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Anti-ganglioside complex antibodies associated with severe disability in GBS☆

K. Kaida [a], D. Morita [b], M. Kanzaki [a], K. Kamakura [a], K. Motoyoshi [a], M. Hirakawa [b], S. Kusunoki [b,*]

[a] *Third Department of Internal Medicine, National Defense Medical College, 3-2 Namiki, Saitama-ken, 359-8513, Japan*
[b] *Department of Neurology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka, 589-8511, Japan*

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Abstract

Ganglioside complexes (GSCs) are known as target antigens in Guillain–Barré syndrome (GBS). To elucidate the clinical importance of the anti-GSC antibodies in GBS, we investigated serum antibodies to GSCs containing two of the gangliosides, GM1, GD1a, GD1b and GT1b, and analyzed clinical features of anti-GSC-positive GBS patients. Thirty-nine (17%) of 234 GBS patients had IgG anti-GSC antibodies. Anti-GSC-positive GBS had antecedent gastrointestinal infection and lower cranial nerve deficits more frequently than control GBS. The presence of antibody specificity to GD1a/GD1b and/or GD1b/GT1b was significantly associated with severe disability and a requirement for mechanical ventilation.
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Keywords: Guillain–Barré syndrome; Ganglioside complex; Antibody; Disability

1. Introduction

Gangliosides concentrate on the surfaces of neurons, with their oligosaccharide portions exposed on the cell surface, and are believed to form clusters extensively in the cell membrane (Hakomori, 2002). Recent studies of plasma membrane molecules have shown that gangliosides appear to be organized in clusters, and to form membrane microdomains together with cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins (Simons and Toomre, 2000). These microdomains are called lipid rafts or detergent-resistant membranes.

Gangliosides in peripheral nerves are often targeted by serum antibodies in patients with Guillain–Barré syndrome (GBS), acute immune-mediated polyradiculoneuropathy, and interaction between anti-ganglioside antibodies and peripheral nerve gangliosides is believed to cause GBS and its variants (Chiba et al., 1993; Willison and Yuki, 2002; Kaida et al., 2003). We recently demonstrated the presence of antibodies to ganglioside complexes (GSCs) in serum of some GBS patients and suggested that anti-GSC antibodies might function in the development of GBS (Kaida et al., 2004). It has hitherto been inferred that ligands of adhesion molecules such as selectins comprise diverse and complex glycoconjugates, called clustered saccharide patches, in which oligosaccharides are packed closely together to form rigid rodlike structures with multivalency and strict binding-specificity (Norgard et al., 1993; Varki, 1994). The discovery of anti-GSC antibodies in GBS serum suggests that clustered glycoepitopes of GSCs or clustered saccharide patches actually exist on the plasma membrane and are involved in immune-mediated events.

In our previous study (Kaida et al., 2004), eight of 100 GBS patients had IgG antibodies to GD1a–GD1b complexes (GD1a/GD1b) and were predisposed to lower cranial nerve palsy and severe disability. However, statistical significance

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* Corresponding author. Tel.: +81 72 366 0221x3553; fax: +81 72 368 4846.
E-mail address: kusunoki-tky@umin.ac.jp (S. Kusunoki).

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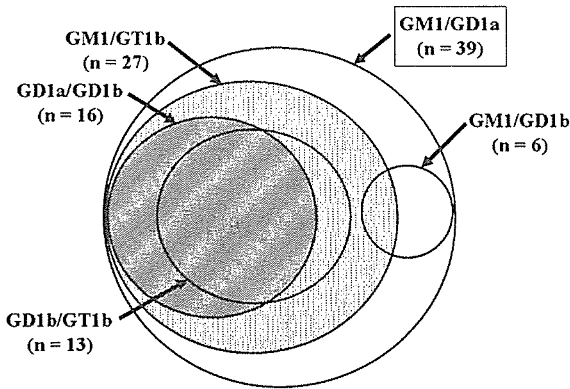

Fig. 1. The Venn diagram shows distribution of anti-GSC(+) group patients based on data in Table 1. All of the anti-GSC(+) group patients had IgG anti-GM1/GD1a antibodies. Anti-GD1a/GD1b-positive patients had also IgG anti-GM1/GD1a and GM1/GT1b antibodies. Ten anti-GSC(+) group patients had IgG anti-GD1a/GD1b and anti-GD1b/GT1b antibodies.

of the association between that antibody and those clinical features has not been demonstrated. In the present study, to clarify the clinical significance of anti-GSC antibodies in GBS, we retrospectively analyzed the clinical features of anti-GSC antibody-positive patients with GBS in a larger population, with a focus on the fine specificity of anti-GSC antibodies and its clinical relevance.

2. Materials and methods

2.1. Patient serum and enzyme-linked immunosorbent assay for anti-ganglioside complex antibodies

Acute phase serum was collected from patients with clinically defined GBS between September 2001 and December 2002 at various general and teaching hospitals throughout Japan. All met the diagnostic criteria of Asbury and Cornblath (Asbury and Cornblath, 1990).

ELISA was done for serum antibodies to gangliosides GM1, GM2, GM3, GD1a, GalNAc–GD1a, GD1b, GD3, GT1b, and GQ1b, as described elsewhere (Kaida et al., 2000). The ELISA for anti-GSC antibodies were performed as described in our previous report (Kaida et al., 2004). GSCs used in the ELISA contained two (0.1 μg each) of four ganglioside antigens, GM1, GD1a, GD1b, and GT1b which

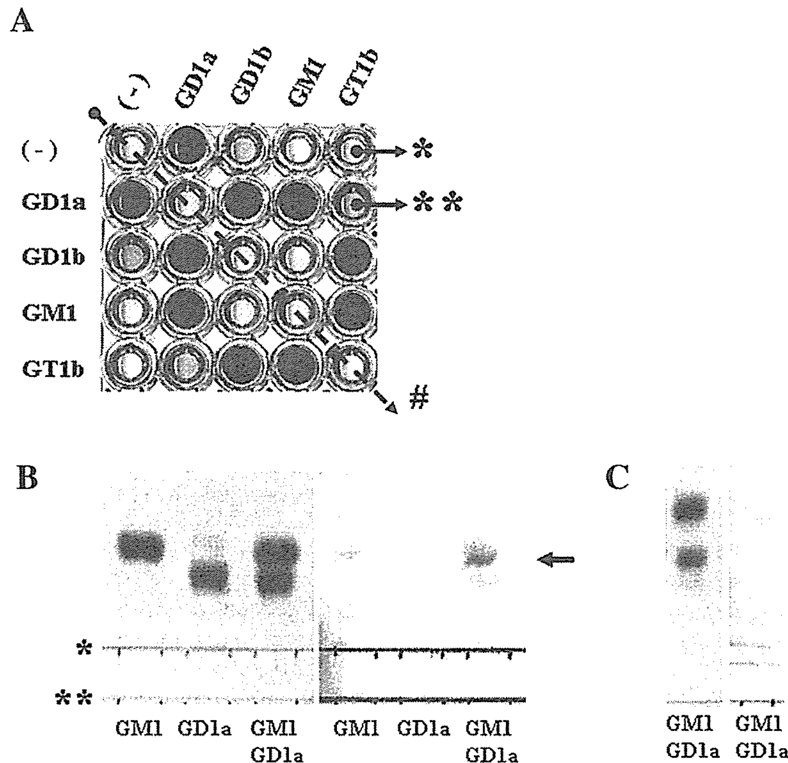

Fig. 2. Representative ELISA results and thin-layer chromatography (TLC) studies for antibodies to GSCs using sera from Anti-GSC(+) group patients. (A) Serum from patient no.16 in the Anti-GSC(+) group was used. A weak reaction is observed in a well containing only GD1a, while strong reactions can be seen in wells containing GD1a/GD1b, GM1/GD1a, GD1b/GT1b, and GM1/GT1b (w/w=0.1/0.1). *=GT1b only (0.2 μg), **=a mixture of GD1a (0.1 μg) and GT1b (0.1 μg). # Five control wells on each plate are indicated by oblique dotted arrows. (B) Serum from patient no.18 was used. TLC bands were visualized with orcinol reagent in the left panels. Immunostaining using anti-GSC antibody-positive sera (diluted 1:100 with 1% BSA in PBS) is shown in the right panels. Immunostaining is shown in the overlapping portions of GM1 and GD1a (arrow). GD1a was applied on the upper line (*) and GM1 on the lower line (**). (C) The immunostaining in the overlapping portions of GM1 and GD1a have disappeared in a solvent system that separated the positions of GM1 and GD1a to a great extent (serum from patient no.18). The developing solvent comprised chloroform, methanol, and 0.2%CaCl2.2H2O as follows; 55/40/10 (v/v, Fig. 2B), 50/45/10 (Fig. 2C).

Table 1
Frequency of antibodies to single gangliosides in anti-GSC(+) and control groups

	Antianglioside antibody	Antibody specificity in anti-GSC(+) group*					
		GM1/GD1a (Anti-GSC(+))	GM1/GD1a GM1/GT1b	GM1/GD1a GM1/GT1b GD1a/GD1b	GM1/GD1a GM1/GT1b GD1b/GT1b	GM1/GD1a GM1/GT1b GD1a/GD1b GD1b/GT1b	Control
		(n=38)	(n=27)	(n=16)	(n=13)	(n=10)	(n=170)
IgG class	anti-GM1	6 (16%)	3 (11%)	3 (19%)	2 (15%)	1 (10%)	27 (16%)
	anti-GD1a	4 (11%)	4 (15%)	4 (25%)[#1]	4 (31%)[#2]	4 (40%)[#3]	10 (6.1%)
	anti-GD1b	28 (74%)[#4]	20 (74%)[#4]	8 (50%)[#3]	8 (62%)[#5]	5 (50%)[#1]	9 (5.5%)
	anti-GT1b	3 (7.9%)	3 (11%)[#6]	3 (19%)[#7]	3 (23%)[#8]	3 (30%)[#9]	3 (1.8%)
	anti-GQ1b	6 (16%)	5 (19%)	1 (6.3%)	1 (7.7%)	1 (10%)	15 (9.1%)
	anti-GalNAc-GD1a	7 (18%)	4 (15%)	1 (6.3%)	0	0	26 (16%)**
IgM class	anti-GM1	2 (5.3%)	2 (7.4%)	2 (13%)	2 (15%)	2 (20%)	21 (13%)
	anti-GalNAc–GD1a	1 (2.6%)	1 (3.7%)	0	0	0	15 (9.4%)**

* The Anti-GSC(+) group was divided into four subgroups based on antibody specficity of anti-GSC antibodies. In the GSC(+) group, IgG antibodies to GM2, GM3, and GD3 and IgM to GD1a, GD1b, GT1b, GQ1b, GM2, GM3, and GD3 were negative. # indicates that there are significant differences between an antibody-positive group and remainder. Remainders include control group patients and remaining anti-GSCantibody-positive patients. #1, p=0.02; #2, p=0.009; #3, p=0.003; #4, p<0.0001; #5, p=0.0004; #6, p=0.03; #7, p=0.007; #8, p=0.004; #9, p=0.002 The chi-square test was used to test differences in proportions when the number in a cell was more than 6. If not, Fisher's exact test was used. **n=166 patients.

were major gangliosides in human nervous system (Yu and Iqbal, 1979). Gangliosides were mixed for 30 min before application to the ELISA. If a serum has both anti-GD1a and anti-GD1b antibody activities, the serum was judged to be anti-GD1a/GD1b antibody-positive when an OD value of anti-GD1a/GD1b antibody was more than the sum of those of anti-GD1a and anti-GD1b antibodies. As for estimation of antibodies to GM1/GD1a, GM1/GD1b, GM1/GT1b, GD1a/GT1b, and GD1b/GT1b, the same criteria were applied. ELISAs were performed in duplicate and mean ODs were calculated.

2.2. ELISA for anti-Campylobacter jejuni antibodies in serum from anti-ganglioside complex antibody-positive patients

IgG and IgM antibodies to *C. jejuni* were investigated in acute phase serum samples from anti-GSC antibody-positive patients with antecedent gastrointestinal infection using an ELISA kit for C. jejuni (Serion ELISA classic, Campylobacter jejuni IgG/IgM; Institut Virion/Serion, Würzburg, Germany). Serum from patients already diagnosed as having had *C. jejuni* enteritis from stool or serum specimens at other hospitals was excluded from the investigation. In cases where patients had positive or borderline ELISA results for IgM or IgG antibodies to *C. jejuni* and had suffered from diarrhea without respiratory symptoms, they were considered to have had antecedent gastrointestinal infection associated with *C. jejuni*.

2.3. Study population and analyses of clinical and electrophysiological features

Patients with IgG antibodies to at least one combination of two of the four gangliosides, GM1, GD1a, GD1b, and GT1b were defined as anti-GSC-positive patients (anti-GSC(+) group) and grouped according to the specificity of the anti-GSC antibodies. We asked the attending physicians to provide detailed clinical data for those subjects for whom clinical information was insufficient. Patients without data concerning neurological signs and symptoms were excluded from the clinical analysis. Anti-GSC-negative patients with detailed clinical data formed the control group. Patients' disabilities were graded using the Hughes Functional Grading Scale (Hughes et al., 1978). Electrophysiological data were evaluated as described previously and categorized as "primary demyelinating," "primary axonal," "unexcitable," "equivocal," or "normal" (Hadden et al., 1998; Kaida et al., 2001). Clinical features and electrophysiological findings were compared between the anti-GSC(+) and control groups.

2.4. Investigations into the specificity of anti-ganglioside complex antibodies

To investigate the specificities of anti-GSC antibodies in anti-GSC antibody-positive serum samples, immunoabsorption and ELISA were performed using mixtures of more than two gangliosides. Anti-GSC antibodies were absorbed on antigen-coated ELISA wells as described previously (Kaida et al., 2001).

2.5. Statistical analysis

Differences in proportions were tested by the Fisher exact probability test or the χ^2 test. The Mann–Whitney test was used for comparisons of age, onset to nadir (days), and peak disabilities among the groups. Two-tailed p-values<0.05 were considered significant throughout. These analyses were performed with SPSS software (12.0, SPSS Inc., Chicago).

3. Results

3.1. Study population

We collected serum from 234 consecutive GBS patients including 100 patients previously published (Kaida et al., 2004). ELISA showed that 39 (17%) of these patients had IgG antibodies to at least one GSC such as GD1a/GD1b, GM1/GD1a, GD1b/GT1b, GM1/GT1b, or GM1/GD1b. Among the 39 anti-GSC-positive patients, all had IgG anti-GM1/GD1a antibodies and 27 had anti-GM1/GT1b antibodies. Sixteen patients had anti-GD1a/GD1b antibodies, 13 had anti-GD1b/GT1b antibodies, and six had anti-GM1/GD1b antibodies. Ten patients had both anti-GD1a/GD1b and anti-GD1b/GT1b antibodies. Distribution of anti-GSC(+) group patients was described with a Venn diagram (Fig. 1). No patients in either group had anti-GD1a/GT1b antibodies. Eight of the 39 anti-GSC-positive patients were already reported in the previous study (Kaida et al., 2004). There were no serum IgM antibodies to GSCs in any of the 39 patients. Representative ELISA results and TLC immunostaining are shown in Fig. 2. Specific immunostaining was found on the overlapping portions of each ganglioside (Fig. 2B). One in the Anti-GSC(+) group was excluded from the analysis of clinical features because detailed clinical data were unavailable. Twenty-five of the 195 anti-GSC-negative patients also were excluded because insufficient clinical data were available; the remaining 170 patients served as the control group.

3.2. Frequencies of antibodies to single ganglioside antigens

Thirty-four of 39 patients in the anti-GSC(+) group had antibodies to single gangliosides (Table 1). There were no significant differences in the frequency of antibodies to single ganglioside antigens between anti-GSC(+) and the control groups, except for IgG anti-GD1b antibody. One of eight ventilated patients in the anti-GSC(+) group had IgG anti-GQ1b antibody. Although the frequencies of IgG antibodies to GD1a, GD1b, or GT1b were significantly higher in three subgroups (Table 1), their OD values were much lower than those of anti-GSC antibodies (data not

Table 2
Clinical features of GBS patients in anti-GSC(+) and control groups

	Antibody specificity in anti-GSC(+) group*					
Clinical features	GM1/GD1a (Anti-GSC(+)) (n=38)	GM1/GD1a GM1/GT1b (n=27)	GM1/GD1a GM1/GT1b GD1a/GD1b (n=16)	GM1/GD1a GM1/GT1b GD1b/GT1b (n=13)	GM1/GD1a GM1/GT1b GD1a/GD1b GD1b/GT1b (n=10)	Control (n=170)
Age**	43.2 (37.7–48.8)	45.3 (38.0–52.6)	43.8 (34.4–53.1)	48 (35.8–60.2)	47.1 (33.2–61.0)	48.9 (45.6–52.1) (n=169)
Sex male/female	25/13	18/9	12/4	9/4	7/3	103/67
Antecedent infection						
Respiratory	19 (50%)	12 (44%)	4 (25%)	3 (23%)	2 (20%)	76 (45%)
Gastro-intestinal	17 (45%)[#1] (*C. jejuni*: 12)	14 (52%)[#2] (*C. jejuni*: 9)	12 (75%)[#3] (*C. jejuni*: 8)	10 (77%)[#4] (*C. jejuji*: 6)	8 (80%)[#5] (*C. jejuni*: 5)	38 (22%)
Onset to nadir (days)**	7.8 (6.1–9.6) (n=33)	7.8 (5.4–10.2) (n=24)	9.2 (4.8–13.6) (n=13)	9.7 (5.2–14.1) (n=12)	10.4 (4.3–16.6) (n=9)	7.8 (7.0–8.7) (n=158)
Cranial nerve deficits	(n=35)	(n=25)	(n=15)			(n=156)
positive	22 (63%)	18 (72%)	11 (73%)	10 (77%)	9 (90%)[#6]	80 (51%)
III, IV, VI	12 (34%)[#6]	11 (44%)[#2]	5 (33%)	6 (46%)[#2]	5 (50%)[#7]	25 (16%)
VII	11 (31%)	9 (36%)	7 (47%)	5 (38%)	5 (50%)	57 (37%)
IX, X	17 (49%)	15 (60%)[#8]	10 (67%)[#9]	9 (69%)[#10]	8 (80%)[#11]	48 (31%)
XI, XII	9 (26%)[#12]	7 (28%)[#10]	6 (40%)[#11]	5 (38%)[#9]	4 (40%)[#6]	13 (8.3%)
Sensory loss	9 (26%) (n=34)	9 (38%) (n=24)	5 (36%) (n=14)	5 (42%) (n=12)	5 (56%) (n=9)	61 (39%) (n=156)
Nerve conduction study	(n=26)	(n=18)	(n=9)	(n=7)	(n=6)	(n=46)
Demyelination	8 (31%)	5 (28%)	3 (33%)	2 (29%)	2 (33%)	18 (39%)
Axonal	6 (23%)	4 (22%)	3 (33%)	3 (43%)	2 (33%)	5 (11%)
Inexcitable	3 (12%)	3 (17%)	1 (11%)	1 (14%)	1 (17%)	4 (8.7%)
Artificial ventilation	8 (22%) (n=36)	7 (27%)[#13] (n=26)	6 (38%)[#9]	6 (46%)[#11]	5 (50%)[#14]	19 (11%)

* The Anti-GSC(+) group was divided into four subgroups based on antibody specficity of anti-GSC antibodies (see Fig 1). Anti-GM1/GD1b antibody-positive group is omitted in this table due to the small number of patients. ** mean (95% confidence interval) # indicates that there are significant differences between an antibody-positive group and remainder. Remainder include Control group patients and remaining anti-GSCantibody-positive patients. #1, $p=0.008$; #2, $p=0.002$, #3, $p<0.0001$; #4, $p=0.0001$; #5, $p=0.0005$; #6, $p=0.02$; #7, $p=0.03$; #8, $p=0.006$; #9, $p=0.009$; #10, $p=0.01$; #11, $p=0.003$; #12, $p=0.007$; #13, $p=0.03$; #14, $p=0.004$.

Table 3
Association of antibody specificities with peak disability

	Antibody specificity in anti-GSC(+) group					
	GM1/GD1a	GM1/GD1a	GM1/GD1a	GM1/GD1a	GM1/GD1a	Control
	(Anti-GSC(+))	GM1/GT1b	GM1/GT1b	GM1/GT1b	GM1/GT1b	
			GD1a/GD1b	GD1b/GT1b	GD1a/GD1b	
					GD1b/GT1b	
Functional score	(n=36)	(n=26)	(n=16)	(n=13)	(n=10)	(n=170)
F-1	2 (5.6%)	1 (3.8%)	0 (0%)	0 (0%)	0 (0%)	6 (3.5%)
F-2	10 (28%)	7 (27%)	2 (13%)	3 (23%)	2 (20%)	48 (28%)
F-3	7 (19%)	3 (12%)	3 (19%)	1 (7.7%)	0 (0%)	42 (25%)
F-4	9 (25%)	8 (31%)	5 (31%)	3 (23%)	3 (30%)	54 (32%)
F-5	8 (22%)	7 (27%)	6 (38%)	6 (46%)	5 (50%)	19 (11%)
F-6	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.6%)
p value* (two-tailed)	0.63 vs. remainder** (n=170)	0.18 vs. remainder** (n=180)	0.008 vs. remainder** (n=190)	0.024 vs. remainder** (n=193)	0.012 vs. remainder** (n=196)	

*The Mann–Whitney test was used to compare functional score of two groups. **Remainder include Control group patients and remaining anti-GSC antibody-positive patients.

shown). TLC immunostaining using anti-GD1a/GD1b-positive sera showed no or little binding to GD1a or GD1b each, but intense binding to in the overlapping portion of GD1a and GD1b.

3.3. *Antecedent Campylobacter jejuni infection in anti-ganglioside complex antibody-positive patients*

Four patients in the anti-GSC(+) group had already been diagnosed as having antecedent *C. jejuni* enteritis at other hospitals. Of the remaining anti-GSC(+) group patients with antecedent gastrointestinal infection, eight were IgG anti-*C. jejuni* antibody-positive, one of whom was IgM anti-*C. jejuni* antibody-borderline. Overall, 12 Anti-GSC(+) group patients had antecedent *C. jejuni* enteritis (Table 2).

3.4. *Clinical and electrophysiological features of anti-ganglioside complex-positive patients*

Clinical features and electrophysiological findings in each group of patients are shown in Table 2. Results of statistical analysis of the clinical features are also shown in Table 2. Anti-GSC(+) group patients more frequently had antecedent gastrointestinal infections, ophthalmoplegia, and lower cranial nerve deficits. Among them, the anti-GD1a/GD1b or anti-GD1b/GT1b-positive patients had significantly higher F-scores at nadir and more frequently needed mechanical ventilation than the remaining subjects including control group patients (Table 3). Of 206 patients who had sufficient clinical data on peak disability, ventilated patients more frequently had IgG anti-GD1a/GD1b antibodies than unventilated ones (6/27, 22%, vs. 10/179, 5.6%) (two-tailed p value=0.009, odds ratio=4.85, χ^2 test). Anti-GD1b/GT1b antibodies also were more frequently found in ventilated patients (6/27, 22%, vs. 7/179, 3.9%) (two-tailed p value=0.003, odds ratio=7.02, χ^2 test). No patients required mechanical ventilation due to any respiratory disorder such as aspiration pneumonia.

In terms of the short-term outcome among patients with a peak F score of more than 2 and adequate follow-up data, improvement by one or more in the F score one month after the onset of GBS was found in 11 (65%) of 17 anti-GSC(+) group and 21 (68%) of 31 control group patients. In the anti-GSC(+) group patients, two (25%) of eight anti-GD1a/GD1b-positive patients and three (43%) of seven anti-GD1b/GT1b-positive patients improved by one or more in the F score one month after the onset of GBS. Anti-GD1a/GD1b-positive patients showed significantly poor recovery compared with the respective remainders (p=0.012, Fisher exact probability). There were no significant differences in treatment between the two groups (Table 4). In this study, because electrophysiological and clinical following-up data were insufficient to statistically compare between anti-GSC(+) and control groups, we could not confirm association of anti-GSC antibodies with electrophysiological results and poor outcome.

Table 4
Therapy in anti-GSC(+) and control groups

Treatment	Anti-GSC(+)	Control
	n=17	n=31
IVIG	10	17
PP	2	3
Combination (IVIG+PP)	3	3
Combination (IVIG+steroid)	2	4
Others	0	4[a]

IVIG = intravenous immunoglobulin.
PP = plasmapheresis, including plasma exchange and immunoadsorption therapy.
[a] Includes prednisolone (1), not done (2), and unknown (1) There were no significant differences in treatment between the two groups.

Table 5
Immunoabsorption study of anti-GSC antibodies with GSCs and single ganglioside antigens using serum from patient No.10

Antigens used for absorption[a]		Absorption rate			
		In anti-GD1a/GD1b antibody activity	In anti-GM1/GD1a antibody activity	In anti-GD1b/GT1b antibody activity	In anti-GM1/GT1b antibody activity
GSCs					
(−)	(Corrected OD)	0.48	0.60	0.67	0.92
GD1a/GD1b		100%	58%	76%	73%
GM1/GD1a		100%	100%	100%	100%
GD1b/GT1b		100%	57%	100%	72%
GM1/GT1b		100%	76%	81%	100%
Single ganglioside antigens					
(−)	(Corrected OD)	0.40	0.68	0.80	0.77
GM1		0%	0%	0%	0%
GD1a		35%	0%	37%	13%
GD1b		58%	5%	20%	11%
GT1b		0%	1%	14%	0%

GSCs = ganglioside complexes.
Uncoated wells served as controls. The serum from the patient No.10 was diluted 1:80 with 1% BSA in PBS.
[a] Each microtiter plate well was coated with a single ganglioside (0.25 μg) or a mixture of 0.2 μg of each ganglioside.

3.5. Immunoabsorption test

Immunoabsorption testing was done with serum from patient no.10 in the anti-GSC(+) group. The serum sample had IgG antibodies to GD1b (a corrected OD value was 0.16 when the serum was diluted 1:40) in addition to anti-GSC antibodies. Results of the immunoabsorption test are shown in Table 5. The serum antibodies reacted more specifically with GSCs than with single ganglioside antigens.

4. Discussion

The present study has shown that 17% of patients with GBS have IgG antibodies to at least one GSC such as GD1a/GD1b, GM1/GD1a, GD1b/GT1b, GM1/GT1b, and GM1/GD1b. The anti-GSC(+) group patients presented more frequently preceding gastrointestinal infection, ophthalmoplegia, and lower cranial nerve deficits than control group patients, although a selection bias in a control group may have influenced the results to some extent. A close association between the presence of anti-GD1a/GD1b and/or anti-GD1b/GT1b antibodies and a need for mechanical ventilation suggest that these antibodies may be useful predictors of a need for mechanical ventilation and a severe course of GBS.

A mixture of GM1 and a phospholipid such as phosphatidic acid, phosphatidylinositol, or phosphatidylserine, intensifies antiganglioside antibody activity in GBS serum (Kusunoki et al., 2003; Hirakawa et al., 2005). Anti-GM1 antibodies, however, appeared not to react with new epitopes generated by mixtures of phospholipids and GM1 because they had no activity toward phospholipids alone, whereas anti-GSC antibodies are likely to react with new epitopes in clustered gangliosides because of the weak activity toward individual ganglioside antigens. Anti-GSC(+) antibodies rarely bound toGM1/GD1b and never to GD1a/GT1b, suggesting that an epitope formed by a combination of [Galβ1–3GalNAc] and [NeuAcα2–3Galβ1–3GalNAc] in the terminal residues of gangliotetraose structures is important for the antibody binding (Fig. 3). When mixtures of three or four gangliosides (GM1, GD1a, GD1b, GT1b) were used as antigens in ELISA, anti-GSC antibody activity was often decreased (data not shown), supporting that epitopes targeted by anti-GSC antibodies are formed in a mixture of two gangliosides and have new specific conformations. We believe that a mixture of two gangliosides is an appropriate antigen for the surveillance of anti-GSC antibodies. The examination of serum antibodies to such complex antigens as GSCs may increase the spectrum of anti-ganglioside antibodies in GBS, enhancing their value as diagnostic markers.

Anti-GD1a/GD1b-and/or anti-GD1b/GT1b-positive patients were more likely to have a poor outcome and be

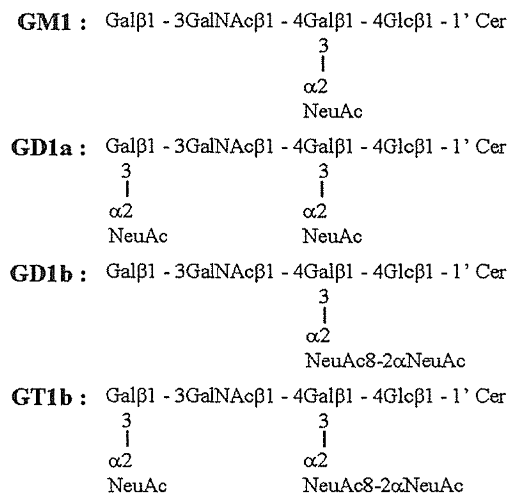

Fig. 3. Carbohydrate structures of GM1, GD1a, GD1b, and GT1b.

refractory to therapy than remaining subjects including control group patients, although more follow-up data are required to confirm the long-term prognosis. Why the anti-GD1a/GD1b-or anti-GD1b/GT1b-positive patients present a severe form of GBS is unclear. The different specificities of the anti-GSC antibodies might influence the peak disability and prognosis in anti-GSC antibody-positive patients. Most of anti-GD1a/GD1b-or anti-GD1b/GT1b-positive sera also react with GM1/GD1a and GM1/GT1b, suggesting that these sera are more multivalent than the antibodies reacting only with GM1/GD1a or GM1/GT1b, or with single ganglioside antigen. Tight binding between such multivalent antibodies and clustered glycoepitopes may correlate with a predisposition to a severe form of the disease.

Antecedent infection plays a critical role in the pathogenesis of GBS. Specific bacterial genes and ganglioside-mimicking structures in LOS of pathogens causing antecedent infection have proven to be essential for the induction of anti-ganglioside antibodies and determine antibody specificities (Ang et al., 2002; Yuki et al., 2004; Godschalk et al., 2004; Koga et al., 2005). Various ganglioside complex-like structures presumably exist in LOS of organisms causing antecedent infection. Cell wall glycoconjugates of *C. jejuni* might share epitopes with the GSCs such as GM1/GD1a or GD1a/GD1b, leading to the induction of anti-GSC antibodies.

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MAJOR ARTICLE

Comprehensive Analysis of Bacterial Risk Factors for the Development of Guillain-Barré Syndrome after *Campylobacter jejuni* Enteritis

Michiaki Koga,[1] Michel Gilbert,[4] Masaki Takahashi,[3] Jianjun Li,[4] Saiko Koike,[2] Koichi Hirata,[1] and Nobuhiro Yuki[1]

[1]Department of Neurology and [2]Institute for Medical Science, Dokkyo University School of Medicine, Tochigi, and [3]Department of Microbiology, Tokyo Metropolitan Institute of Public Health, Tokyo, Japan; [4]Institute for Biological Sciences, National Research Council Canada, Ottawa, Ontario, Canada

Background. Guillain-Barré syndrome (GBS), a postinfectious autoimmune-mediated neuropathy, is a serious complication after *Campylobacter jejuni* enteritis.

Methods. To investigate the bacterial risk factors for developing GBS, genotypes, serotypes, and ganglioside mimics on lipo-oligosaccharide (LOS) were analyzed in *C. jejuni* strains from Japanese patients.

Results. Strains from patients with GBS had LOS biosynthesis locus class A more frequently (72/106; 68%) than did strains from patients with enteritis (17/103; 17%). Class A strains predominantly were serotype HS:19 and had the *cstII* (Thr51) genotype; the latter is responsible for biosynthesis of GM1-like and GD1a-like LOSs. Both anti-GM1 and anti-GD1a monoclonal antibodies regularly bound to class A LOSs, whereas no or either antibody bound to other LOS locus classes. Mass-spectrometric analysis showed that a class A strain carried GD1a-like LOS as well as GM1-like LOS. Logistic regression analysis showed that serotype HS:19 and the class A locus were predictive of the development of GBS.

Conclusions. The high frequency of the class A locus in GBS-associated strains, which was recently reported in Europe, provides the first GBS-related *C. jejuni* characteristic that is common to strains from Asia and Europe. The class A locus and serotype HS:19 seem to be linked to *cstII* polymorphism, resulting in promotion of both GM1-like and GD1a-like structure synthesis on LOS and, consequently, an increase in the risk of producing antiganglioside autoantibodies and developing GBS.

The gram-negative spiral bacterium *Campylobacter jejuni*, which is a major bacterial agent in diarrheal illnesses, has been recognized as the bacterium that most frequently triggers the postinfectious autoimmune-mediated neuropathy called Guillain-Barré syndrome (GBS) [1]. An epidemiological study showed that 1 of 3285 patients with *C. jejuni* enteritis developed GBS [2]. Why such a small number of patients with *C. jejuni* enteritis develop GBS is not clear. Penner serotyping showed that, in Japan and South Africa, GBS-associated strains were more commonly serotypes HS:19 and HS:41 than were enteritis-associated strains [3–5]. Furthermore, HS:2 and the HS:4-complex were the dominant serotypes of strains from patients with Fisher syndrome (FS) [5], a GBS variant presenting the triad of ophthalmoplegia, ataxia, and areflexia [6]. The clustering of strains into particular serotypes is a strong indication that the clonality of *C. jejuni* strains is specifically related to the development of GBS and FS. The actual serodeterminants, however, are still unknown [7]; therefore, use of Penner serotyping schema alone to clarify the critical factors for the development of neurological syndromes would be difficult. It is noteworthy that the clustering of GBS-associated and FS-associated strains into specific serotypes has not been

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Reprints or correspondence: Dr. Michiaki Koga, Dept. of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan (kogamrk@dokkyomed.ac.jp).

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seen worldwide and, in particular, has not been seen in Western countries [8, 9].

Most patients who develop GBS after *C. jejuni* enteritis have IgG autoantibodies in their blood that react with gangliosides (such as GM1, GD1a, and GQ1b) [10]. Many patients with FS have anti–GQ1b IgG autoantibodies that cross-react with GT1a [11, 12]. The *C. jejuni* lipo-oligosaccharide (LOS) is a major candidate for the producer of such autoantibodies [13–15], because its terminal sugar regions mimic the gangliosides GM1, GD1a, and GQ1b [16–18]. The frequency of the GM1 and GD1a epitopes on the LOS of GBS-associated strains is hypothesized to be a risk factor for the development of GBS [19, 20]. Acute motor axonal neuropathy and anti-GM1 antibodies developed in rabbits after inoculation with GM1-like LOS, which indicates that GM1 mimicry of *C. jejuni* LOS is a cause of GBS [14].

Most studies have failed to find a specific *C. jejuni* genotype that was associated with GBS and FS [9, 21–24]. Gilbert et al. [25] reported that *C. jejuni* has 7 classes (A–G) of LOS locus that are based on the organization of the 37 distinct genes found in the LOS biosynthesis loci of 20 strains. This LOS locus typing scheme should help in the identification of the gene content that is responsible for the development of GBS and FS. Godschalk et al. [15], who used clinical isolates from The Netherlands and Belgium, recently reported that the class A locus was overrepresented in GBS-associated strains, compared with enteritis-associated strains (9/17 [53%] vs. 3/21 [14%]), whereas all FS-associated strains had the class B locus (FS-associated strains, 4/4 [100%]; enteritis-associated strains, 7/21 [33%]). The high frequency of the class A locus in GBS-associated strains has been confirmed by Parker et al. [26], who used 16 GBS-associated strains from various countries, although the frequency of this locus in GBS-associated strains could not be compared with that in enteritis-associated strains from each country. Godschalk et al. [15] suspected that the frequent expression of a GM1-like LOS in class A strains and a GQ1b-like LOS in class B strains is responsible for the development of GBS and FS, respectively. Their findings, however, do not clarify which genetic difference leads to the presence of diverse ganglioside mimics (GM1 and GQ1b) in spite of the almost identical gene profiles in class A and B loci [27] or why class C is relatively rare (2/17 [12%]) in GBS-associated strains in spite of the expression of GM1-like LOSs in all 5 strains with the class C locus.

The sialyltransferase gene *cstII* has an Asn/Thr polymorphism at codon 51, which determines substrate specificity; *cstII* (Thr51) has only α-2,3-sialyltransferase activity and is termed "monofunctional" *cstII*, whereas *cstII* (Asn51) has both α-2,3- and α-2,8-sialyltransferase activities and is termed "bifunctional" *cstII* [27]. Both sialyltransferase activities are required for the biosynthesis of the GQ1b and GT1a epitopes on LOS, whereas only α-2,3-sialyltransferase activity is needed for the biosynthesis of the GM1 and GD1a epitopes (figure 1). These findings recently led to our discovery that *cstII* polymorphism is important for the development of GBS and FS after *C. jejuni* enteritis [29]. *cstII* (Thr51) is closely associated with GBS and anti-GM1 and anti-GD1a autoantibodies, and *cstII* (Asn51) is closely associated with FS and anti-GQ1b autoantibodies.

Some of the identified bacterial risk factors for the development of GBS are closely related to each other, especially ganglioside mimics and serotype [19], *cstII* gene content [19, 29], and LOS locus class [15]. Therefore, it is necessary to analyze the risk factors comprehensively in a larger number of clinical isolates. We first examined whether the clustering of GBS-associated and FS-associated strains into a specific LOS locus class would also occur with a large number of Japanese strains; we then analyzed LOS locus classes comprehensively in connection with Penner serotype, *cstII* polymorphism, and ganglioside-like LOSs, to identify risk factors for the development of GBS.

MATERIALS AND METHODS

Strains. From December 1990 to February 2004, 138 *C. jejuni* strains were isolated from patients with GBS ($n = 106$) or FS ($n = 32$), and these strains were used in the present study. Most of the strains were included in our previous study [5]. Two strains, OH4384 and OH4382, were obtained from patients with GBS who were siblings [17, 30], and the others were obtained from patients with GBS who were evenly distributed geographically [5]. Diagnosis of GBS or FS was based on published clinical criteria [31, 32]. A total of 103 strains were isolated from patients throughout Japan who had uncomplicated enteritis, and these strains served as controls. Penner serotypes were determined using the passive hemagglutination technique with a *Campylobacter* antisera "Seiken" Set (Denka Seiken) [5].

LOS locus classification and* cstII *polymorphism. We used a method similar to that of Godschalk et al. [15] to classify the LOS locus (A–F). The presence of each class-specific gene was investigated by polymerase chain reaction (PCR) (table 1). The primer pair used for *orf19d* amplification was the same as that used in the study by Godschalk et al. [15], whereas the other primer pairs were newly designed for the present study. Class G was not examined, because it is considered to be very rare. Strains were judged to be class A when *orf7a/b (cstII)* was present and *orf5IIb (cgtAIIb)* was absent. Similarly, strains were judged to be class F when *orf19d/f* was present and *orf17d* was absent. A single bacterial colony was suspended in 300 μL of sterile distilled water and boiled for 10 min. After centrifugation at 10,000 *g* for 1 min, the supernatant was used as the template in the PCR amplification. Amplification reactions were performed with a total volume of 20 μL, which contained 8 pmol of each primer, 0.4 μL of DNA lysate, 0.5 U of *Taq* DNA

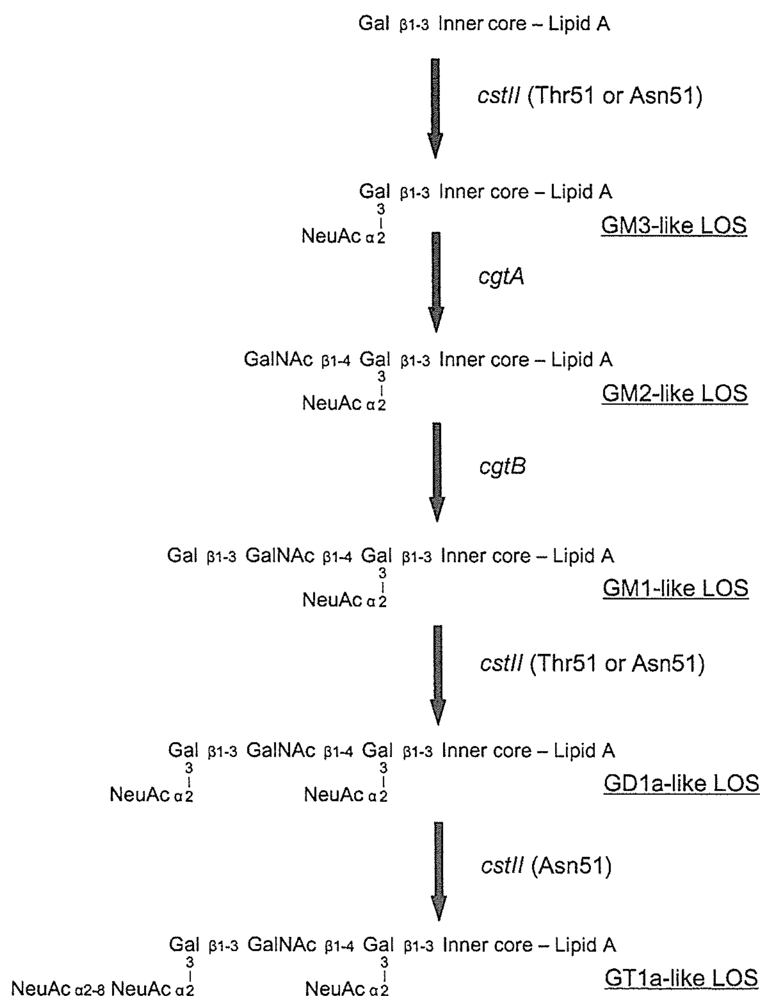

Figure 1. Proposed biosynthesis pathway of *Campylobacter jejuni* lipo-oligosaccharides (LOSs) mimicking gangliosides with a single sialic acid on the inner galactosyl residue [27, 28]. Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid.

polymerase (TaKaRa Ex Taq; Takara Bio), 4 nmol of dNTPs, and buffer (2 mmol/L Mg^{2+}). After the first denaturation step of 5 min at 95°C, the amplification mixture was subjected to 30 cycles of amplification (table 1). Variation at codon 51 of *cstII* was investigated by direct sequencing of the PCR fragment [29].

Ganglioside-like LOS. Crude LOS fractions were prepared from the strains as described elsewhere [33]. The presence of ganglioside epitopes (GM1, GD1a, and GQ1b) on the *C. jejuni* LOS was determined using an ELISA [34]. The reagents used were monoclonal antibodies (mAbs; GB2 [anti-GM1], GB1 [anti-GD1a], and FS3 [anti-GQ1b/GT1a]) [14, 34].

Analysis of* O*-deacylated LOS. *C. jejuni* was grown overnight on a single agar plate, and the cells were treated as described elsewhere [35], with minor modification [34]. The *O*-deacylated LOS sample was analyzed by capillary electrophoresis–electrospray ionization mass spectrometry (CE-ESI-MS) [36].

Sequencing of LOS biosynthesis genes. Isolation of genomic DNA from *C. jejuni* strain CF90-26 was performed with a DNeasy Tissue kit (Qiagen). A 6.1-kb PCR product bearing genes encoding the LOS outer core glycosyltransferases was amplified with an Advantage 2 PCR kit (Clontech Laboratories) and the primers CJ-99 (5′-ATTAAAAAAAGACCTTGGGAATAC-3′) and CJ-147 (5′-AAGGTGTGCTAAGATAAACAAGAC-3′). The 6.1-kb PCR

Table 1. Polymerase chain reactions for the lipo-oligosaccharide gene loci of *Campylobacter jejuni* strains.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

Table 2. Lipo-oligosaccharide (LOS) locus classes of *Campylobacter jejuni* strains.

LOS locus class	Guillain-Barré syndrome–associated strains (n = 106) No. (%)	Guillain-Barré syndrome–associated strains (n = 106) 2-tailed P^a	Fisher syndrome–associated strains (n = 32) No. (%)	Fisher syndrome–associated strains (n = 32) 2-tailed P^a	Enteritis-associated strains, no. (%) (n = 103)
A	72 (68)	<.001	12 (38)	.02	17 (17)
B	18 (17)	.02	15 (47)	.14	33 (32)
C	12 (11)	.13	1 (3)	.03	20 (19)
D	0	.12	0	1.0	3 (3)
E	2 (2)	.001	2 (6)	.36	14 (14)
F	0	.01	0	.34	6 (6)
A/C[b]	0	.49	0	1.0	1 (1)
B/C[c]	0	.49	0	1.0	1 (1)
Unclassified	2 (2)	.06	2 (6)	1.0	8 (8)
A, B, or C	102 (96)	<.001	28 (88)	.06	72 (70)

[a] Compared with enteritis-associated strains (Fisher's exact test).
[b] Overlapping class A and C loci.
[c] Overlapping class B and C loci.

product was sequenced by means of custom-made primers that were used previously to sequence this locus in multiple *C. jejuni* strains [27]. DNA sequencing was performed with a BigDye Terminator mix (Applied Biosystems). Products were analyzed in an ABI 3100 Genetic Analyzer (Applied Biosystems).

Antiganglioside autoantibodies. Serum samples obtained during acute phases of GBS and FS were available from 126 patients (95 patients with GBS and 31 patients with FS). IgG autoantibodies to GM1, GD1a, and GQ1b were measured by ELISA [37]. Serum was considered to be positive for antibody when the titer was ⩾500.

Statistical analysis. Frequency differences between groups were compared using Fisher's exact test. Differences in medians were examined using the Mann-Whitney U test, and Scheffé's test was used in the case of multiple comparisons. The association between the LOS locus class and either GBS or FS was first investigated by univariate analysis, without adjustment for confounding variables. A multiple logistic regression model was then used to determine the relative weighting of each variable. Statistical calculations were made with SPSS (version 12.0J; SPSS). A difference was considered to be statistically significant when $P < .05$.

RESULTS

LOS locus classification. Preliminary analysis of control strains of each LOS locus class confirmed that PCR-based LOS locus classification works well (data not shown). The class A locus was predominant in the GBS-associated strains, and its frequency was significantly higher in GBS-associated strains than in enteritis-associated strains (table 2). The other LOS locus classes were rarer in GBS-associated strains than in enteritis-associated strains. These findings agree with those of Godschalk et al. [15]. In contrast, FS-associated strains most frequently had the class B locus, but, compared with the enteritis-associated strains, the difference did not reach statistical significance, because it also was the most common class found in enteritis-associated strains. In the study by Godschalk et al. [15], all 4 FS-associated strains had the class B locus, whereas, in the present study, a significant number of FS-associated strains had the class A locus. In 12 strains (2 GBS associated, 2 FS associated, and 8 enteritis associated), there was no amplification of any class-specific genes. Two enteritis-associated strains were grouped as having overlapping class A and C or B and C loci.

Sialyltransferase-encoding genes (*cstII* or *cstIII*) are present in class A, B, and C loci [27], and this enables strains with these LOS locus classes to be characterized as a single group. Our data showed that 96% of GBS-associated strains had sialyltransferase-carrying LOS locus classes (A, B, or C), and this percentage was significantly higher than that of enteritis-associated strains (70%) (table 2). FS-associated strains also regularly had these LOS locus classes (88%).

Serotype. Table 3 shows the associations between the LOS locus class and the Penner serotype in GBS-associated, FS-associated, and enteritis-associated strains. LOS locus classes were closely—but not absolutely—associated with the Penner serotype, because strains with each LOS locus class were grouped into several serotypes, as was reported by Parker et al. [26]. Most class A strains were serotype HS:19, whereas the serotypes of class B strains varied. Conversely, most of the HS:19 strains had the class A locus, whereas most of the HS:2 and HS:4-complex strains had the class B or A locus.

cstII *polymorphism.* Class A and B loci are reported to carry the *cstII* gene [27]. Therefore, the association between *cstII* polymorphism and the class A or B locus was examined. Most of the class A strains had the *cstII* (Thr51) genotype (78/

Table 3. Associations between lipo-oligosaccharide (LOS) locus class and Penner serogroup in *Campylobacter jejuni* strains.

LOS locus class	Strains, no.	Serogroup (serotype) A (HS:1/44)	B (HS:2)	D (HS:4/13/16/43/50)	G (HS:8)	O (HS:19)	Y (HS:37)	Other
A[a]	101	0	7 (7)	15 (15)	0	73 (72)	1 (1)	6 (6)
B	66	1 (2)	21 (32)	21 (32)	6 (9)	6 (9)	0	17 (26)
C[b]	33	14 (42)	7 (21)	0	5 (15)	0	0	8 (24)
D	3	0	0	0	0	0	0	3 (100)
E	18	0	0	0	0	0	8 (44)	10 (56)
F	6	0	1 (17)	0	0	0	0	5 (83)
A/C	1	0	1 (100)	0	0	0	0	0
B/C	1	0	0	0	0	0	0	1 (100)
Unclassified	12	2 (17)	2 (17)	0	0	2 (17)	0	6 (50)

NOTE. Data are no. (%) of strains, unless otherwise indicated.
[a] One strain was serotyped as the O/Y serogroup.
[b] One strain was serotyped as the A/G serogroup.

101; 77%), whereas most of the class B strains had the *cstII* (Asn51) genotype (49/66; 74%). FS-associated strains, however, were closely associated with the *cstII* (Asn51) genotype, irrespective of whether the LOS locus class was A or B; 8 (80%) of 10 class A strains and all 12 (100%) class B strains had the *cstII* (Asn51) genotype. This suggests that *cstII* polymorphism, and not LOS locus class, is critical for the development of FS.

Ganglioside-like LOS and antiganglioside autoantibodies. On the whole, reactivity to anti-GM1 mAb was increased in class A and C strains, and that of anti-GD1a mAb was increased only in class A strains (figure 2); this is indicative of a difference in sialyltransferase substrate specificity between classes A *(cstII)* and C *(cstIII)* [25]. Some class B strains had high reactivity to anti-GM1 and anti-GD1a mAbs, but the median optical density was low. Reactivity to anti-GQ1b/GT1a mAb was high overall in class B strains and in some class A strains, but it was not high in strains with loci of other classes.

We defined ganglioside epitopes as being present on LOSs when the OD of mAb in the ELISA was ⩾0.2. There was an obvious difference in ganglioside epitopes between strains with the class A, B, or C locus and strains with the class D, E, or F locus, with epitopes being frequent in the former group and absent in the latter group. For example, the GM1 epitope was judged to be present in 80% of class A strains, 26% of class B strains, and 64% of class C strains but in none of the strains with the class D, E, or F locus. Furthermore, class A strains regularly expressed both the GM1 and has the GD1a epitope, whereas class C strains expressed only the GM1 epitope; the GD1a epitope was detected in 76% of class A strains but in only 3% of class C strains. The GQ1b/GT1a epitope was present in 37% and 50% of class A or B strains, respectively; no strain with the class C, D, E, or F locus had this epitope. Notably, several unclassified strains also had ganglioside-mimicking LOS (figure 2), and this is indicative of an unknown sialyltransferase gene being present at an unclassified locus.

Patients with class A or C strains often were positive for IgG autoantibodies against GM1 (72% and 75%, respectively). Interestingly, the frequency of anti-GD1a IgG autoantibodies was higher in patients with class A strains (51%) than in patients with class C strains (33%). These data agree with the finding that anti-GD1a mAb bound to class A LOS but not to class C LOS. In contrast, patients with class B strains more commonly had anti-GQ1b IgG autoantibodies (44%) than anti-GM1 IgG autoantibodies (25%) or anti-GD1a IgG autoantibodies (25%). Anti-GQ1b IgG autoantibodies were rarely detected in patients with class A (14%) or class C (0%) strains. These data agree with the finding that anti-GQ1b/GT1a mAb regularly bound to class B LOS.

LOS structure and glycosyltransferase genes of strain CF90-26. Because the above data suggested the importance of the GM1 and GD1a epitopes on class A strains, we investigated in detail the LOS structure and gene sequences of the *cstII*, *cgtA*, and *cgtB* glycosyltransferase genes. Elsewhere, we showed that *C. jejuni* strain CF90-26 (a serotype HS:19 class A strain from a patient with GBS who had high anti–GM1 IgG autoantibody titers), which was used in the present study, has a GM1-like structure, on the basis of nuclear magnetic resonance analysis [16], and has the GD1a epitope, on the basis of thin-layer chromatography with immunostaining [38]. CE-ESI-MS analysis of an *O*-deacylated LOS sample from *C. jejuni* strain CF90-26 yielded various masses, and the predominant species was $[M\text{-}4H]^{4-}$ (3645 Da). The differences in observed masses (table 4) were due to lipid A variation, as well as to the presence or absence of a terminal sialic acid (in addition to the sialic acid that is present on the inner galactosyl residue). CE-ESI-MS analysis showed that the absence of the terminal sialic acid resulted in a GM1 mimic, and its presence in a GD1a mimic (figure 3) provided evidence that CF90-26 has both GM1-like and GD1a-like LOSs. The LOS biosynthesis gene sequence in strain CF90-26 (GenBank accession number AY661458) was

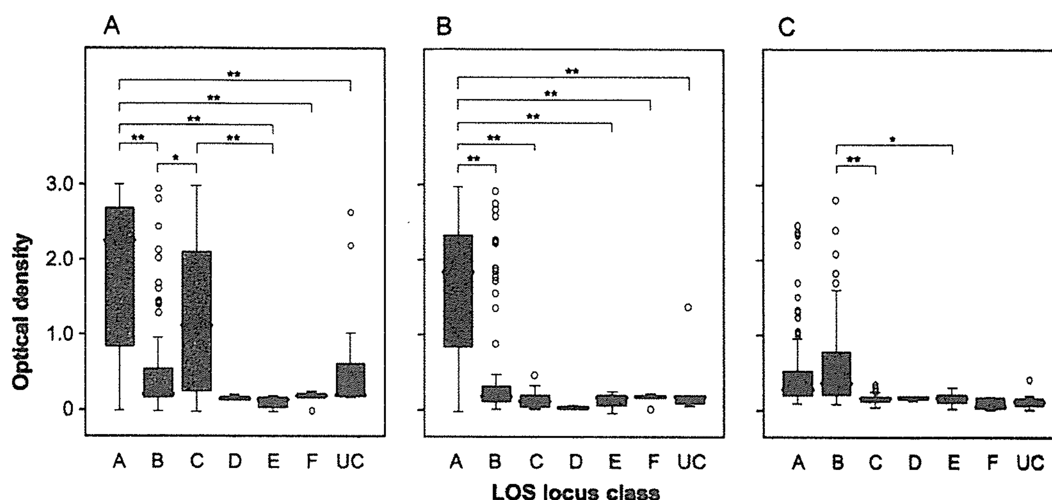

Figure 2. Box and whisker plot of *Campylobacter jejuni* lipo-oligosaccharide reactivity with anti-GM1 (GB2; *A*), anti-GD1a (GB1; *B*), and anti-GQ1b/GT1a (FS3; *C*) monoclonal antibodies in an ELISA. Strains are grouped by lipo-oligosaccharide (LOS) locus class (A–F and unclassified [UC]). Center lines denote medians, boxes denote 25%–75% percentiles, whiskers denote 10% and 90% percentiles, and white circles denote outliers. $^*P < .05$; $^{**}P < .01$ (Scheffé's test).

100% identical to the corresponding region in the *C. jejuni* HS:19 type strain (GenBank accession number AF167344), which also expresses a mixture of GM1 and GD1a mimics in its LOS outer core [17].

Risk factors for development of GBS. Because univariate analysis showed that class A strains were associated with GBS, we compared the features of GBS-associated and enteritis-associated class A strains. Differences remained significant between GBS-associated and enteritis-associated strains in the frequency of the HS:19 serotype, the frequency of *cstII* (Thr51), and LOS binding of anti-GM1 and anti-GD1a IgG autoantibodies (table 5). All HS:19 strains with the class A locus had the *cstII* (Thr51) genotype, except for the 2 GBS-associated strains (OH4382 and OH4384) that were obtained from siblings with GBS and external ophthalmoplegia [30]; these 2 strains were known to carry GD3-like or GT1a-like LOSs [17], as well as the GM1 epitope [39], all of which are present with the *cstII* (Asn51) genotype. Multiple logistic regression modeling was used to adjust the comparisons between GBS-associated and enteritis-associated strains for the class A locus, the HS:19 serotype, the *cstII* (Thr51) genotype, and GM1-like and GD1a-like LOSs. In that analysis, the difference remained significant for the HS:19 serotype (odds ratio [OR], 16.5 [95% confidence interval {CI}, 4.0–68.8]; $P < .001$) and the class A locus (OR, 5.6 [95% CI, 2.1–15.1]; $P = .001$).

DISCUSSION

We confirmed the finding of Godschalk et al. [15] that GBS is associated with the class A locus of *C. jejuni* and provided evidence of the first GBS-related *C. jejuni* characteristic that is common to strains from Asia and Europe. Moreover, we found that strains with the class A locus regularly express both the GM1 and the GD1a epitope on their LOSs; this unique LOS profile among *C. jejuni* strains results in an increased risk of producing anti-GM1 and anti-GD1a IgG autoantibodies and, therefore, developing GBS. Expression of the GM1 and GD1a epitopes in class A strains was enhanced in strains that were also serotype HS:19, and this expression was possibly dependent on the predominance of the *cstII* (Thr51) genotype in HS:19 strains. Of course, microbial properties alone do not sufficiently explain why an autoimmune response is triggered in only a minority of individuals with *C. jejuni* enteritis. Host susceptibility must be much more important. Previous attempts to find common host immunogenetic factors in patients with *C. jejuni* GBS, however, have had negative or conflicting results [40–44].

The class A locus is 11.5 kb and has 13 genes. A and B class loci have the same gene profile, except that the class B locus has *orf5II (cgtAII)*, which may be the result of duplication of *orf5I (cgtAI)* [27]. This raises the question as to why GBS-associated strains primarily have the class A locus. Our findings suggest that nucleotide sequence variation within genes is the answer. In fact, strains with the same LOS biosynthesis

Table 4. Lipid A variants and variable terminal sialic acids of an *O*-deacylated sample from *Campylobacter jejuni* strain CF90-26.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

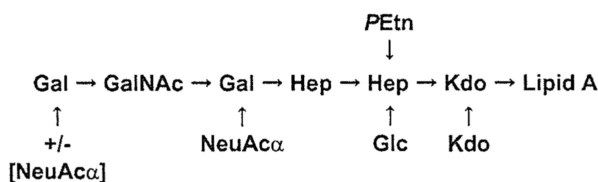

Figure 3. Proposed lipo-oligosaccharide outer core structures as determined on the basis of capillary electrophoresis–electrospray ionization mass spectrometric analysis of *O*-deacylated lipo-oligosaccharide samples from *Campylobacter jejuni* strain CF90-26. Gal, galactose; GalNAc, *N*-acetylgalactosamine; Hep, L-glycero-D-manno-heptose; Glc, glucose; Kdo, 3-deoxy-D-manno-2-octulosonic acid; NeuAc, *N*-acetylneuraminic acid; *P*Etn, phosphoethanolamine.

gene content have been shown to express diverse ganglioside mimics because of phase variation, a single nucleotide deletion, or single or multiple nucleotide mutations of LOS biosynthesis genes [27]. As we reported in the present study, class A strains regularly had *cstII* (Thr51), which is indicative that this genotype is a feature of class A strains and is related to the onset of GBS, because *cstII* (Thr51) encodes an enzyme with α-2,3-sialyltransferase activity that transfers a single α-2,3-sialic acid to both the inner and the terminal galactose residues, resulting in GM1 and GD1a mimics (figure 1) [27].

Candidate enzyme functions of the class A locus have been proposed, and most of them seem to be essential for the biosynthesis of ganglioside-like LOS [28, 45–47]. This suggests that the content of a single gene of *C. jejuni* is insufficient for the development of GBS; the entire gene content of the class A locus is essential. Nachamkin et al. [19] reported that the 3 glycosyltransferase genes that are necessary for ganglioside-like LOS biosynthesis—*cstII/cstIII* (sialyltransferase), *cgtA* (β-1,4-*N*-acetylgalactosaminyltransferase), and *cgtB* (β-1,3-galactosyltransferase)—[28] are more often present in GBS-associated strains than in enteritis-associated strains. These 3 genes are present in class A, B, and C loci [27], which agrees with our finding that 96% of GBS-associated strains had the class A, B, or C locus. This strongly suggests that the class A, B, or C locus is required to induce the development of GBS, although the class C locus is a much weaker risk factor than is the class A locus.

Interestingly, class A strains regularly express GM1-like and GD1a-like LOSs, whereas class C strains express GM1-like LOS only. GM1 and GD1a are candidate target antigens for the circulating autoantibody [10]. Our results suggest that multiple ganglioside mimicry is more effective for developing GBS than is single ganglioside mimicry. This disagrees with the findings of Nachamkin et al. [19] that the expression of the GD1a epitope alone is associated with GBS. The reason for this discrepancy is not known. We have shown in the present study, however, that patients from whom class A strains were isolated often had both anti-GM1 and anti-GD1a IgG autoantibodies; in addition, the inoculation of rabbits with CF90-26 LOS (which has GM1 and GD1a mimics) caused acute motor axonal neuropathy that was accompanied by anti-GM1 antibodies, not by anti-GD1a antibodies [14]. The assumption that GM1 mimicry, in addition to GD1a mimicry, is responsible for the development of GBS, therefore, is reasonable.

On the basis of chemical analysis, the coexistence of the GM1 and GD1a epitopes on the outer core of the LOS of the HS:19 serotype reference strain has been reported [17]. Elsewhere, we showed by use of mAb immunostaining that both epitopes were present in a GBS-associated HS:19 strain (CF90-26) [38], and we confirmed that finding in the present study by use of mass-spectrometric analysis. Furthermore, the nucleotide sequence of the 6.1-kb PCR product that included *cstII*, *cgtA*, and *cgtB* (as well as downstream and upstream sequences) was identical to the corresponding region in the *C. jejuni* HS:19 type strain that also expresses mixed GM1 and GD1a mimics [17]. This finding confirms that the DNA sequence, as well as

Table 5. Comparison of Guillain-Barré syndrome–associated and enteritis-associated *Campylobacter jejuni* strains with the class A lipo-oligosaccharide (LOS) locus.

Characteristic	Guillain-Barré syndrome–associated strains (n = 72)	Enteritis-associated strains (n = 17)	2-tailed P^a	OR (95% CI)
Serotype HS:19	66 (92)	6 (35)	<.001	20.2 (5.5–73.9)
cstII polymorphism				
Thr51	66 (92)	10 (58)	.002	7.7 (2.1–27.6)
Asn51	6 (8)	7 (41)	.002	0.13 (0.036–0.47)
Median OD ± SD of mAb to LOS[b]				
GM1-like LOS	2.402 ± 0.897	1.106 ± 1.107	.002	
GD1a-like LOS	1.976 ± 0.830	0.558 ± 1.215	.03	
GQ1b/GT1a-like LOS	0.086 ± 0.431	0.126 ± 0.500	.11	

NOTE. Data are no. (%) of strains, unless otherwise indicated. CI, confidence interval; mAb, monoclonal antibody; OR, odds ratio.

[a] Fisher's exact test or Mann-Whitney *U* test.

[b] ODs were measured by an ELISA with mAbs GB2 (GM1), GB1 (GD1a), and FS3 (GQ1b/GT1a).

the makeup of glycosyltransferase genes, is responsible for determining the type of ganglioside mimic that is formed on LOSs.

In the present study, we found that most of the FS-associated strains had the class A or B locus, which supports the finding of van Belkum et al. [48] that *cstII* was present in all 8 strains with GQ1b-like LOS that they tested. Godschalk et al. [15] found that all 4 of the FS-associated strains that they tested had the class B locus, whereas a significant number of FS-associated strains that we tested in the present study had the class A locus. Furthermore, the differences between the class A and the class B locus were not important in our FS-associated strains, whereas the *cstII* (Asn51) genotype was critical. *cstII* (Asn51) has both α-2,3- and α-2,8-sialyltransferase activities [27], which are essential for transferring the disialyl moiety to the outer core of LOS, thereby mimicking GQ1b and GT1a gangliosides. Our findings suggest that the ganglioside-like LOS synthesis gene contents of *cstII, cgtA,* and *cgtB,* which are common to the class A and B loci, are important for triggering an autoimmune response and that *cstII* polymorphism is the determinant of autoantibody reactivity and neurological presentations in GBS and FS.

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Isolated abducens nerve palsy as a regional variant of Guillain–Barré syndrome

Muneto Tatsumoto, Masaaki Odaka, Koichi Hirata, Nobuhiro Yuki *

Department of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi, 321-0293, Japan

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Abstract

The authors reviewed clinical profiles and laboratory findings for 100 cases of abducens nerve paresis without impairment of the other cranial nerves, limb weakness, and ataxia throughout the clinical course. Review of the medical records of 9300 patients referred to our neuoroimmunological laboratory for serum anti-ganglioside antibody testing. Information was obtained from each primary physician on symptoms of preceding infection; initial symptoms; neurological signs during the illness; the clinical course; treatment provided; and outcome. Isolated abducens nerve paresis was present in 100 patients and bilateral paresis in 29. Tentative diagnoses made by the primary physicians on request of anti-ganglioside antibody testing were abducens nerve palsy ($n = 68$), Fisher syndrome ($n = 14$), acute ophthalmoparesis without ataxia ($n = 14$). Symptoms of infection anteceded in 63. Tendon reflexes were absent or decreased in 27. Distal paresthesias were experienced by seven. Serum anti-GQ1b antibody was positive in 25. These findings suggest that some cases of isolated abducens nerve palsy can be categorized as a regional variant of Guillain–Barré syndrome or mild form of Fisher syndrome.
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Keywords: Isolated abducens nerve paresis; Guillain–Barré syndrome; Fisher syndrome; Anti-GQ1b antibody

1. Introduction

Most cases of isolated abducens nerve palsy are attributable to vascular disorders, trauma, or tumors, as well as to palsy after infection [1]. A case of "sixth nerve paresis with paresthesias" was described as a regional Guillain–Barré syndrome (GBS) variant, as were cases of pharyngeal–cervical–brachial weakness; paraparesis; bifacial weakness with paresthesias; and lumbar polyradiculopathy [2,3]. The patient in the original case described by Ropper had sinusitis and showed *unilateral* sixth nerve paresis, minimal facial weakness, mild proximal limb weakness, areflexia in the legs, and paresthesias in the toes and feet, whereas the other patients showed *bilateral* weakness [2]. Cardinal clinical features of the former were an antecedent illness and areflexia as well as sixth nerve paresis and acral paresthesias. We therefore searched for patients who showed abducens nerve paresis without impairment of the other cranial nerves and had neither limb weakness nor ataxia throughout the clinical course. Whether some cases of isolated abducens nerve paresis should be categorized as a regional variant of GBS is discussed.

2. Patients and methods

2.1. Patients and clinical data

One of the authors (M.T.) reviewed the medical records of 9300 patients referred to our neuoroimmunological laboratory for serum anti–ganglioside antibody testing from hospitals throughout Japan between May 1994 and January 2004. The patients' clinical features were reviewed by application form as well as medical records at both admission and discharge obtained from each primary physician. When they did not contain adequate information, questionnaires

* Corresponding author. Tel.: +81 282 86 1111x2360; fax: +81 282 86 5884.
E-mail address: yuki@dokkyomed.ac.jp (N. Yuki).

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were sent to the physicians by fax. Patients were selected who showed abducens nerve paresis without impairment of the other cranial nerves and had neither limb weakness nor ataxia during the clinical course of the illness. Patients, who had diabetes mellitus, vascular disorders, trauma, or tumors, as well as to palsy after infection, were excluded in this study. Information was obtained from each primary physician on symptoms of preceding infection; initial symptoms; neurological signs during the illness; the clinical course; CSF, brain MRI, and motor nerve conduction study findings; treatment provided; and outcome. Presence of symptoms of preceding infection was defined as a history of symptoms of infection within four weeks before onset of neurological symptoms. CSF samples were tested at the various hospitals. Albuminocytological dissociation was defined as a raised protein concentration (more than 45 mg/dl) associated with a count of ten or fewer mononuclear leukocytes per microliter.

2.2. Anti-GQ1b IgG antibody assay

Serum IgG antibody to GQ1b was measured by an enzyme-linked immunosorbent assay as reported elsewhere [4]. Serum was judged positive when the absorbance value was 0.1 or more at the dilution of 1 : 500. Specificity of this test was investigated in serum samples from 1549 patients who had been referred between January and December 2003.

3. Results

3.1. Specificity of anti-GQ1b IgG antibody test

Of 1549 consecutive patients, anti-GQ1b IgG was positive in 205: Fisher syndrome (FS, $n=133$), acute ophthalmoparesis without ataxia ($n=18$), ataxic GBS ($n=12$), Bickerstaff's brainstem encephalitis ($n=11$), GBS ($n=10$), GBS with ophthalmoplegia ($n=8$), pharyngeal–cervical–brachial weakness ($n=8$), overlapping FS and GBS ($n=4$), and isolated abducens nerve palsy ($n=2$) [5–7]. Because autoantibody was negative in all patients with amyotrophic lateral sclerosis ($n=33$), multiple sclerosis ($n=3$), and myasthenia gravis ($n=10$), the specificity of the test was 100% for the cutoff point in our assay. One hundred thirty-three of the 149 patients with typical FS had anti-GQ1b IgG, a sensitivity of 89% in FS.

Table 1
Clinical profiles of patients with isolated abducens nerve palsy

	$n=100$	
Median age (range)	42 (1–81)	
Sex (M/F)	51/49	
Antecedent infectious illness	(%)	
Upper respiratory tract infection	38 (38)	
Diarrhea	14 (14)	
Headache	19 (19)	
Fever	27 (27)	
Neurological findings		
Cranial nerve impairment		
Bilateral ophthalmoplegia	29 (29)	
Tendon reflex absent or decreased	27 (27)	
Distal paresthesias	7 (7)	
Labolatory findings		
Anti-GQ1b IgG antibody positive	25 (25)	
		Available data
CSF albuminocytological dissociation	10 (16)	$n=64$
MRI abnormality	1 (3)	$n=35$
Nerve conduction study abnormality	5 (21)	$n=24$

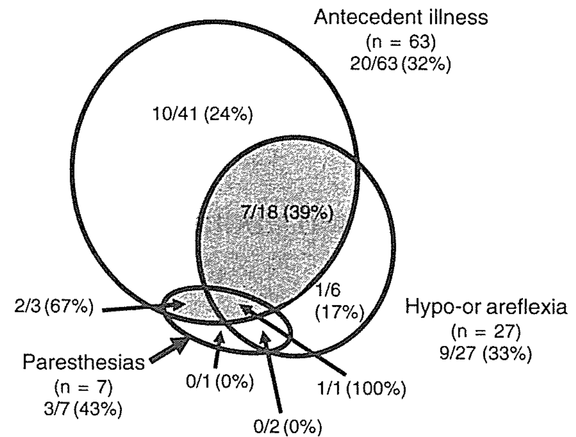

Fig. 1. Anti-GQ1b IgG-positive patients with isolated abducens nerve palsy ($n=100$). Positive anti-GQ1b serology percentages are higher in those who had both antecedent illness and distal paresthesias and in those who had both an antecedent illness and hypo- or areflexia (gray areas).

3.2. Clinical features and laboratory data

Isolated abducens nerve paresis was present in 100 patients and bilateral paresis in 29 (Table 1). Tentative diagnoses made by the primary physicians on request of anti-ganglioside antibody testing were abducens nerve palsy ($n=68$), FS ($n=14$), acute ophthalmoparesis without ataxia ($n=14$), Bickerstaff's brainstem encephalitis ($n=2$), atypical FS ($n=1$), and GBS ($n=1$). Of 100 patients, tendon reflexes were absent or decreased in 27, distal paresthesias were experienced by seven, and symptoms of infection were antecedent in 63 (Table 1). Upper respiratory infectious symptoms proceeded in 38 patients and diarrhea in 14. Only one patient had an antecedent illness, distal paresthesias, and areflexia.

Of the 100 patients with isolated abducens nerve palsy, anti-GQ1b IgG was present in 25 (25%) of the 100; 20 (32%) of the 63 patients with an antecedent illness; 9 (33%) of the 27 patients with hypo- or areflexia; and 3 (43%) of the 7 patients with distal paresthesias. Percentages of positive anti-GQ1b serology were high in the four patients who had both an antecedent illness and distal paresthesias, and in the 19 patients who had both an antecedent illness and hypo- or areflexia (Fig. 1). CSF albuminocytological dissociation was present in 10 (16%) of the 64 tested; 10 (16%) of the 64

patients with an antecedent illness; 3 (17%) of the 18 patients with hypo- or areflexia; and 2 (33%) of the 6 patients with distal paresthesias. Brain MRI information was available for 35 patients. Abducens nerve enhancement was found in only one patient. Although detailed information on motor nerve conduction studies was available for 24 patients, only five had abnormal findings, the predominant process being axonal degeneration in two and demyelination in three.

Steroids were given to 19 patients, intravenous immunoglobulin (IVIg) to five, plasmapheresis to three, a combination of steroids and plasmapheresis to two, and plasmapheresis followed by IVIg to one. Six months after disease onset, all 100 patients given or not given specific treatment had returned to normal activities.

4. Discussion

Anti-GQ1b IgG antibody identifies a cluster of closely related syndromes that have in common the presence of external ophthalmoplegia or cerebellar-like ataxia [7]. This was confirmed in our consecutive samples. The autoantibody is not a simple serological marker, rather it is considered an effector molecule that produces the syndromes subsequent to infection by microorganisms carrying a GQ1b epitope [7]. We found one case of "sixth nerve paresis with paresthesias" associated with an antecedent illness and hyporeflexia. Anti-GQ1b IgG was detected in the patient, a strong indication that this regional variant is an autoimmune disorder similar to FS.

With and without anti-GQ1b antibody, as stated by Ropper [2], isolated abducens nerve palsy associated with antecedent illness, acral paresthesias, hypo- or areflexia, and CSF albuminocytological dissociation should be categorized as a regional variant of GBS. Twenty five percentages of the 100 patients in this study, with or without an antecedent illness, areflexia, or paresthesias had anti-GQ1b IgG, indicative that some cases of isolated abducens nerve palsy are due to autoimmune etiology. These cases could be called "acute ophthalmoparesis without ataxia" or an incomplete form of "sixth nerve paresis with paresthesias" [2,6]. In other words, such cases should be categorized as a regional variant of GBS. The percentage of positive anti-GQ1b serology was higher when patients complained of both an antecedent illness and paresthesias, or of both antecedent illness and hypo- or areflexia. When such patients are seen, physicians should bear in mind the possibility of a regional variant of GBS. Interestingly, the primary physicians had done so, and had sent us serum samples for anti-GQ1b IgG testing.

A cellular CSF with a raised protein concentration is a valuable laboratory finding in autoimmune-mediated abducens nerve palsy, but the frequency found was low. We recently reported CSF albuminocytological dissociation in 25% of 91 FS patients and 44% of 195 GBS patients during the first week of illness [8]. During subsequent weeks, it increased to 84% in FS and 75% in GBS. In contrast, anti-GQ1b IgG was positive in 81% of 73 FS patients during the first week of illness. These findings suggest that when physicians encounter patients who have acute onset, isolated abducens nerve palsy anti-GQ1b serology is as useful for diagnosing autoimmune-mediated abducens nerve palsy as is serial lumbar punctures.

Some patients with isolated abducens nerve palsy respond favorably to plasmapheresis and IVIg [6], but a definitive therapy has yet to be established. The present study supported the idea of Ropper [2] that the condition is a regional variant of GBS. In contrast, corticosteroids alone are not effective in GBS [9]. Therefore, corticosteroids may not be recommended in the treatment of isolated abducens nerve palsy. All 50 FS patients given or not given specific treatments were almost free of ataxia and had returned to normal activities six months after onset [10]. Therefore neither plasmapheresis nor IVIg may be required for treatment of patients with autoimmune-mediated isolated abducens nerve palsy, although either may facilitate a faster recovery. Most of our patients who received neither treatment showed complete remission 6 months after onset.

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