contrast, $GM1^+$ cells showed no neurite extension and no or a very faint response of TrkA phosphorylation. Activation of ERK1/2 was also scarcely detectable. Thus, expression of GM1 showed suppressive effects on NGF-induced differentiation.

Recent progress in the analysis of membrane microdomains indicates that even neuronal cells have caveolae-like domains containing Shc, Ras, caveolin, and TrkA (28), and GEM/rafts are structurally unique components of plasma membranes, crucial for neural development and function (29). Differentiation signals from NGF could be transduced only through the NGF receptors localized in GEM/rafts showing a different behavior from that of epidermal growth factor receptor (30). Moreover, association and interaction of caveolin with TrkA and $p75^{NTR}$ were demonstrated as a mechanism affecting the neurotrophin-induced signals (31, 32). Then, the most intriguing issue is how the regulation of growth/differentiation factor receptors is performed by gangliosides. Among the three models presented by Miljan *et al.* (33), *i.e.* ganglioside modulation of ligand binding, ganglioside regulation of receptor dimerization, and ganglioside implication with receptor activation state and subcellular localization, the latter two seemed likely in the results of our study.

There have been a number of reports to indicate the changes in the intracellular localization of receptors and signal molecules following the modification of components in lipid rafts (34). In Swiss3T3 cells, overexpression of GM1 synthase gene induced clear reduction of cell growth. It also resulted in the reduction of phosphorylation levels of PDGFR and ERK1/2 after PDGF treatment (8). The mechanisms behind these phenotypic changes should be the dramatic changes in the intracellular localization of receptor molecules in the cells, and this was also the case in PC12 as demonstrated in this study. Originally, NGF induces neuronal differentiation through the TrkA/Ras/MEK/ERK pathway in PC12 cells (35–40). Our results indicated that overexpression of GM1 modulated the initial events in TrkA activation just after binding of NGF, *i.e.* TrkA dimerization and phosphorylation. This means that TrkA outside of GEM/rafts is hard to be activated in $GM1^+$ cells, suggesting that appropriate physicochemical circumstances of GEM/rafts are needed for the early step of its activation. Not only TrkA but also $p75^{NTR}$ and Ras underwent translocation from GEM/rafts to non-raft compartment. Although the roles of $p75^{NTR}$ are not clear, it might be important in the full reaction of PC12 cells to NGF, and its cotransfer with TrkA may enhance the reduction in NGF signals in $GM1^+$ cells. Recently, new roles of $p75^{NTR}$ have been reported not only in the apoptosis but in the myelination (41, 42). Moreover, Ras translocation to the non-raft fraction might also enhance the suppressive effects. H-Ras is believed to localize in GEM/rafts (43, 44). Our study, however, indicated that intracellular localization of Ras could be changed with the modification of carbohydrate moiety of expressed glycosphingolipids. Mechanisms for this regulation remain to be investigated.

Dynamic changes in the subcellular localization of receptors during the ligand binding and subsequent receptor activation/polymerization have been observed in various systems (45). In the case of c-Ret receptor, GDNF binding to GFRα (glycosylphosphatidylinositol-anchored) in GEM/rafts recruit c-Ret molecule to the microdomain (46). EGF/EGFR binding in GEM/rafts results in the translocation out of the microdomain. In the case of fibroblast growth factor receptor, the primary signaling out side of GEM/rafts causes secondary engagement of GEM/raft signaling components. In contrast with these systems, Trk receptors usually show constant localization and activation in GEM/rafts during the signal transduction. We also confirmed no apparent changes in the subcellular localization of NGF receptors after NGF treatment (data not shown). Cell type-specific factors might determine which pattern of regulation individual receptor molecules undergo. Structures of receptors are also very important in the regulation of the intracellular localization. However, little is known about the universal principle determining the behavior of receptors inside/outside of GEM/rafts, and it remains to be analyzed.

Epidermal growth factor (EGF) induces cell proliferation through the EGF receptor/Ras/MEK/MAPK pathway, too. The reason why these two factors, NGF and EGF, can induce different cellular events via the same signaling pathway has been explained based on the different kinetics of ERK1/2 activation after their binding to the individual receptors (46). Therefore, the response of the $GM1^+$ cells to EGF might be very interesting, and remains to be analyzed.

As for the reason for the difference in the effects of exogenous GM1 and cDNA-derived GM1, four possibilities should be considered. First, exogenously added GM1 may exert effects on the cell surface molecules (such as TrkA) in quite a different way from that of cis-existing glycolipids, although some portion of the added GM1 is certainly incorporated into the membrane and expressed (data not shown). Second, the expression levels of GM1 may vary resulting in the different effects on TrkA molecular function as observed in the experiments of *in vtro* kinase of the precipitated TrkA (Fig. 8). The artificial control of the expression levels of the transfected gene is not so easy at this moment, although it is needed to establish multiple transfectants with various levels of GM1 expression to confirm the dose-dependent effects in the future. Third, the altered NGF signal may come not from increased GM1, but from other changes of glycolipid components in the transfectant cells, *i.e.* increased fucosyl-GM1 or decreased GT1b. These additional changes should not be present in the cells treated with exogenous GM1. Fourth, differences in the duration of the exposure of cells to GM1 might be crucial to determine the fates of cells as suggested by the experiments for alcohol toxicity (47).

Although an important role of glycosphingolipids in the formation of the membrane microdomains has been discussed (48–50), the quantitative effects of glycosphingolipids have not been well understood. Little is also known about the effects of ganglioside expression on the membrane fluidity. The effects of exogenous gangliosides on the cell membrane fluidity have been examined in a few system using the technique of FRAP. HL-60 cells showed decreased fluidity after GM3 treatment (51). In the analysis of the ethanol effects on the mobility and viability of embryonal neural crest cells, added GM1 decreased membrane fluidity, resulting in the protection of the cell death (52). Furthermore, the membrane fluidity of the reconstituted liposomes increased with dioleoylphosphatidylcholine (liquid crystal phase) compared with distearoylphosphatidylcholine (gel phase), resulting in the increased kinase activity of EGFR (47). Taken together, it seems reasonable that overexpression of GM1 results in the decreased membrane fluidity and in the reduction of the differentiation signal with NGF/TrkA as demonstrated in this study. However, how the membrane fluidity associates with the nature of the GEM/raft is not well understood and remains to be investigated.

In conclusion, our findings suggest that glycosphingolipids produced and expressed in the cells play critical roles in the modulation of the quality and quantity of signals for the cell differentiation/proliferation and probably for death. Although the mechanisms by which glycosphingolipids regulate the signals have been poorly understood, the results presented here clearly indicate one example of the modes for such regulation. Namely, the microdomain is important as a place for the interaction between glycolipids and signaling molecules, and carbo-

hydrate moieties in the glycosphingolipids contain much more influence than expected on the intracellular localization of various receptors and signal molecules. This means that we need to recognize glycolipids as critical factors determining the cell fates and to understand their roles in malignant tumors and neuronal degeneration when we construct strategies to apply glycosphingolipids and their synthetic machineries for the treatment of cancers and neurological degenerative diseases.

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Correspondence

Anti-GQ1b antibody as a factor predictive of mechanical ventilation in Guillain-Barré syndrome

To the Editor: I read with interest the article by Kaida et al.[1] on the role of anti-GQ1b antibody as predicting mechanical ventilation in Guillain-Barré syndrome (GBS). We have known that bulbar dysfunction[2] and neck weakness[3] are independent predictors of mechanical ventilation. Laboratory markers of predictive value were lacking until Kaida's challenging first step. We may anticipate that the anti-GT1a antibody is related to bulbar palsy. In their research, Kaida et al. show that anti-GQ1b antibody is an independent predictor with a multiple logistic regression model. They found more patients have ophthalmoplegia or bulbar palsy in the ventilation group, but did not mention whether ophthalmoplegia is an independent predictor.

The anti-GQ1b antibody is associated with ophthalmoplegia, while anti-GT1a antibody is associated with bulbar palsy. Although in the ex vivo mouse diagram, anti-GQ1b-positive Miller-Fisher syndrome (MFS) serum induces a temporary dramatic increase of spontaneous quantal acetylcholine release and then makes transmission blockade at NMJ,[4] there is no evidence that anti-GQ1b antibodies can cause respiratory failure in MFS or GBS. No similar effects of anti-GT1a antibodies were found until recently. Anti-GQ1b IgG antibodies were absorbed by GT1a in 98% of the tested sera,[5] while anti-GT1a IgG cross reacted with GQ1b in 75% of the tested sera.[6] It is well known that patients with anti-GT1a IgG present with a variety of clinical conditions. A major part of this clinical variation was due to the coexistence of anti-GQ1b IgG. Anti-GT1a-positive patients frequently had ophthalmoplegia, bulbar palsy, ataxia, and areflexia. These features were also seen in patients with anti-GQ1b IgG.

There was no significant difference between the two groups with respect to the frequency of clinical findings. Whereas the subgroup who had anti-GT1a IgG without GQ1b reactivity frequently had preceding diarrhea as well as oropharyngeal, neck, and limb weakness. The distinctive clinical features and antibody profile indicate a specific subgroup within GBS.[6]

Because of the cross-reactivity of the two anti-ganglioside antibodies, they should not be used as independent variables in logistic regression model. If there is no evidence that anti-GQ1b and GT1a antibodies are associated with respiratory failure in GBS, we would like to see the link between clinical and laboratory data in before making conclusions, especially the overlapping of ophthalmoplegia and bulbar palsy and the cross-reactivity between anti-GQ1b and GT1a antibodies.

Li Haifeng, MD, PhD, *Shandong Province, P.R. China*

Reply from the Authors: We thank Dr. Haifeng for the comments on our article.[1] He questions whether or not ophthalmoplegia and bulbar palsy are independent predictors. Our analyses on cranial nerve involvement, using a multiple logistic regression model, showed that neither ophthalmoplegia ($p = 0.07$) nor bulbar palsy ($p = 0.23$) was an independent variable, but facial diplegia was an independent variable ($p = 0.001$). We would like to add the results as a postscript.

As they pointed out, most of the IgG anti-GQ1b antibodies also bind to GT1a and vice versa.[7] Clinical significance of the IgG activities against the respective antigens, GQ1b and GT1a, is therefore not easy to analyze. Actually, frequency of IgG anti-GT1a antibody, as well as that of IgG anti-GQ1b antibody, was significantly higher in GBS-AV(+) group than in GBS-AV(−) group. However, analyses using a multiple logistic regression model indicated that only IgG anti-GQ1b antibody is an independent predictor for artificial ventilation, being a definite result.

On the other hand, our results do not necessarily deny the possible association between IgG anti-GT1a antibody and respiratory failure. The association between the need for mechanical ventilation and the respective antibodies should be further investigated using the serum samples from a larger number of patients with GBS with IgG antibodies monospecific to either GQ1b or GT1a. In the meantime, basic research on the role of each antibody in the pathogenetic mechanisms of neurological diseases should be performed. Before that, it is practical to use the IgG anti-GQ1b antibodies, most of which cross-react with GT1a, as a marker predictive of the mechanical ventilation in GBS.

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The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome

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Molecular mimicry of *Campylobacter jejuni* lipo-oligosaccharides (LOS) with gangliosides in nervous tissue is considered to induce cross-reactive antibodies that lead to Guillain-Barré syndrome (GBS), an acute polyneuropathy. To determine whether specific bacterial genes are crucial for the biosynthesis of ganglioside-like structures and the induction of anti-ganglioside antibodies, we characterized the *C. jejuni* LOS biosynthesis gene locus in GBS-associated and control strains. We demonstrated that specific types of the LOS biosynthesis gene locus are associated with GBS and with the expression of ganglioside-mimicking structures. *Campylobacter* knockout mutants of 2 potential GBS marker genes, both involved in LOS sialylation, expressed truncated LOS structures without sialic acid, showed reduced reactivity with GBS patient serum, and failed to induce an anti-ganglioside antibody response in mice. We demonstrate, for the first time, to our knowledge, that specific bacterial genes are crucial for the induction of anti-ganglioside antibodies.

Introduction

Autoimmune diseases are often preceded by an infectious illness. Molecular mimicry between microbial antigens and structures in host tissue has been implicated as a mechanism for triggering a cross-reactive immune response after an infection (1). There is strong but indirect evidence for the pathogenic role of molecular mimicry in Guillain-Barré syndrome (GBS), an acute peripheral polyneuropathy and the most frequent cause of acute neuromuscular paralysis (2). Therefore, GBS is an excellent model disease to study both microbial and host factors involved in molecular mimicry.

The most frequently identified triggering agent of GBS is *Campylobacter jejuni*, a spiral-shaped Gram-negative bacterium (3, 4). *C. jejuni* is the leading causative agent of bacterial gastroenteritis worldwide, and it has recently also been associated with neoplastic disease of the gut (5). Acute-phase sera of GBS patients contain high titers of antibodies directed against gangliosides, membrane glycolipids that are highly enriched in nervous tissue (6). Biochemical and serological studies have identified various ganglioside-mimicking structures in the lipo-oligosaccharides (LOS) of the *Campylobacter* cell wall (7), and cross-reactive antibodies between *Campylobacter* LOS and gangliosides have been demonstrated in serum from GBS patients (6). Ganglioside-mimicking structures are found more frequently in neuropathy-associated *C. jejuni* strains than in strains isolated from patients with diarrhea (8). An important feature in ganglioside mimicry is the presence of sialic acid (*N*-acetylneuraminic acid) in both LOS and gangliosides (9).

Recently, we described a collection of geographically clustered but genetically heterogeneous *C. jejuni* strains isolated from Dutch patients with GBS or its variant, Miller Fisher syndrome (MFS) (10, 11). Characterization of the isolates by phenotypic and molecular methods showed that no clustering of GBS/MFS-associated strains occurred when these were compared with control strains (10, 12).

The availability of a database with detailed serological and clinical data of the Dutch GBS/MFS patients provides a unique opportunity for a systematic search for bacterial GBS/MFS-associated virulence factors and correlations with specific immune responses and clinical presentation. We recently reported the association between the presence of the *campylobacter sialic acid transferase-II* (*cst-II*) gene and the expression of a GQ1b-like structure in the bacterial LOS (13). Based on these findings, we hypothesized that the presence of certain *C. jejuni* genes involved in LOS biosynthesis may be crucial for the induction of the anti-ganglioside immune response that leads to GBS. Therefore, we analyzed the LOS biosynthesis gene locus of GBS/MFS-associated *C. jejuni* strains. We found that specific types of the LOS biosynthesis locus are associated with GBS, and this finding led to the identification of potential GBS marker genes in *C. jejuni*. Functional analysis of *Campylobacter* gene knockout mutants, including mouse immunization experiments, demonstrated that these genes are crucial for the induction of anti-ganglioside antibodies.

Results

Specific classes of the LOS biosynthesis gene locus are associated with neuropathy and ganglioside mimicry. In *C. jejuni*, the genes involved in LOS biosynthesis are clustered in the LOS biosynthesis gene locus (LOS locus) (14). Variation in gene content of the LOS locus is an important mechanism for variation in LOS structure between different *C. jejuni* strains. Previously, we had described 3 different gene compositions or "classes" of the LOS locus in *C. jejuni* (15).

Nonstandard abbreviations used: Cm^r, chloramphenicol resistance; *cst-II*, *campylobacter sialic acid transferase-II*; GBS, Guillain-Barré syndrome; LOS, lipo-oligosaccharides; MFS, Miller Fisher syndrome; *orf*, *open reading frame*.

Conflict of interest: The authors have declared that no conflict of interest exists.

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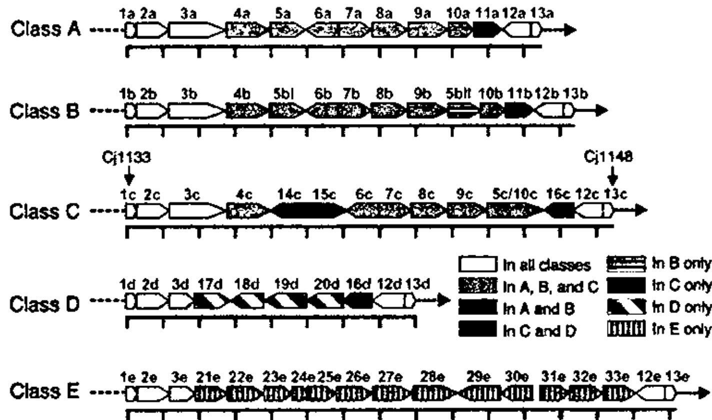


Figure 1
Genetic organization of the 5 different classes of the *C. jejuni* LOS biosynthesis locus. The distance between the scale marks is 1 kb. The direction of the arrows indicates the direction of transcription. Corresponding homologous genes have the same number with a letter for the LOS locus class added. For *orf1c* and *orf13c* the corresponding Cj gene numbers of the "genome" strain NCTC 11168 are given. The 5 LOS classes are based on DNA sequences of the following strains (GenBank accession number): class A: OH4384 (AF130984), OH4382 (AF167345), HS:4 (AF215659), HS:10 (AF400048), HS:19 (AF167344), HS:41 (AY044868); class B: HS:23 (AF401529), HS:36 (AF401528); class C: NCTC 11168 (AL139077), HS:1 (AY044156), HS:2 (AF400047); class D: LIO87 (AF400669); class E: 81116 (AF343914 and AJ131360). The proposed functions for the *orf*s are described in Supplemental Table 1.

Since then, the DNA sequences of several additional *C. jejuni* LOS loci were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and there are now 5 distinct classes (Figure 1).

To study whether certain classes are more prevalent among neuropathy-associated *C. jejuni* strains, we determined the class of LOS locus (classes A–E) in a collection of 21 neuropathy-associated and 21 control *C. jejuni* strains isolated from patients with uncomplicated enteritis. All the strains used in this study were positive for 1 of the 5 identified LOS locus classes. In addition, we analyzed the individual class A/B and class C genes by PCR RFLP and hybridization analysis and found that the LOS gene content in the strains was in agreement with their class of LOS locus (data not shown). The class A LOS locus was overrepresented in the GBS-associated strains compared with the control strains (53% vs. 14%, $P = 0.02$; Table 1). In contrast, all 4 MFS-associated strains contained a class B locus, which was detected in only 33% of control strains ($P = 0.03$; Table 1) and 18% of GBS-associated strains ($P < 0.01$).

Studying the expression of ganglioside-like structures in relation to the class of LOS locus, we found that GM1-like structures were associated with a class A locus ($P < 0.01$), whereas GQ1b-like structures were predominantly expressed by strains with a class B locus ($P < 0.01$; Table 2). Although the class A locus was associated with both GBS and the presence of a GM1-like structure, a GM1-like structure was not detected more frequently in GBS-associated strains (13 of 17 GBS strains vs. 12 of 21 control strains, $P = 0.3$). In 8 of 11 strains with a class D or E locus, ganglioside-like structures were not detected, which is in accordance with the absence of genes involved in the biosynthesis or transfer of sialic acid in these classes (Table 2; Figure 1). These results indicate that genes that are unique to the class A and B loci and genes that are involved in sialic acid biosynthesis or transfer may be crucial for the induction of neuropathogenic cross-reactive antibodies and may thus be considered as GBS marker genes.

Mutagenesis of putative GBS marker genes. To study whether potential GBS marker genes are indeed crucial for the induction of potentially neuropathogenic cross-reactive antibodies, we constructed *Campylobacter* knockout mutants of 3 potential GBS marker genes. *Open reading frame 11* (*orf11*) is unique to the class A and B loci (Figure 1). Its function is unknown, but sequence homology with an enzyme of *Rhodobacter capsulatus* suggests that *orf11* encodes an acetyltransferase (16). The *cst-II* gene (*orf7*) encodes an enzyme (Cst-II) that is involved in the transfer of sialic acid to the LOS backbone. *Orf10* encodes a CMP-sialic acid synthetase and is involved in the biosynthesis of the sialic acid donor used by Cst-II.

Cst-II and *orf11* single mutants and an *orf10/orf11* double mutant were produced in 2 class A GM1-expressing GBS-associated *C. jejuni* strains (GB2 and GB11) by insertion of a chloramphenicol resistance (Cm^r) cassette (Figure 2). The cassette was inserted with the same transcriptional polarity as that of the mutated genes. For all mutants, possible double crossover events were verified by PCR. Southern blot analysis confirmed single integration of the Cm^r cassette into the correct position on the chromosome (data not shown).

LOS structure analysis of WTs and mutants. To determine the effect of the gene inactivations on the LOS structure, we performed mass spectrometry analysis and identified a mixture of GM1- and GD1a-like structures for both GB2 and GB11 WT LOS (Figure 3). The sets of triply charged ions corresponding to GM1- and GD1a-like structures were of similar intensities (data not shown), which suggests that these 2 structures are present in approximately equimolar amounts, assuming that the ionization efficiency is proportional to the amount of a species in the sample. Both the *cst-II* and the *orf10/orf11* mutants of GB2 and GB11 expressed a mixture of 3 nonsialylated structures. The main fraction consisted of asialo-GM3, but asialo-GM2 and asialo-GM1 structures were also present (Figure 3). No structural differences were observed between the WTs and the *orf11* mutants. This indicates that the truncated LOS structures as observed in the *orf10/orf11* mutants were caused by the inactivation of *orf10* and not *orf11*. Detailed results of the mass spectrometry analysis can be found in Supplemental Table 2 (supplemental material available at http://www.jci.org/cgi/content/full/114/11/1659/DC1).

Table 1
LOS biosynthesis loci in *C. jejuni* strains from patients with GBS, MFS, and uncomplicated enteritis

	No. of strains associated with:			
LOS locus class	GBS ($n = 17$)	MFS ($n = 4$)	Enteritis ($n = 21$)	*P*
A ($n = 12$)	9	0	3	0.02[A]
B ($n = 14$)	3	4	7	0.03[B]
C ($n = 5$)	2	0	3	NS
D ($n = 4$)	2	0	2	NS
E ($n = 7$)	1	0	6	NS

[A]GBS vs. enteritis. [B]MFS vs. enteritis.

Table 2
LOS biosynthesis loci and presence of ganglioside-like structures in *C. jejuni* strains

LOS locus type	No. of strains with a ganglioside-like structure in the LOS[A] GM1 (*n* = 27)	GQ1b (*n* = 9)	Other (*n* = 30)	None (*n* = 8)	*P*
A (*n* = 12)	12	1	10	0	<0.01[B]
B (*n* = 14)	9	8	14	0	<0.01[C]
C (*n* = 5)	5	0	3	0	NS
D (*n* = 4)	0	0	1	3	0.02[D]
E (*n* = 7)	1	0	2	5	<0.01[D]

[A]A strain can express more than 1 ganglioside-like structure in its LOS. Therefore, the sum of strains in a row (GM1 + GQ1b + other + none) is higher than the actual number of strains with the corresponding LOS locus type. [B]GM1 vs. non-GM1. [C]GQ1b vs. non-GQ1b. [D]None vs. any.

Sialic acid mutants have a reduced reactivity with patient serum. Analysis of the LOS by gel electrophoresis revealed faster-migrating LOS cores for the GB11 *cst-II* and *orf10/orf11* mutants compared with the WT, confirming that these mutants have a truncated LOS (Figure 4A). The *orf11* mutant LOS showed migration patterns identical to those of WT LOS. Results for the GB2 mutant were identical to those obtained with GB11 (data not shown). These findings are concordant with the structures found by mass spectrometry.

To determine whether the gene inactivations influenced the reactivity of the LOS with the antibodies in the serum from GBS patients, we performed an immunoblot analysis with the serum of the GBS patient from whose stools *C. jejuni* GB11 was isolated (GB11 patient) and WT and mutant purified LOS fractions. Previous studies showed that this serum contains high levels of anti-GM1 antibodies (8). Reactivity of GB11 patient serum was reduced for the *cst-II* and *orf10/orf11* mutants (Figure 4B) but remained the same for the *orf11* mutant when compared with the WTs. For the *cst-II* and *orf10/orf11* mutants, the reactivity with cholera toxin, a ligand for GM1-oligosaccharide structures, was almost completely lost (Figure 4C). These results indicate that genes involved in sialic acid biosynthesis and transfer are important for the expression of ganglioside-like LOS structures that are capable of inducing potentially pathogenic autoantibody responses. Although strain GB11 and the "genome" strain NCTC 11168 both express GM1 mimics in their LOS (this was confirmed by their reactivity with cholera toxin; Figure 4C), the GB11 patient serum did not react with the LOS of NCTC 11168 (Figure 4B). This observation has been described previously (17). This observation illustrates the fine specificity of the antibody response in patient GB11, as the GB11 and NCTC 11168 strains have different LOS structures with a common GM1 mimic (Figure 3).

Sialic acid mutants fail to induce anti-ganglioside antibody responses in mice. To demonstrate that the truncated LOS structures in the mutants do not induce autoantibodies, we immunized GD2/GM2 synthase knockout mice. These mice that lack complex gangliosides are immune-naive hosts that can be used to raise high-titer anti-ganglioside antibody responses (18). Immunization with WT GB11 lyophilized whole bacteria resulted in high IgG serum responses against GD1a, a ganglioside containing 2 sialic acid residues, in 2 of 2 mice (Figure 5A). We could not detect antibody reactivity against GM1 (data not shown). None of the mice injected with the *cst-II* and *orf10/orf11* mutants produced anti-GD1a antibodies (Figure 5, B and C). However, all 6 mice produced anti-asialo-GM1 antibodies, as can be expected from the truncated LOS structures in the *cst-II* and *orf10/orf11* mutants (Figure 5, F and G). Two of 3 mice immunized with the *orf11* mutant strain produced GD1a antibodies (Figure 5D). Low asialo-GM1 titers were also found in 3 of 3 mice immunized with the *orf11* mutant (Figure 5H). These results indicate that genes involved in sialylation of the LOS are crucial for the induction of cross-reactive anti-ganglioside antibodies.

Discussion

We have shown that specific types of the *C. jejuni* LOS biosynthesis gene locus ("LOS locus") are clearly associated with immune-mediated neuropathy and with the presence of ganglioside-mimicking structures in the LOS. Moreover, using *Campylobacter* gene knockout mutants, we demonstrate here, for the first time to our knowledge, that genes involved in sialylation of the LOS are crucial for the induction of anti-ganglioside antibodies.

Diversity in the content of genes involved in LOS biosynthesis results in the variety of LOS structures that is observed in *C. jejuni*. In this study, we identified 2 new classes of the LOS locus, in addition to 3 previously described classes (15, 19). At the time of writing, DNA sequences of additional *C. jejuni* strains are accessible through GenBank, and new LOS gene combinations not reported earlier can be identified. However, all our strains had a positive PCR test for 1 of the 5 LOS locus classes described in this study, which indicates that our tests completely covered the spectrum of different LOS loci present in this collection of *Campylobacter* strains.

The expression of ganglioside-mimicking structures in *Campylobacter* LOS is considered to be essential for the induction of autoantibodies that lead to GBS. Indeed, we previously reported that ganglioside-mimicking structures are present more frequently in neuropathy-associated strains than in strains isolated from patients with uncomplicated enteritis (8). In contrast, most of the *Campylobacter* strains whose LOS structure has been chemically characterized and reported in the literature express ganglioside-mimicking structures. The number of strains that have been analyzed, however, is small and may not be representative. Our study demonstrates that *C. jejuni* needs specific gene combinations to express ganglioside mimics. Only 3 of the 5 identified classes of the LOS locus, i.e., classes A, B, and C, contain genes that are involved in the biosynthesis and transfer of sialic acid, an essential component of gangliosides. We showed that these LOS loci are present in the large majority of strains expressing ganglioside mimics, including the neuropathy-associated strains, whereas class D and E loci are more frequently found in strains lacking ganglioside mimics. In 3 class D/E strains,

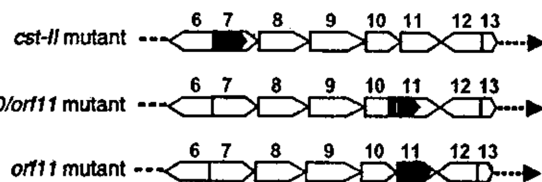


Figure 2
Schematic representation of the mutants used in this study. *Orf6* to *orf13* of the class A LOS locus are displayed. The positions of the 0.7-kb Cm^r cassette are indicated with gray arrows. Both *orf10* and *orf11* are inactivated in the *orf10/orf11* mutant.

Strain	Structure	Ganglioside mimic
WT GB2/GB11	Gal-GalNAc-Gal-Hep-Hep- (branches: NeuAc, Glc)	GM1
	Gal-GalNAc-Gal-Hep-Hep- (branches: NeuAc, NeuAc, Glc)	GD1a
cst-II and *orf10/orf11* mutants GB2/GB11	Gal-Hep-Hep- (branch: Glc)	No
	GalNAc-Gal-Hep-Hep- (branch: Glc)	No
	Gal-GalNAc-Gal-Hep-Hep- (branch: Glc)	No
orf11 mutants GB2/GB11	like WT	like WT
NCTC 11168	Gal-GalNAc-Gal-Gal-Hep-Hep- (branches: NeuAc, Gal, Glc, Glc)	GM1
	GalNAc-Gal-Gal-Hep-Hep- (branches: NeuAc, Gal, Glc, Glc)	GM2

Figure 3
Molecular mimicry between gangliosides and LOS outer cores of WT and mutant *C. jejuni* strains. LOS outer core structures of GB2 and GB11 WT and mutant strains were determined by mass spectrometry analysis. The LOS outer core structure of the "genome" strain NCTC 11168 was described by St. Michael et al. (35). Note that all strains express a mixture of different LOS structures. The *cst-II* and *orf10/orf11* mutants of GB2 and GB11 expressed not ganglioside mimics but a mixture of 3 nonsialylated structures. No structural differences were observed between the WTs and the *orf11* mutants.

ganglioside-like structures other than GM1 or GQ1b were detected (Table 2). The sera used in the serological assay for the detection of these "other" ganglioside-like structures may also react with asialo-GM1-like structures lacking sialic acid. Therefore, the detection of an "other" ganglioside-like structure does not necessarily imply that the strain is able to synthesize and transfer sialic acid. However, we cannot exclude the possibility that some strains have both class D or E genes and sialic acid biosynthesis genes that have diverged from their homologues in classes A, B, and C.

Interestingly, we found that the class A locus is associated specifically with GBS and the presence of a GM1-like structure, whereas the class B locus is associated with MFS and the presence of a GQ1b-like structure. The presence of anti-GM1 antibodies has been shown to be associated with a preceding *Campylobacter* infection (3). We found that GM1-like structures can be expressed by strains with various LOS locus classes. However, the association of a class A LOS locus with both GBS and the presence of a GM1-like structure suggests that class A strains expressing GM1-like LOS structures are more likely to induce GBS compared with non-A strains with a GM1-like LOS.

MFS, a variant of GBS, has consistently been associated with preceding *C. jejuni* infection. Several authors have demonstrated that GQ1b-like structures occur more frequently in MFS-associated strains and are associated with anti-GQ1b antibody reactivity and the presence of oculomotor symptoms in patients with GBS or MFS (8, 20). Although GQ1b-like structures were detected in both class A and class B strains, we found that only the class B locus was significantly associated with the presence of a GQ1b-like structure and that all our MFS-associated strains contained a class B locus. However, not all class B strains expressed GQ1b-like structures. This may be explained by mechanisms such as phase variation or point mutations that lead to gene inactivations resulting in the absence of expression of GQ1b-like structures in some class B strains (21, 22). In conclusion, we have now identified specific molecular substrates in *C. jejuni* that are involved in the induction of GBS or MFS.

To our knowledge, this is the first study that analyzed the LOS locus of a large collection of neuropathy-associated and control *C. jejuni* strains. Previously, it was demonstrated that 2 closely related GBS-associated strains contained a class A locus (16). Very recently, we demonstrated that the strain GB11 that we used in this study has probably acquired its class A locus through horizontal gene transfer (17). Another recent report did not find an association between GBS and LOS biosynthesis genes using a whole-genome DNA microarray (23). This microarray, however, only contained LOS genes present in a class C locus and would therefore not be able to detect the association described here.

Based on the results of the LOS locus analysis, we hypothesized that genes unique to the class A and B loci or genes that are involved in sialic acid biosynthesis and transfer are crucial for the induction of neuropathogenic cross-reactive antibodies. To test this hypoth-

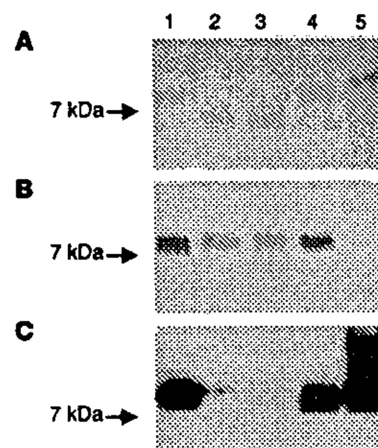


Figure 4
SDS-PAGE analysis of LOS from GB11 WT and mutants. The genome strain NCTC 11168 was included as a control. Lane 1, WT GB11; lane 2, GB11 *cst-II* mutant; lane 3, GB11 *orf10/orf11* mutant; lane 4, GB11 *orf11* mutant; lane 5, NCTC 11168. (**A**) Silver staining of the LOS revealed faster-migrating LOS cores for the GB11 *cst-II* and *orf10/orf11* mutants compared with the WT, indicating that these mutants have a truncated LOS. The *orf11* mutant LOS showed migration patterns identical to those of WT LOS. (**B**) A Western blot incubated with GB11 patient serum showed a reduced reactivity for the *cst-II* and *orf10/orf11* mutants but unchanged reactivity for the *orf11* mutant when compared with the WTs. (**C**) For the *cst-II* and *orf10/orf11* mutants, the reactivity with cholera toxin, a ligand for GM1-oligosaccharide structures, was almost completely lost. Reactivity with the *orf11* mutant remained unchanged.

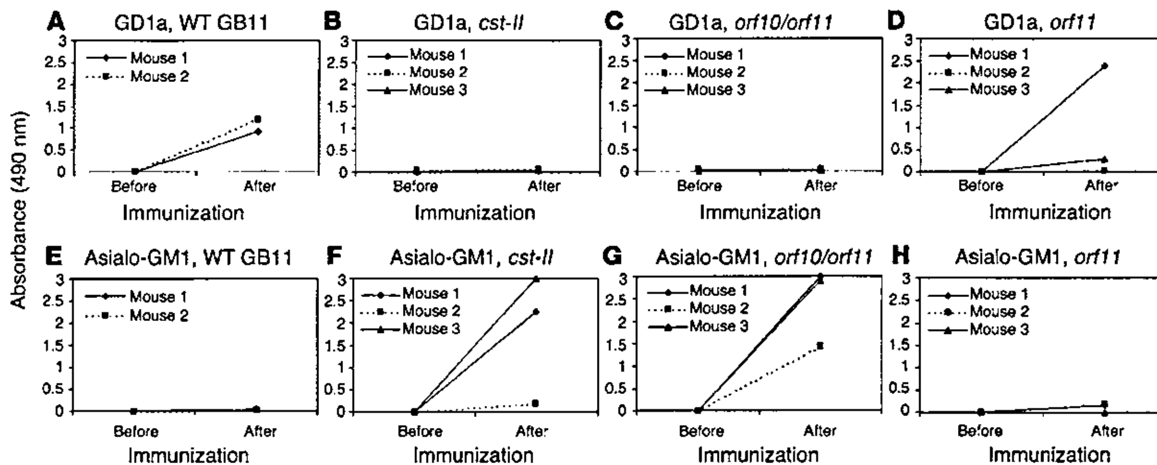


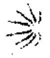
Figure 5
Anti-glycolipid antibodies in serum of mice before and after immunization with WT and mutant GB11 *C. jejuni* strains. (**A** and **D**) When sialic acid was present on the LOS core (WT and *orf11* mutant), anti-GD1a antibodies were induced in mice. (**B** and **C**) Anti-GD1a antibody responses were not detected in mice immunized with nonsialylated LOS (*cst-II* and *orf10/orf11* mutants). (**F** and **G**) Nonsialylated LOS (*cst-II* and *orf10/orf11* mutants) induced high asialo-GM1 antibody responses. (**E** and **H**) Asialo-GM1 antibody responses were absent or low after immunization with the *orf11* mutant and WT.

esis, we constructed a *Campylobacter* knockout mutant of *orf11*, encoding a putative acetyltransferase with unknown function in LOS biosynthesis and the only gene that is unique to class A and B loci. Several genes are involved in the sialylation of *Campylobacter* LOS (Supplemental Table 1). Inactivation of either of these genes will probably lead to identical changes in LOS structure; i.e., truncated LOS lacking sialic acid. We constructed knockout mutants of 2 such genes: *orf10*, encoding a CMP-sialic acid synthetase, and *cst-II*. *Cst-II*, present in class A and B loci, encodes a bifunctional sialic acid transferase that has been characterized in detail and that has been associated with the presence of a GQ1b-like structure (13, 15, 24). *Cst-II* has a sialyltransferase homologue in the class C locus, called *cst-III* (53% identity), but the bifunctional (α-2,3/α-2,8) sialyltransferase activity that is probably necessary for the biosynthesis of GQ1b-like structures has so far only been detected for *cst-II* (15). *Orf10* was inactivated along with the adjoining *orf11* in a so-called double mutant (Figure 2). Because the *orf11* single mutants did not show any differences compared with the WTs, we assumed that the differences observed in the *orf10/orf11* double mutant were the result of the inactivation of *orf10*. Mutants were constructed of 2 class A GBS-associated strains that expressed GM1-like structures in their LOS. We were unsuccessful in our efforts to construct mutants of MFS-associated strains expressing GQ1b-like structures, since, unfortunately, these strains did not appear to be transformable (data not shown).

Mass spectrometry analysis revealed a mixture of 2 different sialylated ganglioside-mimicking structures in the WT GB2 and GB11 strains. Inactivation of *cst-II* and *orf10* resulted in a mixture of 3 truncated LOS structures lacking sialic acid. Two of these structures lacked not only sialic acid, when compared with the WT, but also the outer Gal or Gal-GalNAc residues of the LOS backbone (Figure 3). This observation is consistent with the lower activity of the β-1,4-*N*-acetylgalactosaminyltransferase (CgtA) on nonsialylated acceptors that was previously reported for CgtA from *C. jejuni* OH4384 (16). Inactivation of *orf11* did not result in differences in LOS structure compared with that in the WT strains. Therefore, the function, if any, of this gene in LOS biosynthesis and in the pathogenesis of GBS remains unknown. In summary, mass spectrometry analysis revealed that genes involved in sialylation are necessary for the biosynthesis of ganglioside mimics in *Campylobacter* LOS. Other studies have demonstrated the role of *cst-III* (*orf7c*), *neuB1* (*orf8c*), and *neuC1* (*orf9b* and *orf9c*) in the synthesis of ganglioside mimics (21, 25, 26).

Inactivation of *cst-II* and *orf10* resulted in reduced reactivity of GB11 patient serum with mutant LOS, providing support for their importance in the induction of autoantibodies in GBS. Although residual reactivity was seen, this was probably caused by anti-asialo-GM1 antibodies or anti-GM1 and anti-GD1b antibodies that cross-react with asialo-GM1 structures in the mutant LOS. It has previously been demonstrated that the GB11 patient serum contains antibodies directed against GM1 (high titer), asialo-GM1 (low titer), and GD1b (high titer), which all contain the terminal disaccharide Gal-GalNAc (8). Although nonsialylated glycoconjugates are present on nerve tissue, so far, anti-asialo-GM1 antibodies have not been associated with GBS. The complete absence of reactivity of cholera toxin with *cst-II* and *orf10/orf11* mutant LOS further confirms the crucial role of *cst-II* and *orf10* in the biosynthesis of ganglioside mimics.

The mouse immunization experiments conducted in this study provide additional evidence for the necessity of sialylation genes for the induction of autoantibodies. Both *cst-II* and *orf10/orf11* mutants failed to induce anti-ganglioside antibodies in mice, in contrast to the WT strains. Mice injected with the *cst-II* and *orf10/orf11* mutants produced serum antibodies against the nonsialylated glycoconjugate asialo-GM1, but these antibodies have not yet been associated with GBS. Anti-GD1a but no anti-GM1 antibodies were detected in mice immunized with the GB11 WT strain. This is in contrast with earlier findings that GB11 also expresses a GM1-like LOS structure and that GB11 patient serum contains high levels of anti-GM1 antibodies. However, it has been observed previously that immunization with both GD1a and GM1 induced primarily anti-GD1a responses in mice, whereas rabbits only had



anti-GM1 antibodies (27, 28). Thus, anti-ganglioside antibody responses may be species dependent.

The associations that we found are not absolute. As host factors also play an important role in the pathogenesis of GBS, infection with a *C. jejuni* strain that expresses ganglioside-like structures is not sufficient to trigger GBS. In addition, a variety of ganglioside mimics, produced by different LOS classes, have been associated with GBS with diverse clinical manifestations (8, 20, 29). Furthermore, it is not clear at present whether all bacterial factors involved in the development of GBS/MFS have been identified.

After the demonstration of anti-ganglioside antibodies in GBS/MFS patient sera, and the subsequent identification of ganglioside mimics in *Campylobacter* LOS and of the specific host responses toward these epitopes, we now also demonstrate that the microbial gene repertoire is an important factor in the initiation of postinfectious autoimmune disease. The markers described in the current communication may facilitate the search for mechanisms of microbial pathogenicity and be helpful in the development of new molecular diagnostic tools for identifying *C. jejuni* strains with an increased ability to induce GBS/MFS. Furthermore, increased insight into the biosynthesis of ganglioside-mimicking structures may ultimately lead to the development of new treatment strategies and interventions (30).

Methods

C. jejuni strains. Seventeen GBS-associated and 4 MFS-associated *C. jejuni* strains used in this study were isolated from GBS/MFS patients from The Netherlands and Belgium between 1991 and 1999 (10). GB13 and GB26 were cultured from the diarrheal stools of family members of 2 GBS patients (31). Twenty-one control *C. jejuni* strains were isolated from sporadic Dutch enteritis patients without neurological symptoms between 1990 and 1999 (8). Ten reference *C. jejuni* Penner serotypes (HS:1, HS:2, HS:3, HS:4, HS:10, HS:19, HS:23, HS:35, HS:36, and HS:64) and the "genome" strain NCTC 11168 (19) were also included as controls for the PCR tests. Characterization of the isolates by phenotypic and molecular methods showed that no clustering of GBS/MFS-associated strains occurred when these were compared with control strains (10, 12).

Growth conditions and DNA isolation. For PCR analysis, *C. jejuni* strains were cultured for 24–48 hours on blood agar plates in a microaerobic atmosphere at 37°C. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega Corp.).

For other experiments, *C. jejuni* WT strains were grown at 37°C on Mueller-Hinton agar plates supplemented with Modified Preston Campylobacter Selective Supplement (MPCSS; product code SR0204E; Oxoid Ltd.) in a microaerobic atmosphere. The mutant strains were grown on Mueller-Hinton agar plates supplemented with MPCSS and chloramphenicol, at a concentration of 20 μg/ml. For cloning, the *E. coli* strain JM109 (Promega Corp.) was grown on 2YT agar with 40 μg/ml ampicillin and 20 μg/ml chloramphenicol.

Serological methods. The ganglioside mimics in the LOS of the various strains were determined using polyclonal and monoclonal anti-ganglioside antibodies as described previously (8). An inventory was made for GM1-like, GQ1b-like, and, collectively, other ganglioside-like structures.

Determination of the LOS locus class. To determine the class of LOS locus in *C. jejuni* strains, we developed specific primer sets for the classes A/B, C, D, and E, based on the DNA sequence of a gene unique to the LOS locus class(es) involved (Supplemental Table 1). To discern between classes A and B, we developed a primer set that was based on the DNA sequence of *orf5-II*. PCR assays were performed using a Thermocycler 60 (Biomed GmbH) with a program consisting of 40 cycles of 1 minute at 94°C, 1 minute at 52°C, and 2 minutes at 74°C. Per reaction, approximately 50 ng of template DNA was used in a buffer system consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM of each of the deoxyribonucleotide triphosphates (Promega Corp.), and 0.2 U Super Taq polymerase (HT Biotechnology Ltd.). Timing needed to be adjusted for some of the amplifications. The selective PCR tests were validated with a panel of *C. jejuni* strains with a known LOS locus class.

Cloning and mutagenesis. The target genes (*cst-II*, *orf10/orf11*, and *orf11*) and approximately 700 bp of flanking sequence were amplified and cloned into the pGem-Teasy vector (Promega Corp.). Inverse PCR was used to introduce a *BamHI* restriction site and a deletion of approximately 800 bp in the target genes (32). Inverse PCR products were digested with *BamHI* and ligated to the *BamHI*-digested chloramphenicol resistance (Cm^r) cassette of pAV35 (33). After sequencing, the resulting constructs were electroporated into different *C. jejuni* strains, and recombinants were selected on Mueller-Hinton plates containing chloramphenicol (33). Junction PCR, with primers up- and downstream of the area involved in the homologous recombination, and primers in the Cm^r cassette, was performed to confirm double crossover events and to assess the orientation of the resistance cassette.

Mass spectrometric analysis. Overnight growths from 1 agar plate were treated as described by Szymanski et al. (34) except that we used proteinase K at 60 μg/ml, RNase A at 200 μg/ml, and DNase I at 100 μg/ml. The O-deacylated LOS samples were analyzed by capillary electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS) as described by St. Michael et al. (35).

SDS-PAGE and Western blotting. Fresh overnight cultures of GB11 WT and mutant strains were harvested in PBS and lysed by sonification. To visualize both protein and LOS fractions, the cell lysates were electrophoresed on 11% SDS-PAGE gels. To visualize the LOS fraction only, cell lysates were treated with proteinase K (1 mg/ml) for 2 hours at 65°C and electrophoresed on 15% SDS-PAGE gels. Gels were silver-stained to visualize LOS cores, or the gels were blotted onto nitrocellulose. GB11 patient serum was used at a dilution of 1:2,500, and peroxidase-conjugated cholera toxin (Sigma-Aldrich) was used at a concentration of 1 μg/ml.

Mouse immunization experiments. This research was approved by the Animal Care and Use Committee, Dokkyo University School of Medicine (approval no. 00-22). Mice were treated according to the Guidelines for the Care and Use of Laboratory Animals of Dokkyo University School of Medicine. Mice lacking GM2/GD2 synthase as well as GM1 and GD1a (36) were immunized i.p. several times at 2-week intervals with 1 mg of each lyophilized *C. jejuni* dissolved in 100 μl keyhole limpet hemocyanin solution, mixed with 100 μl CFA. One week after the final inoculation, blood samples were taken from the tail vein. Serum IgG antibodies to GM1, GD1a, and asialo-GM1 were measured by an ELISA (37). The mean absorbance value for triplicate reference wells without antigen was subtracted from the mean value for triplicate sample wells with the antigen.

Statistical analysis. Statistical analysis was performed with InStat (version 2.05a; GraphPad Software Inc.) using 2 × 2 contingency tables. Fisher's exact tests were executed and 2-sided P values determined. Associations were considered significant at $P < 0.05$.

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Post-infectious acute ataxia and facial diplegia associated with anti-GD1a IgG antibody

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Sir,
Neurological features of uncommon variants may make it difficult to recognize Guillain–Barré syndrome (GBS). We studied a patient with acute ataxia, facial diplegia and anti-GD1a IgG antibody.

Two weeks after having fever and cough, this 42-year-old man developed painful paresthesias in both hands, perioral, lingual numbness, and severe myalgia (day 1). Within next 3 days, he noted dysarthria, difficulty chewing, and unsteady gait. On day 5, examination showed facial diplegia and tongue paralysis. Eye movements and other cranial nerves were intact. Limb weakness was minimal. Tendon jerks were decreased or absent. Plantar responses were flexor. Finger-nose and heel-shin tests showed ataxia. There was distal impairment of light touch and pin-prick perception. Position and vibration sense were impaired in both feet. Romberg sign was present. Stool culture did not reveal *Campylobacter jejuni*. Antibodies to *Borrelia burgdorferi*, cytomegalovirus and Epstein–Barr virus were not detected. Chest radiography did not reveal lymphadenopathy. On day 6, cerebrospinal fluid was acellular but protein content was 160 mg/dl. An enzyme-linked immunosorbent assay revealed elevated anti-GD1a IgG antibody titer of 2000 (normal < 500) in serum obtained on day 6. Tests for IgG and IgM antibodies against GM2, GM1, GM1b, GalNAc-GD1a, GD1b, GT1a and GQ1b were negative. Thin-layer chromatography with immunostaining confirmed the anti-GD1a antibody (Fig. 1). On day 25, electrophysiological examination showed prolonged distal motor latencies with delayed, inconstantly evoked F waves in median, peroneal, tibial nerves. He was given intravenous immunoglobulins (0.4 g/kg/day for 5 days). Neurological symptoms first ameliorated then worsened. Starting on day 28, he had six plasmaphereses. After first exchange, he began to recover. On day 180, there were no residual neurological abnormalities.

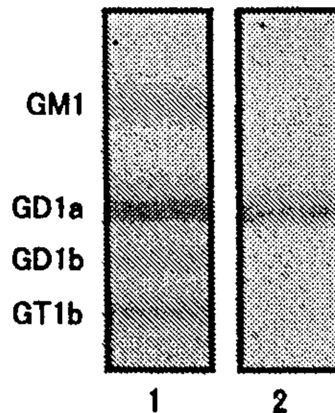

Figure 1 Thin-layer chromatogram with immunostaining. Bovine brain ganglioside mixtures were layered on thin-layer chromatography plate and developed with chloroform-methanol-0.2% calcium chloride in water (5:4:1). The plate next were stained with orcinol/sulfuric acid for hexose (lane 1) and IgG from our patient on day 6 (lane 2). Anti-GD1a IgG antibody clearly is present.

Seventeen months after onset, titer of anti-GD1a IgG antibody was 500.

In this patient, antecedent infection, acute, monophasic neurologic illness and cerebrospinal fluid albumino-cytologic dissociation could be considered a GBS variant. Limb and gait ataxia without ophthalmoplegia were thought to be more severe than that could be expected from the mild proprioceptive sensory impairment. These findings were compatible with features of ataxic GBS (Richter, 1962). Severe bifacial but minimal limb weakness, hyporeflexia, and limb paresthesias are characteristic of GBS regional variant, facial diplegia and paresthesias, according to Ropper (1994). Our patient's condition could be considered overlap version of these syndromes. We could find three similar cases (Shuaib and Becker, 1987). Despite treatment related fluctuating course (Kleyweg and van der Meché, 1991), both intravenous immunoglobulin and plasmapheresis were effective. The anti-GD1a IgG antibody, a diagnostic marker of GBS in generalized forms (Yuki *et al.*, 1993; Ho *et al.*, 1999), can be taken as evidence of GBS in our case. In ataxic GBS, no association with that antibody was described, whereas IgG antibodies to GQ1b and GT1a (Yuki *et al.*, 2000a), GD1b (Yuki *et al.*, 2000b), GM1b and GalNAc-GD1a (Odaka *et al.*, 2004) were reported. Anti-GD1a and anti-GT1a IgG antibodies were present in one patient with acute facial diplegia and hyperreflexia (Susuki *et al.*, 2004). Our patient's findings suggest a range of unusual GBS variants and underscore usefulness of anti-ganglioside antibody assays.

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report

Longitudinal changes of antiganglioside antibodies before and after Guillain-Barré syndrome onset subsequent to *Campylobacter jejuni* enteritis

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AUTHORS' SUMMARY

Antiganglioside antibodies frequently are present in sera from patients with Guillain-Barré syndrome during the acute phase, but such antibodies have not been detected before the onset of the syndrome. We describe the first case of Guillain-Barré syndrome subsequent to *Campylobacter jejuni* infection, in which longitudinal changes in antiganglioside antibody titers were measured before and after the onset of limb weakness. Serum antibody titers against GM1 (IgM/IgG), GM1b (IgM/IgG), GalNAc-GD1a (IgM/IgG), and GD1b (IgG) were highest on the day of onset, but negative before onset. Anti-*C. jejuni* IgG and IgA antibodies titers paralleled those of the antiganglioside antibodies, indicating that *C. jejuni* infection triggered antiganglioside antibody production. We speculate that antiganglioside antibodies are the primary effectors of nerve damage in Guillain-Barré syndrome.

Case report

A 12-year-old girl had diarrhea and a respiratory infectious illness. Two days after their resolution, she developed gait disturbance (day 1) and limb weakness, which gradually worsened. She could not write on day 3 nor stand on day 6. She had been treated for 3 months for Hashimoto's disease before the onset of these symptoms. On examination (day 6), there was no abnormality in the cranial nerves. Muscle strength in the upper limbs was graded 4 on the Medical Research Council scale, and 3–4 for the proximal and 2–3 for the distal muscles of the lower limbs. Deep tendon reflexes were absent in all limbs. There was numbness of the lower extremities, but vibration and position senses were intact. A stool culture was negative for *C. jejuni.* Cerebrospinal fluid findings showed 31 mg/dL protein on day 5 and 96 mg/dL on day 14 with normal cellularity. Electrophysiological examination showed reduced muscle action potential amplitude with normal motor conduction velocity, findings indicative of primary axonal neuropathy. From day 6, she was given human immunoglobulin (0.4 g/kg intravenously daily) for 5 days. Her strength improved gradually from day 13. On day 18, she was able to walk around the room by holding on to the furniture, and on day 21 she could walk without support. She was discharged on day 22. Thyroid function was normal during the course of her illness.

Sera were obtained 89, 68, 54, and 27 days before the onset of symptoms and 1, 5, 20, 56, and 112 days after their onset. The sera before the onset of Guillain-Barré syndrome had been stored at –80 °C after measurement of thyroid hormone. IgG and IgA antibody titers to *C. jejuni* were 16,000 (normal, less than 2000) and 8000 (normal, less than 500), respectively, at onset. The marked decrease during the recovery phase suggested recent *C. jejuni* infection (Table 1). Anti-GM1, anti-GM1b, anti-GD1a, anti-GalNAc-GD1a, or anti-GD1b IgM or IgG antibodies titers were highest on day 1 and/or day 5, whereas all were negative before symptom onset (Table 1). No clear pattern of isotype switching from IgM to IgG during the illness was found. Except for the anti-GM1b IgM antibody titer, titers did not change before or after intravenous immunoglobulin treatment (day 5). Four months after symptom onset, the titers had decreased to less than 500.

Discussion

Electrophysiological and pathological studies done on animal nerve preparations have shown that antiganglioside antibodies are disturbed in the peripheral nerves. Cumulative evidence supports the speculation that antiganglioside antibodies against peripheral nerves are involved in the development of Guillain-Barré syndrome *(1).* IgG antibodies against GM1, GM1b, GD1a, or GalNAc-GD1a are present in Guillain-Barré syndrome during the acute phase and are markedly associated with axonal Guillain-Barré syndrome with preceding *C. jejuni* infection. Whether these antiganglioside antibodies are the primary effectors of nerve damage, however, is not clear. The highest antiganglioside IgM or IgG antibody titers were detected at disease onset in our patient with Guillain-Barré syndrome subsequent to *C. jejuni* infection and decreased with the clinical course of the illness. No antiganglioside antibodies were detected before the onset of Guillain-Barré

Table 1

Anti-ganglioside and anti-*C. jejuni* antibody titers of patients before and after onset of limb weakness

Days from onset to serum sampling	Anti-*C. jejuni*			IgM antibody against				IgG antibody against			
	IgM	IgG	IgA	GM1	GM1b	GalNAc-GD1a	GD1b	GM1	GM1b	GalNAc-GD1a	GD1b
-89	-	-	-	-	-	-	-	-	-	-	-
-68	-	-	-	-	-	-	-	-	-	-	-
-54	-	-	-	-	-	-	-	-	-	-	-
-27	-	-	-	-	-	-	-	-	-	-	-
1	-	16 000	8 000	2 000	16 000	16 000	-	32 000	32 000	16 000	16 000
5	-	16 000	2 000	2 000	8 000	16 000	-	32 000	32 000	16 000	16 000
20	-	8 000	500	1 000	4 000	8 000	-	4 000	4 000	4 000	2 000
56	-	2 000	-	1 000	2 000	4 000	-	2 000	4 000	4 000	2 000
112	-	-	-	-	-	-	-	-	-	-	-

-: negative

Cutoff level of IgM, IgG, and IgA antibodies to *C. jejuni* respectively are less than 500, 2,000, and 500

syndrome. We previously established an animal model of axonal Guillain-Barré syndrome based on inoculation with a bovine brain ganglioside mixture or isolated GM1 ganglioside *(2)*. In this model, the serum anti-GM1 IgM antibody titer increased first (2 to 3 weeks after the first sensitization), followed by the anti-GM1 IgG antibody titer (3 to 4 weeks after the first inoculation). The IgG antibody titer gradually increased, and neurological deficits, such as flaccid paresis of the hind limbs, were seen 1 to 3 weeks (median, 1 week) after the IgG antibody titer peaked. The observation that during the course of the illness these titers were highest at the onset of Guillain-Barré syndrome suggests that, as in the animal model, antiganglioside antibody titers peak before the onset of neurological symptoms. Our reported case and disease model support the speculation that antiganglioside antibodies are the primary effectors of nerve damage and have a pathogenic role in the development of Guillain-Barré syndrome, rather than being merely a secondary phenomenon. Antiganglioside antibodies are useful diagnostic markers, even during the very early stage of Guillain-Barré syndrome.

C. jejuni is the reported causal agent in one-third of patients with Guillain-Barré syndrome, and molecular mimicry between the gangliosides and lipo-oligosaccharide of the bacterium is considered to contribute to the production of antiganglioside antibodies *(3)*. GM1, GM1b, GD1a, and GalNAc-GD1a epitopes are expressed in the lipo-oligosaccharides of *C. jejuni* isolated from patients with Guillain-Barré syndrome, and a single strain of *C. jejuni* has several ganglioside-like lipo-oligosaccharides. These findings provide a possible explanation for the detection of several species of antiganglioside IgG antibodies in our patient. She had neither anti-*C. jejuni* nor anti-ganglioside antibodies before the onset of Guillain-Barré syndrome but showed parallel longitudinal changes in antibody titers after disease onset, which suggests that infection by *C. jejuni* bearing a ganglioside-like lipo-oligosaccharide triggered the production of antiganglioside antibodies, thereby eliciting Guillain-Barré syndrome. ■

EDITORIAL BOARD COMMENT

A strong association between *C. jejuni* infection and Guillain-Barré syndrome has been established. This case report contributes to the understanding of the pathogenesis of Guillain-Barré syndrome. The emergence of antibodies to both *C. jejnuni* and gangliosides concomitantly with the appearance of symptoms of Guillain-Barré syndrome strongly indicates a causal relationship in the present case. The authors suggested that lipopolysaccharides of the bacterium induce the production of antibodies to several gangliosides, due to molecular similarities between these substances. There were no antibodies prior to disease onset, but the latest serum sample was taken 27 days before the appearance of Guillain-Barré syndrome, and symptoms of *C. jejnuni* infection occurred in the interval between these events. Nevertheless, the development of antibodies must have occurred over a short period of time. The polymerase chain reaction technique has been recently introduced to identify *C. jejuni* infection in patients with the Guillain-Barré syndrome, since culture underestimates the *C. jejuni* infection, and the specificity of serology remains uncertain.

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Various immunization protocols for an acute motor axonal neuropathy rabbit model compared

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Abstract

Various ganglioside immunization protocols were examined to refine the procedure for establishing an animal model of acute motor axonal neuropathy. The most effective was subctaneous injection of an emulsion of 2.5 mg of bovine brain ganglioside mixtures, *keyhole lympet hemocyanin*, and complete Freund's adjuvant to Japanese white rabbits, repeated at 3-week intervals. Under that protocol, all the rabbits developed marked flaccid paralysis associated with plasma anti-GM1 IgG antibody. This acute motor axonal neuropathy rabbit model also could be reproduced by the use of incomplete Freund's adjuvant, methylated bovine serum albumin, and New Zealand white rabbits. These results provide useful information for the confirmation of and further research on the model.
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Keywords: Acute motor axonal neuropathy; Rabbit model; Immunization protocol; Adjuvant

Guillain-Barré syndrome (GBS) is a post-infectious autoimmune polyneuropathy. GBS consists of two phenotypes; primary demyelinating and axonal forms. In Japan, 38% of GBS patients have been classified electrophysiologically as having acute motor axonal neuropathy (AMAN), the axonal form of GBS [9]. AMAN and IgG antibodies against gangliosides such as GM1 are closely associated [9]. We produced an AMAN disease model by sensitization of Japanese white (JW) rabbits with a bovine brain ganglioside (BBG) mixture or isolated GM1 [11,17]. Because complete Freund's adjuvant (CFA) was repeatedly used, however, it is unlikely that that immunization procedure would be approved as an animal research protocol for use in institutions in the United States, therefore alternative approaches for reproducing the model are required [5]. Whether a similar model can be induced in another rabbit breed, e.g., the New Zealand white (NZW), or in other animal species is an important issue. Despite frequent use of intravenous immunoglobulin or plasma exchange, GBS still involves considerable morbidity and mortality [10], and the need to develop new treatments for it is clear. One of the most effective strategies for evaluating new treatments is to test them on an animal model. We examined various ganglioside immunization protocols for an AMAN animal model and obtained useful information for future investigations.

This research was approved by the Animal Care and Use Committee, Dokkyo University School of Medicine (approval numbers 0146, 99-237, 99-233, and 0180). The animals were treated according to the Guidelines for the Care and Use of Laboratory Animals at Dokkyo University School of Medicine.

We used BBG in this study, because BBG was most suitable as the immunogen for the experiments of AMAN model. Sensitization with BBG could efficiently induce acute tetraplegia due to axonal neuropathy [11,17]. Target molecule for IgG antoantibody was GM1, whereas the BBG used contains several gangliosides (GM1 21%, GD1a 40%, GD1b 16%, GT1b 19%). Moreover, BBG can be easily prepared, as described elsewhere with modification [15]. In brief, bovine brain tissues were homogenized, and lipid was extracted

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with chloroform/methanol/water (8:4:3, v/v). The lipid extract was concentrated and dialyzed to remove salts then applied to a column of Iatrobeads (Iatron Laboratories Inc., Tokyo, Japan) to eliminate free sialic acid. Neutral glycolipids were eluted with chloroform/methanol (7:3, v/v). The purity of the extracted ganglioside mixture was confirmed by thin-layer chromatography.

The sensitization, serological and pathological studies were done as described elsewhere [17]. Male and female JW (Kbs:JW) and male NZW (Kbs:NZW) rabbits, weights 2.0–2.5 kg, were obtained from Oriental Bioservice Kanto Inc. (Tsukuba, Japan). Five milligrams of the BBG mixture, prepared as described above, was injected subcutaneously to the back at 3-week intervals until limb weakness developed or maximal sensitization (five times). The rabbits were checked daily for neurological signs using a clinical scale [7] (Table 1). At the endpoint, 15 weeks after beginning sensitization, all the rabbits were killed. Anti-GM1 IgG antibody was tested for by an enzyme-linked immunosorbent assay of plasma obtained within 1 week of the onset of limb weakness, or at death if the rabbits showed no limb weakness. Specimens of the sciatic nerve and cauda equina were evaluated pathologically. Five groups (three rabbits each) were prepared (Table 2): *Group 1*, BBG was dissolved in 0.5 ml of *keyhole lympet hemocyanin* (KLH) (2 mg/ml; Sigma, St. Louis, MO) in phosphate-buffered saline and emulsified with 0.5 ml of CFA (Sigma), then injected to male JW rabbits repeatedly [17]; *Group 2*, 0.5 ml of CFA was used for the first inoculation and 0.5 ml of incomplete Freund's adjuvant (IFA; Sigma) for subsequent sensitizations; *Group 3*, 2 mg of methylated bovine serum albumin (BSA; Sigma) was used instead of KLH; *Group 4*, the same immunogen as in *Group 1* was injected to male NZW rabbits instead of JW; *Group 5*, the same immunogen as in *Group 1* was injected to female JW rabbits.

Table 1
AMAN rabbit model clinical scale

Clinical features
Abnormal posture
Neck weakness
Slip of forelimbs
Slip of hindlimbs
Dragging forelimbs
Dragging hindlimbs
Unable to walk
Slow righting reflex
No righting reflex
Ptosis
Tremor of limbs or trunk induced by pulling tail
Spontaneous tremor of limbs or trunk
Animal appeared systemically unwell

Each clinical feature is scored 1. The total is the clinical score (maximum score, 13). Disease onset is defined as a daily clinical score of 4 or more. AMAN, acute motor axonal neuropathy.

Male JW rabbits were immunized according to the reported procedure [17] with 0.5, 1, 2.5, or 5 mg of BBG (Cronassial^{TM}; Fidia, Padova, Italy). The severity of clinical symptoms at nadir was evaluated.

Ten 8-week-old male Lewis rats purchased from Oriental Bioservice Kanto Inc. were injected intraperitoneally with 250 μg of BBG (Cronassial^{TM}) together with CFA and KLH. Three 7-week-old female guinea pigs (Std:Hartley) obtained from Japan SLC Inc. (Hamamatsu, Japan) were injected subcutaneously to the back with 1 mg of BBG (prepared in our laboratory as described above) together with CFA and KLH. Five sensitizations were made at 3-week intervals.

Table 2
AMAN model rabbits produced by various immunization methods

Group	Rabbits	Breed	Sex	Carrier protein	Adjuvant	Innoculation times	Onset of disease (day)	Clinical scale at nadir	Anti-GM1 IgG titer	Days from onset to sacrifice	Axonal degeneration
1	Bg-1	JW	M	KLH	CFA	2	35	8	16000	22	Moderate
	Bg-2	JW	M	KLH	CFA	3	57	10	16000	26	Moderate
	Bg-3	JW	M	KLH	CFA	3	60	10	16000	30	Moderate
2	Bg-4	JW	M	KLH	CFA, IFA	5	94	6	16000	27	Moderate
	Bg-5	JW	M	KLH	CFA, IFA	5	90	6	≥64000	29	Moderate
	Bg-6	JW	M	KLH	CFA, IFA	4	77	6	8000	22	Moderate
3	Bg-7	JW	M	mBSA	CFA	5			16000	–	Mild
	Bg-8	JW	M	mBSA	CFA	5	97	8	32000	25	Moderate
	Bg-9	JW	M	mBSA	CFA	5			4000	–	Mild
4	Bg-10	NZW	M	KLH	CFA	3	61	8	8000	31	Moderate
	Bg-11	NZW	M	KLH	CFA	5	97	7	32000	30	Moderate
	Bg-12	NZW	M	KLH	CFA	5			16000	–	Mild
5	Bg-13	JW	F	KLH	CFA	3	57	8	≥64000	28	Moderate
	Bg-14	JW	F	KLH	CFA	3	55	10	16000	23	Moderate
	Bg-15	JW	F	KLH	CFA	2	32	8	≥64000	22	Moderate

For the clinical scale, see Table 1. Bg-7, Bg-9, and Bg-12 showed no obvious weakness. In those rabbits, anti-GM1 IgG antibody was assayed in sera obtained 15 weeks after the beginning of sensitization. Wallerian-like degeneration was evaluated in sciatic nerve specimens: mild, <10% of degenerated fibers; moderate, 10–40%; severe, >40% on toluidine blue-stained cross sections. AMAN, acute motor axonal neuropathy; JW, Japanese white rabbit; NZW, New Zealand white rabbit; KLH, *keyhole lympet hemocyanin*; mBSA, methylated bovine serum albumin; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

In *Group 1* treated by the reported method [17], all three rabbits developed marked limb weakness (Table 2). In *Group 2*, in which CFA was used for the first immunization and IFA thereafter, all three rabbits developed limb weakness, but the periods between the beginning of sensitization and neurological onset (77, 90, and 94 days) were longer than those (35, 57, and 60 days) for the paralyzed rabbits in *Group 1*. Moreover, the severity of paralysis tended to be milder than in *Group 1*. In *Group 3*, in which methylated BSA was used instead of KLH, only one (Bg-8) of the three rabbits developed limb weakness. The interval between the first immunization and onset of limb weakness (97 days) also was longer than the intervals for the paralyzed rabbits in *Group 1*. In *Group 4*, two of the three male NZW rabbits developed tetraparesis, and one (Bg-11) took 97 days from the first sensitization to the onset of paralysis. In *Group 5*, all three female JW rabbits had outcomes similar to those in *Group 1*. All the rabbits, including those who showed no apparent weakness, had elevated plasma anti-GM1 IgG antibody titers. A pathology examination confirmed there was Wallerian-like degeneration in the sciatic nerves of all the rabbits, even those without paralysis (Fig. 1). The cauda equina had occasional clusters of small fibers with various degrees of myelination, indicative of axonal sprout regeneration.

Induction rates for AMAN rabbits paralyzed by sensitization with BBG were dose dependent (Table 3). On sensitization with the 2.5 mg of BBG, all six rabbits developed marked flaccid limb weakness.

Neither the rats nor guinea pigs sensitized with BBG developed limb weakness. At the endpoint, 15 weeks after beginning sensitization, no anti-GM1 IgG antibody was detected in plasma obtained from the rats. Neither anti-GM1 nor anti-GD1a IgG antibody was present in the plasma from guinea pigs.

Two reports have described immunization of animals with GM1, but induction of paralysis associated with anti-ganglioside antibodies was not sufficient to establish the AMAN model in both. In one [6], a conjugate of lipid micelles, consisting of ganglioside and methylated BSA, was emulsified with CFA and injected intradermally to foot pads of JW rabbits. Four of eight rabbits injected with the GD1a ganglioside developed flaccid limb paresis, but only a small or

Table 3
Relationship between immunogen amount and limb weakness severity

Immunogen BBG (mg)	Number of rabbits	Symptom severity		
		Normal	Mild	Marked
0.5	3	2	1	
1.0	6	1	2	3
2.5	6			6
5.0	3		1	2

Symptom severity: normal, no limb weakness; mild, limb weakness present but able to walk; marked, unable to walk due to limb weakness. BBG, bovine brain ganglioside.

negligible amount of anti-ganglioside antibody was detected. The sciatic nerve showed non-inflammatory degeneration, myelin breakdown, and phagocytic cells containing myelin debris. GM1 induced rigid or spastic paralysis and considerable amounts of anti-GM1 antibodies in the rabbits, but the induction rate was lower than that for GD1a. In another report [12], NZW rabbits were immunized with GM1 and methylated BSA or the Gal β1-3 GalNAc conjugate of BSA. CFA was used for the first immunization, thereafter IFA. Although anti-GM1 antibodies were detected in the sera and there was mild axonal degeneration in the sciatic nerve, no rabbit developed limb weakness. In contrast, the sensitization of JW rabbits with GD1b and KLH emulsified with CFA did induce sensory neuropathy associated with anti-GD1b antibody [4]. According to the procedure of Kusunoki et al. [4], we tried sensitization with BBG. All the rabbits inoculated with 2.5 or 5 mg of BBG developed limb weakness under our protocol ([11,17], present study). Furthermore, 9 of 11 [17] and 5 of 7 [11] GM1-sensitized rabbits developed flaccid limb paresis. Paralysis was highly induced because our immunization procedure used very effective factors (repeated use of CFA, KLH, and JW rabbits) as discussed below.

On repeated injection of CFA (*Group 1*), all the rabbits developed severe limb weakness. The possibility that systemic undesirable side effects caused by the booster CFA [1] worsened the severity of disease in our model is unlikely. No control animals injected repeatedly with KLH and CFA, but not gangliosides, experienced limb weakness, systemic disease, or unexpected death after injection [11,17]. None of the AMAN rabbits given a booster CFA injection (*Groups 1, 3, 4,*

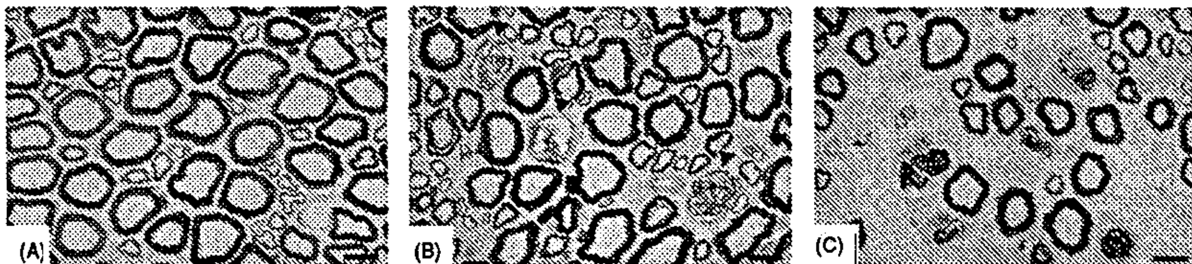

Fig. 1. 0.5% toluidine blue-stained transverse sections. (A) Sciatic nerve from a normal rabbit. (B) Sciatic nerve from rabbit Bg-12 (*Group 4* in Table 2; immunized to male NZW rabbit). Myelin ovoid produced by Wallerian-like degeneration of a myelinated fiber is occasionally noted (arrow heads), whereas the rabbit did not show obvious neurological symptoms. (C) Sciatic nerve from rabbit Bg-5 (*Group 2* in Table 2; CFA was used for the first immunization and IFA thereafter). Degenerated nerve fibers are frequently seen. No inflammatory cells are seen in the endoneurium. Scale bars = 10 μm.

and *5*) had developed obvious additional lesions in the lung, liver, or other organs at the time of death. Repeated use of CFA therefore did induce weakness efficiently. In our first report [17], immunogens were injected both subcutaneously and intraperitoneally. In subsequent experiments ([11], present study), immunization was obtained by subcutaneous injection alone, one of the normal routes for CFA injection [1]. The AMAN model was reproducible when CFA was used for the first immunization and IFA thereafter (*Group 2*), but paralysis tended to be milder, and more sensitizations were needed for neurological onset than in *Group 1*. When the lipo-oligosaccharide (LOS) from *Campylobacter jejuni*, the most frequent antecedent pathogen of GBS [3], together with CFA was injected to rabbits repeatedly, they developed high anti-GM1 IgG antibody titers and flaccid limb paresis [16], whereas neither anti-GM1 IgG nor limb weakness was induced by the other adjuvants; the Ribi Adjuvant System (RIBI ImmunoChem Research Inc., Hamilton, MT) or TiterMax® Gold (CytRx Corporation, Norcross, GA) (Yuki, unpublished observation).

Both KLH and methylated BSA are widely used as carrier molecules to enhance immune responses to administered antigens. In our study, neither was conjugated. Both were emulsified with gangliosides, therefore neither was used as the carrier protein. The effectiveness of KLH, however, was apparent. When methylated BSA was used instead of KLH (*Group 3*), anti-GM1 IgG antibody titers were elevated in all three rabbits, but only one rabbit developed paralysis. KLH may strengthen the pathogenic role of anti-GM1 antibodies as well as accelerate their induction, possibly because KLH and GM1 have a Gal β1-3 GalNAc epitope in common [14]. Immunization with KLH and Freund's adjuvant (initially CFA, 21 days later IFA) in Lewis rats induced anti-GM1 antibody production [14], but that result could not be reproduced in a subsequent study [13]. In our earlier study, no rabbits inoculated with KLH and CFA had anti-GM1 antibody activities [17]. After pre-sensitization with KLH, rats immunized with the LOS from *C. jejuni* had high anti-GM1 IgM (no IgG) antibody titers, whereas sensitization with KLH or LOS alone failed to induce antibodies [13].

Reactions to active ganglioside immunization differed with the rabbit breed or animal species, but why is not clear. One of three NZW rabbits (*Group 4*) did not develop obvious limb paresis despite having elevated anti-GM1 IgG antibody and mild Wallerian-like degeneration in the sciatic nerve. The other two rabbits did develop limb weakness, but the intervals between the first sensitization and onset tended to be longer and limb weakness milder than for the JW rabbits paralyzed under the same protocol (*Group 1*). We tried only two rabbit breeds, JW and NZW. Trials with other breeds are required to develop a more suitable AMAN model. We failed to produce an AMAN animal model of rats and guinea pigs by active immunization with BBG. Nagai et al. [6] reported induced limb weakness in guinea pigs immunized with GD1a, but we could not confirm it.

Differences in the ganglioside source did not affect the results. Because some patients develop axonal GBS after BBG therapy [2,8], we used Cronassial™ (BBG: [11,17], present study) or Sygen™ (GM1: [17]), which had been available on the Italian market, as the immunogen. Gangliosides from other sources, the BBG prepared in our laboratory (present study), and the GM1 purchased from Sigma [11] also induced an AMAN model, indicative of the universality of ganglioside effects. The appropriate amount of immunogen to use was also examined. We concluded that at least 2.5 mg of BBG is required to induce the AMAN rabbit model by our immunization procedure.

Because of small sample size, we could not do statistical analysis. The tendency in efficiency of each protocol for sensitization of rabbit with BBG, however, was apparent. The alternative protocols in which CFA is used for the first immunization and IFA thereafter or NZW rabbits are used instead of JW rabbits also induced AMAN model, although they were not as effective as our reported method [11,17]. An anti-GM1 IgG- and complement-mediated attack on the nodal axolemma of motor nerve fibers causes AMAN. The features of BBG-immunized rabbits are very similar to those of patients with AMAN [11,17]. We believe this disease model helps to clarify the molecular pathogenesis of AMAN. Furthermore, the therapeutic efficacy of intravenous immunoglobulin treatment was confirmed in the AMAN rabbit [7], indicative that our model can be used in studies to evaluate new GBS treatments. We hope that the present findings will encourage others to undertake further research using our AMAN model.

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Does *Campylobacter jejuni* infection elicit "demyelinating" Guillain–Barré syndrome?

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Abstract—*Background: Campylobacter jejuni* enteritis is the most common antecedent infection in Guillain–Barré syndrome (GBS). *C. jejuni*-related GBS is usually acute motor axonal neuropathy (AMAN), but previous reports described many cases of the demyelinating subtype of GBS (acute inflammatory demyelinating polyneuropathy [AIDP]) after *C. jejuni* infection. *Objective:* To investigate whether *C. jejuni* infection elicits AIDP. *Methods:* In 159 consecutive patients with GBS, antibodies against *C. jejuni* were measured using ELISA. Antecedent *C. jejuni* infection was determined by the strict criteria of positive *C. jejuni* serology and a history of a diarrheal illness within the previous 3 weeks. Electrodiagnostic studies were performed weekly for the first 4 weeks, and sequential findings were analyzed. *Results:* There was evidence of recent *C. jejuni* infection in 22 (14%) patients. By electrodiagnostic criteria, these patients were classified with AMAN (n = 16; 73%) or AIDP (n = 5; 23%) or as unclassified (n = 1) in the first studies. The five *C. jejuni*-positive patients with the AIDP pattern showed prolonged motor distal latencies in two or more nerves and had their rapid normalization within 2 weeks, eventually all showing the AMAN pattern. In contrast, patients with cytomegalovirus- or Epstein–Barr virus-related AIDP (n = 13) showed progressive increases in distal latencies in the 8 weeks after onset. *Conclusion:* Patients with *C. jejuni*-related Guillain–Barré syndrome can show transient slowing of nerve conduction, mimicking demyelination, but *C. jejuni* infection does not appear to elicit acute inflammatory demyelinating polyneuropathy.

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Guillain–Barré syndrome (GBS) is classified into demyelinating and axonal categories by electrophysiologic and pathologic criteria.[1-3] In Western countries, GBS is usually caused by acute inflammatory demyelinating polyneuropathy (AIDP) with electrodiagnostic and pathologic evidence of peripheral nerve demyelination.[4-6] In contrast, a pure motor axonal form of GBS, acute motor axonal neuropathy (AMAN), is frequently seen in eastern Asian countries such as China and Japan.[3,7,8]

Campylobacter jejuni enteritis is the most common antecedent infection in GBS[8,9] and is often associated with antiganglioside antibodies.[8,10] The *C. jejuni* lipo-oligosaccharides have a ganglioside GM1-like structure indicative of molecular mimicry between neural tissue and bacterial components.[11] Ganglioside GD1a has been shown to be expressed on the lipo-oligosaccharides of *C. jejuni*.[12] *C. jejuni* infection is likely to induce antiganglioside antibodies and thereby GBS.

Whereas electrodiagnostic and pathologic studies have shown that *C. jejuni* infection is significantly associated with primary axonal dysfunction, the relationship between *C. jejuni* infection and neurophysiology is still the subject of debate.[8,13] In northern China, positive *C. jejuni* serology was found for 76% of 21 AMAN patients and for 42% of 12 AIDP patients, suggesting that *C. jejuni* infection elicits AMAN more frequently than AIDP, but a considerable number of AIDP cases also occur after *C. jejuni* infection.[3] In a large study conducted in North America and Europe involving 229 GBS patients, 52 patients had positive serology for *C. jejuni*, and 56% of them showed demyelinating neurophysiology.[13] A study in Japan investigating 86 GBS patients showed that of the 20 *C. jejuni*-positive patients, 70% had AMAN and 15% had AIDP.[8] These results raise the possibility that *C. jejuni* infection can elicit AMAN and AIDP.

To investigate whether *C. jejuni* infection really elicits AIDP, we conducted serial electrodiagnostic studies in *C. jejuni*-positive GBS patients who were selected by strict criteria and blind serologic assay and compared results with those of patients with typical AIDP after infection by cytomegalovirus (CMV) or Epstein–Barr virus (EBV).

Methods. *Patients.* One hundred fifty-nine consecutive patients with GBS who fulfilled the clinical criteria for GBS and were seen at Chiba University Hospital in Japan between 1994 and 2002 were studied.[14] The first electrophysiologic studies were

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