 17 patients with GBS (9 with ataxia and 8 without ataxia) who had GD1b-mono IgG, with the use of an ELISA in which wells were coated with a mixture of GD1b and each of nine gangliosides (GM1, GM2, GM3, GD1a, GD3, GT1a, GT1b, GQ1b, and GalNAc-GD1a). The binding activities of the anti-GD1b IgG antibodies against such mixture antigens were compared between ataxic and nonataxic patients.

**Results:** The reactivities to antigens, such as GD1b combined with GD1a, GT1b, GQ1b, and GalNAc-GD1a, were significantly reduced in ataxic compared with nonataxic patients. Sera from all nonataxic patients had antibody activities to GSCs not containing GD1b.

**Conclusions:** The addition of another ganglioside may cause conformational change of GD1b. Given the inhibition of the binding ability of the anti-GD1b IgG antibodies by such a conformational change, the anti-GD1b IgG antibodies in ataxic patients may interact closely with GD1b. IgG antibodies highly specific for GD1b may induce ataxia in Guillain-Barré syndrome.

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## GLOSSARY

**GBS** = Guillain-Barré syndrome; **GSCs** = ganglioside complexes; **OD** = optical density.

Antiganglioside antibodies are known to play an important role in the pathogenesis of Guillain-Barré syndrome (GBS). Clinical features of GBS tend to depend upon the specificity of antiganglioside antibodies.<sup>1,2</sup> Many case reports suggest that IgG anti-GD1b antibodies are closely associated with ataxia in GBS.<sup>3-9</sup> Some of them are reported to show sensory ataxia,<sup>3,6,7</sup> while others cerebellar-like ataxia.<sup>4,5,8,9</sup> Its close association is also supported by an experimental model of ataxic neuropathy using rabbits sensitized with GD1b.<sup>10,11</sup> In this animal model, it is indicated that monospecific anti-GD1b IgG plays a pathogenic role in the development of experimental ataxic neuropathy.<sup>11</sup> Moreover, immunohistochemical studies have shown that GD1b is localized on the dorsal root ganglia neurons.<sup>12,13</sup> GD1b-positive neurons exhibited larger diameters than negative ones,<sup>11</sup> supporting the view that antibodies monospecific to GD1b cause ataxic neuropathy. Our clinical investigation, on the other hand, demonstrated that four of nine patients with GBS who had only IgG anti-GD1b antibodies in the screening

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assay exhibited ataxia.<sup>14</sup> In other words, anti-GD1b antibody is not necessarily associated with ataxia.

Recently, antibodies specific to a mixture of two ganglioside antigens were found in a proportion of patients with GBS and its variant, Fisher syndrome.<sup>15,16</sup> It has recently been shown that two gangliosides actually form clusters in the plasma cell membrane.<sup>17</sup> The antibodies may bind to such clusters in the neuronal or Schwann cell membranes, where two gangliosides can form conformational epitopes. We described such novel antigens as ganglioside complexes (GSCs) and the antibodies as anti-GSC antibodies.<sup>15</sup> In GSC, each ganglioside may interact with another to undergo conformational change. If this assumption is correct, the binding activities of the antibodies specific to GD1b itself should be weaker toward GSC comprising GD1b than to GD1b alone.

In the present study, we investigated the antibody activities of the anti-GD1b IgG-positive GBS sera against GSCs comprising GD1b. We found that the anti-GD1b IgG antibody activities against a mixture antigen comprising GD1b were reduced compared with those against GD1b alone. Moreover, the reduction rates of the activities were more pronounced in ataxic compared with nonataxic patients. These findings indicate that the antibodies highly specific to GD1b are closely associated with development of ataxia in GBS.

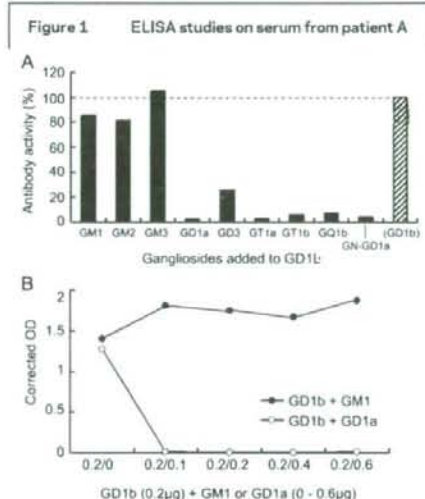
**METHODS Representative serum and antiganglioside antibody assay.** A 70-year-old woman (patient A) with preceding upper respiratory infection presented with numbness in her upper and lower extremities (glove and stocking type), acute ataxic gait, and mild limb weakness, and then became bedridden. Deep tendon reflexes were not elicited. On day 14 that her signs got to nadir, her limb weakness did not exceed Medical Research Council grade 4, and her sensation of vibration and position was markedly involved. Impairment of deep sensation was considered to cause her gait disturbance. Instability of blood pressure was seen only early in the course of the disease. CSF on day 14 showed a protein level of 48 mg/dL and a cell count of 2/μL. Sensory nerve action potentials of median and sural nerves could not be evoked. Motor nerve conduction studies were not performed. GBS was diagnosed based on a standard criteria.<sup>18</sup> After a 5-day course of high-dose IV immunoglobulin treatment (0.4g/kg/day), her ataxic gait and sensory disturbance were gradually improved and she could walk independently 2 months after onset of the disease. Her acute phase serum was investigated for antiganglioside antibodies by an ELISA, as described elsewhere.<sup>18</sup> The ELISA was performed for antibodies to 10 single ganglio-

side antigens: GM1, GM2, GM3, GD1a, GD1b, GD3, GT1a, GT1b, GQ1b, and GalNAc-GD1a, and demonstrated that she had only IgG anti-GD1b antibody. Her serum was also investigated with ELISA for IgG antibodies to a mixture of GD1b (0.2 μg) and one (0.2 μg) of the above nine gangliosides except for GD1b. Antibody activity to each mixture of antigens was compared with that to GD1b alone, and expressed as a percentage.

The antibody activity of the patient's serum to GD1b combined with varied amounts of GM1 or GD1a (0.1, 0.2, 0.4, and 0.6 μg) was examined by ELISA. Each experiment was performed in duplicate.

**Antibody activities to GSCs containing GD1b in anti-GD1b-positive GBS sera.** Among the GBS serum samples collected in our laboratories, after conventional ELISA screening using the above 10 individual ganglioside antigens, we selected consecutive serum samples with IgG anti-GD1b antibody without antibody activity to any of the other ganglioside antigens tested (GD1b-mono IgG). In order to precisely evaluate antibody activity and clinical features, when the corrected optical density (OD) of the anti-GD1b antibody was less than 0.3 or functional scores (F-score) at the nadir of patients were less than 2, the patients' sera were excluded from further analyses.

We then investigated IgG antibodies to a mixture of GD1b (0.2 μg) and one of the other gangliosides such as GM1, GM2, GM3, GD1a, GD3, GT1a, GT1b, GQ1b, and GalNAc-GD1a



(A) IgG antibody activities to GD1b combined with one of several gangliosides are expressed relative to the response to single GD1b antigen (shaded square, 100%). The antibody activities are shown as the average of the two independent assays. The antibody activities were markedly decreased in wells containing GD1b and one of GD1a, GD3, GT1a, GT1b, GQ1b, or GalNAc-GD1a. (GN-GD1a represents GalNAc-GD1a). (B) The antibody activities to a mixture of GD1b (0.2 μg) combined with a varied amount of GM1 or GD1a (0 to 0.6 μg). Black circles describe changes of activities to a mixture of GD1b and GM1, and white circles indicate changes of activities to a mixture of GD1b and GD1a. Each study was performed in duplicate and the mean of the corrected optical density is shown. The antibody activities were completely inhibited when GD1a was added to GD1b.

**Table 1** Clinical features and antiganglioside complex antibodies in anti-GD1b-positive Guillain-Barré syndrome

Patient no.	Age, y/sex	Antecedent infection	Loss of deep sensation	Cerebellar signs	Ophthalmoplegia	Ptosis	Deficits of other cranial nerves	Antiganglioside complex antibodies
<b>Ataxia: n = 9, age 58 (25-70)*</b>								
1	70/F	R	(+)			(-)	9, 10, 11	(-)
2	58/F	R	(-)			(-)	7	(-)
3	56/M	R†		(+)	(-)	(-)	7, 9, 10, 11, 12	(-)
4	58/F	(-)	(+)			(-)	(-)	(-)
5	25/M	GI		(+)		(-)	9, 10	(-)
6	63/M	R	(+)			(-)	7, 9, 10	GM1/GD1a, GM1/GT1b
7	51/M	R	(+)				(-)	GM1/GT1b
8*	36/M	R				(-)	5	(-)
9	67/F	R	(+)				(-)	(-)
<b>Nonataxia: n = 8, age: 35 (19-48)*</b>								
10	48/F	R					7	GM1/GD1a, GM1/GT1b
11	36/M	R					(-)	GM1/GD1a, GM1/GT1b
12	26/F	R				(-)	7	GM1/GT1b, GM1/GT1a, GQ1b/GM1
13	36/F	GI					7, 9, 10	GM1/GD1a, GM1/GT1b
14	39/M	R				(-)	7, 9, 10, 11, 12	GM1/GT1b
15	33/F	R				(-)	(-)	GM1/GT1b
16	22/F	R			(-)	(-)	9, 10	GM1/GD1a, GM1/GT1b, GQ1b/GD1a
17	19/M	(-)				(-)	(-)	GQ1b/GD1b, GT1a/GD1b

\*Age = median (range) years. Patients with ataxia were older than those without ataxia ( $p = 0.003$ , unpaired  $t$  test).

†Mycoplasma infection diagnosed serologically (complement-fixation reaction: 1:128).

\*His case history did not distinguish between sensory ataxia and cerebellar ataxia. Patient 1 (ataxia group) is patient A.

R = respiratory; GI = gastrointestinal.

(0.2  $\mu\text{g}$ ) with ELISA, which was conducted in duplicate. Antibody activities to the mixture antigens were compared with those to single GD1b antigen in each serum sample. Moreover, these sera were investigated for antibodies to GSCs consisting of two of the above gangliosides except for GD1b.

**Association between the affinity of anti-GD1b antibodies and the development of ataxia.** We analyzed the clinical features of patients with GD1b-mono IgG and subdivided them into two groups based on the presence or absence of ataxia; i.e., the ataxia and nonataxia groups. Patients who exhibited sensory or cerebellar ataxia irrespective of limb weakness during the course of the disease were categorized into the ataxia group. Between the two groups, we analyzed the association of the clinical features and antibody activities to GSCs containing GD1b. In statistical comparison with antibody activities to each GSC between the two groups, unpaired  $t$  test was used. Two-tailed  $p$  values of  $< 0.05$  were considered significant. These analyses were performed with SPSS software (12.0, SPSS Inc., Chicago).

**RESULTS Specificity of the anti-GD1b antibody in the representative serum.** The antibody activity of patient A's serum was markedly decreased when an antigen mixture of GD1b and each of the gangliosides, such as GD1a, GD3, GT1a, GT1b, GQ1b, and GalNAc-GD1a, was used instead of GD1b alone (figure 1A). The addition of GM1 had no effect on anti-GD1b activity even if GM1 was added up to 0.6  $\mu\text{g}$  (figure 1B). In combination

with GD1a, on the other hand, anti-GD1b activity was strongly inhibited.

**Clinical features and antiganglioside complex antibodies in anti-GD1b-positive GBS.** The subjects were 17 GD1b-mono IgG-positive GBS patients whose corrected ODs of IgG anti-GD1b antibody were more than 0.3 and whose functional grading scales were 2 or more. The clinical features and anti-GSC antibodies of the subjects are shown in table 1. The ataxia group patients were significantly older than those in the nonataxia group. Six of the nine patients in the ataxia group had deep sensory disturbance and two patients exhibited cerebellar dysfunction. For the remaining one patient, neither the sensory type nor cerebellar type could be determined. Antecedent respiratory infection, ptosis without external ophthalmoplegia, and cranial nerve deficits were frequent in both patient groups. It is noteworthy that all of the nonataxia group patients had anti-GSC antibodies and that, among the anti-GSC antibodies, the target GSCs did not contain GD1b.

Immunoabsorption test on serum from Patient 11 was performed using GD1b, GM1/GD1a, and GM1/GT1b antigens, as described previously.<sup>20</sup> The percentage absorption of anti-GD1b antibody activ-