of microbial mimics of the target antigen; and (4) reproduction of the disease in an animal model. As reviewed here, GBS subsequent to *C. jejuni* enteritis fulfills all four criteria and provides the first verification that molecular mimicry is a cause of human autoimmune diseases.

Epidemiological association of *C. jejuni* with Guillain–Barré syndrome

An epidemiological study in England established the relationship between GBS and antecedent *C. jejuni* infection [3]. Serological assessments are useful for epidemiological studies, but there are neither standard methodologies as to which antigens to use nor standards of judgement. Because *C. jejuni* isolation is the standard for the diagnosis of this infection, epidemiological studies of a large number of *C. jejuni* isolates from GBS patients are required. Epidemiological features of more than 100 Japanese patients with GBS, from whom *C. jejuni* had been isolated, have been reported [4•]. *C. jejuni*-isolated GBS peaked in 10–30-year-old individuals, and the male : female ratio was 1.7 : 1. The dominance of young adult, male patients with *C. jejuni*-isolated GBS may be related to the preponderance of young adult and male patients who had had *C. jejuni* enteritis. The median latent period between antecedent symptoms and the onset of neuropathy was 10 days. Diarrhoea or abdominal pain preceded symptoms in 90% of those studied, and was often accompanied by fever.

On the basis of electrodiagnostic and pathological criteria, GBS is divided into acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN). Whereas electrodiagnostic studies have shown that *C. jejuni* infection is associated significantly with primary axonal dysfunction, the relationship between it and neurophysiology has been the subject of debate. To investigate whether *C. jejuni* infection does elicit AIDP, serial electrodiagnostic studies were conducted on *C. jejuni*-positive GBS patients [5•]. The presence of antecedent *C. jejuni* infection was determined by strict criteria; a positive *C. jejuni* serology and a history of diarrhoeal illness within the previous 3 weeks. Based on the electrodiagnostic criteria, 22 *C. jejuni*-positive patients were classified as having AMAN ($n = 16$, 73%), AIDP ($n = 5$, 23%), or were unclassified ($n = 1$) in the first studies. The five *C. jejuni*-positive patients with the AIDP pattern showed prolonged motor distal latencies but rapid normalization within 2 weeks. Eventually all showed the AMAN pattern. In contrast, patients with cytomegalovirus or Epstein–Barr virus-related AIDP had progressive increases in distal latencies during 8 weeks after onset. Patients with *C. jejuni*-related GBS had a transient slowing of nerve conduction that mimicked demyelination, but *C. jejuni* infection did not produce AIDP.

Autoantibodies against gangliosides in acute motor axonal neuropathy

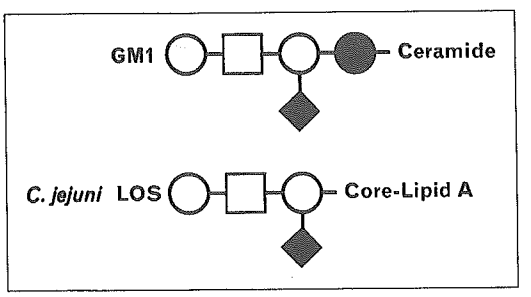
Autoantibodies against self-gangliosides are present in patients with GBS [6]. IgG antibodies to GM1 and GD1a are associated with AMAN or *C. jejuni* infection. Serial electrodiagnostic studies were performed on GBS patients who had IgG antibodies to either ganglioside [7]. Besides the simple axonal degeneration pattern, those patients showed transient conduction slowing/a block in distal or proximal nerve segments, mimicking demyelination, but their antiganglioside antibodies were not associated with AIDP.

C. jejuni mimic of gangliosides

Lipo-oligosaccharide is a major cell-surface structure in *C. jejuni* that is recognized by the host. A *C. jejuni* strain (CF90-26) isolated from an AMAN patient carrying anti-GM1 IgG antibody expressed an oligosaccharide structure [Gal β1–3 GalNAc β1–4 (NeuAc α2–3) Galβ], which protruded from the lipo-oligosaccharide core (Fig. 1) [8]. This terminal structure was identical to that of the terminal tetrasaccharide of the GM1 ganglioside. Another *C. jejuni* strain (16971.94GSH) isolated from a patient with GBS carried a GM1-like lipo-oligosaccharide, but neither antiganglioside antibodies nor electrodiagnosis was investigated [9]. A *C. jejuni* strain (ATCC43446) from an enteritis patient also had GM1 and GD1a-like lipo-oligosaccharides [10].

C. jejuni strains (OH4382 and OH4384) isolated from two patients with GBS, respectively, carried GD3 and GT1a-like lipo-oligosaccharides [10]. Both patients had external ophthalmoplegia, and one was comatose [11]. Overlapping GBS and Fisher syndrome and overlapping GBS and Bickerstaff's brainstem encephalitis, respectively, were diagnosed in these patients according to our diagnostic criteria [12]. Whether the patients carried anti-GQ1b IgG antibody was not tested, but inoculations of the lipo-oligosaccharides of both *C. jejuni* strains induced anti-GQ1b antibodies in mice [13].

Figure 1. Molecular mimicry of GM1 ganglioside and *Campylobacter jejuni* lipo-oligosaccharide



LOS, Lipo-oligosaccharide. ○ Galactose; ▪ *N*-Acetylgalactosamine; ◆ *N*-Acetylneuraminic acid; ● Glucose.

Animal models of acute motor axonal neuropathy

Gangliosides extracted from bovine brain tissue have been widely used in western Europe and South America as therapeutic agents for various neurological disorders. After receiving bovine brain ganglioside, some patients developed AMAN, and anti-GM1 IgG antibody was detected in them [14]. An AMAN model was established by sensitization of Japanese white rabbits with a bovine brain ganglioside mixture that included GM1 or with an isolated GM1 [15]. The rabbits developed high anti-GM1 IgG antibody titres, then flaccid limb weakness of acute onset with a monophasic course. Pathological findings in their peripheral nerves showed predominant Wallerian-like degeneration with neither lymphocytic infiltration nor demyelination. IgG was deposited on the axons of the ventral roots, internodal axolemmas, and nodes of Ranvier. Cauda equina and spinal nerve root specimens from the paralysed rabbits showed macrophage infiltration in the periaxonal space [16]. Surrounding myelin sheaths were almost intact. These findings correspond well with pathological findings for human AMAN [17,18]. This AMAN rabbit model was also reproducible in New Zealand white rabbits [19].

The most straightforward way to verify whether molecular mimicry between microbes and autoantigens causes GBS is to establish a GBS model by the immunization of animals with components of antecedent infectious agents. An AMAN model was established by the immunization of Japanese white rabbits with *C. jejuni* lipo-oligosaccharide bearing a GM1-like structure [20••]. On sensitization with this GM1-like lipo-oligosaccharide, rabbits developed high anti-GM1 IgG antibody titres and subsequent flaccid limb weakness. Their nerve roots had occasional macrophages in the periaxonal spaces surrounded by almost intact myelin sheaths. Axons of these nerve fibres showed various degrees of degeneration. Demyelination and remyelination were rare. These findings, compatible with the features of human AMAN, are evidence that rabbits inoculated with *C. jejuni* lipo-oligosaccharide constitute a valid AMAN model. This is the first definitive replica of a human autoimmune disease produced by immunization with the mimic of an infectious agent associated with epidemiological evidence of microbial infection.

Progress towards fulfilling the postulates of Witebsky *et al.*

Documentation of the autoimmune aetiology of a human disease is difficult. Witebsky *et al.* [21] developed standards for such documentation: (1) It should be possible to demonstrate circulating antibodies, active at body temperature in the serum of patients who have the disease, or cell-bound antibodies (in contemporary terms, this is cell-mediated immune reactivity); (2) The antigen against which the antibody is directed should be characterized or even isolated; (3) Antibodies should be produced against the same antigen in experimental animals; (4) Pathological changes that appear in the corresponding tissue from an actively sensitized animal should be similar or identical to those found in the human disease. Although a number of human diseases are believed to have an autoimmune aetiology or, at least, an autoimmune component, clearly only a few formally fulfil the criteria of Witebsky *et al.* [21]. Myasthenia gravis does, whereas other diseases such as multiple sclerosis do not.

As reported, GM1 is one of the autoantigens for IgG antibodies found in some patients with AMAN [6]. The IgG class of the autoantibody against GM1 was produced in experimental animals. Pathological changes that appeared in the peripheral nerves were identical to those in human AMAN [15,16]. More data are needed to satisfy the postulates of Witebsky *et al.* [21] involving the induction of clinical and pathological diseases by the passive transfer of anti-GM1 IgG antibody. This systemic transfer of anti-GM1 IgG antibody from AMAN patients did not induce paralysis in mice, but the autoantibody did block muscle action potentials in a rat muscle–spinal cord co-culture [20••].

Passive transfer attempted with systemically administered mouse anti-GD1a IgG antibody did not cause nerve fibre degeneration despite high circulating autoantibody titres [22••]. Half of a population of mice given an intraperitoneal implant of anti-GD1a IgG antibody-secreting hybridoma, however, developed a patchy, predominantly axonal neuropathy that affected a small number of nerve fibres. Mice implanted with the hybridoma had a leaky blood–nerve barrier compared with those that received systemically administered anti-GD1a IgG antibody. These findings suggest that in addition to circulating anti-ganglioside antibodies, such factors as antibody accessibility and nerve fibre resistance to antibody-mediated injury are important in the development of a neuropathy. *Ex vivo* nerve-muscle preparations from GD1a-overexpressing, GD3 synthase knockout mice were exposed to mouse anti-GD1a IgG antibody in the presence of a source of complement [23•]. Dense antibody and complement deposits were found only on presynaptic motor axons accompanied by severe ultrastructural damage and electrophysiological blockade of motor nerve terminal functions. Identical paralysing effects were observed on testing human anti-GD1a-positive sera.

C. jejuni genes associated with Guillain–Barré syndrome

An epidemiological study performed in Sweden showed that one of 3285 *C. jejuni* enteritis patients developed GBS [24]. Only a minority of those who have *C. jejuni* enteritis develop GBS, possibly because of both bacterial

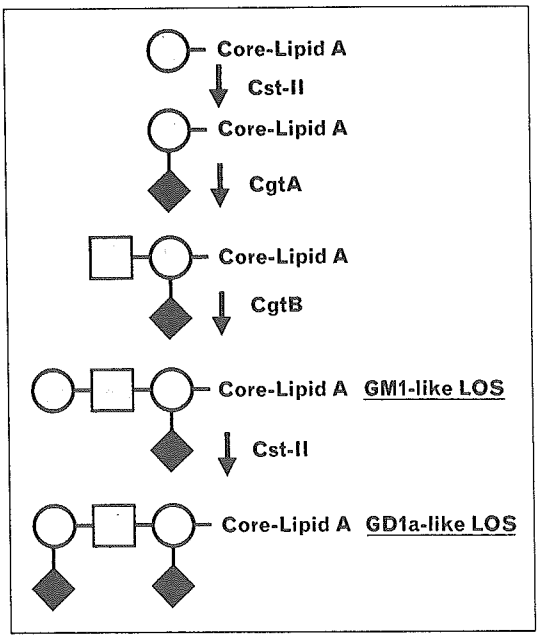
and host factors. Penner's serotyping revealed that in Japan and South Africa, respectively, HS:19 and HS:41 were more common in GBS isolates than in enteritis isolates [4•,25]. In contrast, no clustering of a specific serotype was found in GBS-related isolates in England and the Netherlands [3,26]. Previous studies failed to find a specific *C. jejuni* genotype for GBS [26,27].

As stated, ganglioside mimicry of *C. jejuni* lipo-oligosaccharide is a cause of GBS. Ganglioside-like lipo-oligosaccharide is synthesized by sialyltransferase Cst-II, *N*-acetylgalactosaminyl-transferase CgtA, and galactosyltransferase CgtB (Fig. 2) [28]. Compared with gastroenteritis-related isolates, GBS-related *C. jejuni* isolates have a strong association with the expression of GD1a mimicry [29]. The presence of some genes (*cst-II*, *cgtA*, and *cgtB*) involved in ganglioside mimicry is also associated with GBS-related strains. These lipo-oligosaccharide biosynthesis genes cluster at the lipo-oligosaccharide biosynthesis gene locus [30]. A specific type of gene locus, which includes these three genes, is associated with GBS and with the GM1-like lipo-oligosaccharide [31••].

The *cst-II* gene encodes an enzyme that transfers sialic acid to the lipo-oligosaccharide and another gene, possibly an enzyme, which synthesizes sialic acid [28]. Because both genes are involved in lipo-oligosaccharide sialylation, they are essential for ganglioside-like lipo-oligosaccharide synthesis. Mutants of *C. jejuni* that lack these genes have been made and analysed [31••]. Mass spectrometry analysis identified a mixture of GM1 and GD1a-like structures in wild-type *C. jejuni* strains isolated from GBS patients. The mutants expressed such non-sialylated structures as asialo-GM3, asialo-GM2, and asialo-GM1-like lipo-oligosaccharides. The knockout mutants, unlike the wild types, had decreased reactivity to the sera of GBS patients. GM2/GD2 synthase knockout mice, which lack GM1 and GD1a, are immune-naive hosts that can be used to obtain high-titre antiganglioside antibody responses. Immunization with the wild-type strain induced an anti-GD1a IgG antibody response in these mice, whereas immuniztion with the mutant strains did not. This shows that the genes involved in lipo-oligosaccharide sialylation are essential for the induction of antiganglioside antibodies.

In contrast, no host susceptibility genes associated with the development of GBS have yet been identified. Lipopolysaccharide receptors CD14 and Toll-like receptor 4 are important in antigen presentation and intracellular signaling, but the functional polymorphisms in *CD14* and *TLR4* are not associated with susceptibility to *C. jejuni*-associated GBS [32]. Fas polymorphisms are associated with the presence of antiganglioside antibodies in GBS [33].

Figure 2. Enzymatic synthesis of GM1 and GD1a-like lipo-oligosaccharides



○ Galactose; ▣ *N*-Acetylgalactosamine; ◆ *N*-Acetylneuraminic acid. LOS, Lipo-oligosaccharide.

Pathogenesis of acute motor axonal neuropathy subsequent to *C. jejuni* enteritis

C. jejuni, which simultaneously carries the lipo-oligosaccharide biosynthesis genes *cst-II*, *cgt-A*, and *cgt-B*, may express GM1 or GD1a-like lipo-oligosaccharide on its cell surfaces. Infection by such *C. jejuni* strains could induce anti-GM1 or anti-GD1a IgG production in patients who have certain immunogenetic backgrounds. Anti-GM1 or anti-GD1a IgG antibody would bind to GM1 or GD1a at the nodes of Ranvier. Complements would be deposited, and the nodes would lengthen. Macrophages would be recruited to some nodes as a result of complement activation. These local events at the nodes would disrupt the junctional complexes between the axolemma and myelin terminal loops at the paranodes. As a result, IgG and complement would enter the periaxonal space at the internodes. Complement activation within the periaxonal space would recruit macrophages. Complements with and without macrophages would then induce axonal degeneration of the motor fibres.

Conclusion

Convincing evidence has shown that molecular mimicry between GM1 and *C. jejuni* is a cause of AMAN. Bacterial lipo-oligosaccharide biosynthesis genes appear to be essential for the production of antiganglioside antibodies

and the subsequent development of AMAN. The susceptibility genes of the host have now to be clarified.

Acknowledgements

Supported in part by a grant-in-aid for Scientific Research (B) (KAKENHI 14370210) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Health Science Research Grant (Research on Brain Science) from the Ministry of Health and Welfare of Japan; a grant from the Human Frontier Science Program (RGP0038/2003-C).

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1 Jacobs BC, Rothbarth PH, van der Meché FGA, *et al.* The spectrum of antecedent infections in Guillain–Barré syndrome: a case–control study. Neurology 1998; 51:1110–1115.

2 • Ang CW, Jacobs BC, Laman JD. The Guillain–Barré syndrome: a true case of molecular mimicry. Trends Immunol 2004; 25:61–66.
This review shows that GBS is an excellent paradigm and an attractive model for the clarification of both the host and microbial aspects of molecular mimicry.

3 Rees JH, Soudain SE, Gregson NA, Hughes RAC. *Campylobacter jejuni* infection and Guillain–Barré syndrome. N Engl J Med 1995; 333:1374–1379.

4 • Takahashi M, Koga M, Yokoyama K, Yuki N. Epidemiology of *Campylobacter jejuni* isolated from patients with Guillain–Barré and Fisher syndromes in Japan. J Clin Microbiol 2005; 43:335–339.
Because there are many fewer false-positives in culture-confirmed GBS than in serology-confirmed GBS, this report provides precious information on the epidemiological features of GBS subsequent to *C. jejuni* enteritis.

5 • Kuwabara S, Ogawara K, Misawa S, *et al.* Does *Campylobacter jejuni* infection elicit "demyelinating" Guillain–Barré syndrome? Neurology 2004; 63:529–533.
This study shows that both strict infection serology criteria and sequential neurophysiological studies are crucial for this type of research.

6 Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain 2002; 125:2591–2625.

7 Hiraga A, Kuwabara S, Ogawara K, *et al.* Patterns and serial changes in electrodiagnostic abnormalities of axonal Guillain–Barré syndrome. Neurology 2005; 64:856–860.

8 Yuki N, Taki T, Inagaki F, *et al.* A bacterium lipopolysaccharide that elicits Guillain–Barré syndrome has a GM1 ganglioside-like structure. J Exp Med 1993; 178:1771–1775.

9 Prendergast MM, Lastovica AJ, Moran AP. Lipopolysaccharides from *Campylobacter jejuni* O:41 strains associated with Guillain–Barré syndrome exhibit mimicry of GM1 ganglioside. Infect Immun 1998; 66:3649–3655.

10 Aspinall GO, McDonald AG, Pang H, *et al.* Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with the Guillain–Barré syndrome. Biochemistry 1994; 33:241–249.

11 Yuki N, Tsujino Y. Familial Guillain–Barré syndrome subsequent to *Campylobacter jejuni* enteritis. J Pediatr 1995; 126:162.

12 Odaka M, Yuki N, Hirata K. Anti-GQ1b IgG antibody syndrome: clinical and immunological range. J Neurol Neurosurg Psychiatry 2001; 70: 50–55.

13 Goodyear CS, O'Hanlon GM, Plomp JJ, *et al.* Monoclonal antibodies raised against Guillain–Barré syndrome-associated *Campylobacter jejuni* lipopolysaccharides react with neuronal gangliosides and paralyze muscle–nerve preparations. J Clin Invest 1999; 104:697–708.

14 Illa I, Ortiz N, Gallard E, *et al.* Acute axonal Guillain–Barré syndrome with IgG antibodies against motor axons following parenteral gangliosides. Ann Neurol 1995; 38:218–224.

15 Yuki N, Yamada M, Koga M, *et al.* Animal model of axonal Guillain–Barré syndrome induced by sensitization with GM1 ganglioside. Ann Neurol 2001; 49:712–720.

16 Susuki K, Nishimoto Y, Yamada M, *et al.* Acute motor axonal neuropathy rabbit model: immune attack on nerve root axons. Ann Neurol 2003; 54:383–388.

17 McKhann GM, Cornblath DR, Griffin JW, *et al.* Acute motor axonal neuropathy: a frequent cause of acute flaccid paralysis in China. Ann Neurol 1993; 33:333–342.

18 Hafer-Macko C, Hsieh S-T, Li CY, *et al.* Acute motor axonal neuropathy: an antibody-mediated attack on axolemma. Ann Neurol 1996; 40:635–644.

19 Susuki K, Nishimoto Y, Koga M, *et al.* Various immunization protocols for an acute motor axonal neuropathy rabbit model compared. Neurosci Lett 2004; 368:63–67.

20 •• Yuki N, Susuki K, Koga M, *et al.* Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipo-oligosaccharide causes Guillain–Barré syndrome. Proc Natl Acad Sci U S A 2004; 101:11404–11409.
This study provides convincing evidence that molecular mimicry is a cause of GBS subsequent to *C. jejuni* enteritis. Moreover, it is the first to verify that molecular mimicry is a cause of human autoimmune disease.

21 Witebsky E, Rose NR, Terplank K, *et al.* Chronic thyroiditis and autoimmunization. JAMA 1957; 164:1439–1447.

22 •• Sheikh KA, Zhang G, Gong Y, *et al.* An anti-ganglioside antibody-secreting hybridoma induces neuropathy in mice. Ann Neurol 2004; 56:228–239.
This in-vivo investigation shows that anti-GD1a IgG antibody can cause peripheral neuropathy and that blood–nerve barrier dysfunction is required to cause it.

23 • Goodfellow JA, Bowes T, Sheikh K, *et al.* Overexpression of GD1a ganglioside sensitizes motor nerve terminals to anti-GD1a antibody-mediated injury in a model of acute motor axonal neuropathy. J Neurosci 2005; 25:1620–1628.
This elegant ex-vivo study shows that anti-GD1a IgG antibody can cause axonal neuropathy at motor nerve terminals where the blood–nerve barrier is deficient.

24 McCarthy N, Giesecke J. Incidence of Guillain–Barré syndrome following infection with *Campylobacter jejuni*. Am J Epidemiol 2001; 153:610–614.

25 Lastovica AJ, Goddard EA, Argent AC. Guillain–Barré syndrome in South Africa associated with *Campylobacter jejuni* O:41 strains. J Infect Dis 1997; 176(Suppl. 2):S139–S143.

26 Endtz HP, Ang CW, van Den Braak N, *et al.* Molecular characterization of *Campylobacter jejuni* from patients with Guillain–Barré and Miller Fisher syndromes. J Clin Microbiol 2000; 38:2297–2301.

27 Leonard EE II, Tompkins LS, Falkow S, Nachamkin I. Comparison of *Campylobacter jejuni* isolates implicated in Guillain–Barré syndrome and strains that cause enteritis by a DNA microarray. Infect Immun 2004; 72:1199–1203.

28 Gilbert M, Brisson JR, Karwaski MF, *et al.* Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384: identification of the glycosyltransferase genes, enzymatic synthesis of model compounds, and characterization of nanomole amounts by 600-MHz ^{1}H and ^{13}C NMR analysis. J Biol Chem 2000; 275:3896–3906.

29 Nachamkin I, Liu J, Li M, *et al. Campylobacter jejuni* from patients with Guillain–Barré syndrome preferentially expresses a GD1a-like epitope. Infect Immun 2002; 70:5299–5303.

30 Gilbert M, Karwaski MF, Bernatchez S, *et al.* The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*: biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. J Biol Chem 2002; 277:327–337.

31 •• Godschalk PCR, Heikema AP, Gilbert M, *et al.* The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain–Barré syndrome. J Clin Invest 2004; 114:1659–1665.
The authors demonstrate for the first time that specific bacterial genes are required for the induction of anti-ganglioside antibodies

32 Geleijns K, Jacobs BC, van Rijs W, *et al.* Functional polymorphisms in LPS receptors *CD14* and *TLR4* are not associated with disease susceptibility or *Campylobacter jejuni* infection in Guillain–Barré patients. J Neuroimmunol 2004; 150:132–138.

33 Geleijns K, Laman JD, van Rijs W, *et al.* Fas polymorphisms are associated with the presence of anti-ganglioside antibodies in Guillain–Barré syndrome. J Neuroimmunol 2005; 161:183–189.

Antecedent infections in Fisher syndrome

A common pathogenesis of molecular mimicry

M. Koga, MD, PhD; M. Gilbert, PhD; J. Li, PhD; S. Koike, AS; M. Takahashi, DVM, PhD; K. Furukawa, MD, PhD; K. Hirata, MD, PhD; and N. Yuki, MD, PhD

Abstract—*Objective:* To assess the production mechanism of anti-GQ1b autoantibody in Fisher syndrome (FS). *Methods:* The authors conducted a prospective case-control serologic study of five antecedent infections (*Campylobacter jejuni*, cytomegalovirus, Epstein–Barr virus, *Mycoplasma pneumoniae*, and *Haemophilus influenzae*) in 73 patients with FS and 73 sex- and age-matched hospital controls (HCs). Serologic evidence in FS patients of *C. jejuni* (21%) and *H. influenzae* (8%) infections was present significantly more often than in the HCs. None of the five pathogens examined was found in the 49 (67%) patients with FS. Anti-GQ1b IgG antibody was detected in most FS patients infected with *C. jejuni* or *H. influenzae*. Mass spectrometry analysis identified a *C. jejuni* strain (CF93-6) carrying a GT1a-like lipo-oligosaccharide (LOS) that had been isolated from an FS patient. Immunization of complex ganglioside-lacking knockout mice with the GT1a-like LOS generated IgG class monoclonal antibodies (mAbs) that reacted with GQ1b and GT1a. Thin-layer chromatography with immunostaining showed that anti-GQ1b mAb bound to the *C. jejuni* LOS (50% of the 20 FS-related strains) more commonly than in the Guillain–Barré syndrome (GBS)–related (7% of 70) or enteritis-related (20% of 65) strains. Anti-GM1 and anti-GD1a mAbs also reacted with the LOS from some FS-related strains (both 20%), but binding frequencies were higher in the GBS-related strains (74 and 57%). The GQ1b epitope was detected in 4 (40%) of the 10 FS-related *H. influenzae* strains but was absent in strains from patients with GBS (n = 4) and uncomplicated respiratory infections (n = 10). *Conclusions: C. jejuni* and *H. influenzae* are related to Fisher syndrome (FS) development, and production of anti-GQ1b autoantibody is mediated by the GQ1b-mimicking lipo-oligosaccharides on those bacteria. The causative agents remain unclear in the majority of patients with FS.

NEUROLOGY 2005;64:1605–1611

Fisher syndrome (FS) is characterized by the acute onset of ophthalmoplegia, ataxia, and areflexia.[1] Its pathogenesis has been actively investigated,[2] and most patients with FS are found to have serum anti-GQ1b IgG autoantibodies during the acute phase of the illness that are cross-reactive with GT1a.[3-6] FS occurs subsequent to a wide variety of infections, most of which have been described in case reports, but there has been no case-control study of the antecedent infections. Previously, we reported positive serology for recent *Campylobacter jejuni* infection of 18% of 65 FS patients,[7] but no other Guillain–Barré syndrome (GBS)–related agent in FS has been investigated in a large number of patients. We also reported that in 7% of 70 FS retrospective cases, there was serologic evidence of recent *Haemophilus influenzae* infection.[8] A case-control study is needed to confirm its association with FS because the incidence of this infection is relatively rare.

C. jejuni strains isolated from FS patients had lipo-oligosaccharides (LOSs) that bore a terminal trisaccharide epitope mimicking GQ1b, GT1a, or GD3.[9-11] Immunization of mice with GT1a- or GD3-like LOS produced a monoclonal antibody (mAb) reactive against GQ1b and GT1a.[12] This raised the possibility that anti-GQ1b IgG antibody production is mediated by the trisaccharide epitope on bacterial LOSs. To verify this, it is necessary to prove that in a large number of isolates, FS is related to *C. jejuni* strains bearing a GQ1b-, GT1a-, or GD3-like LOS. We also reported that an *H. influenzae* type b serostrain had a GT1a-like LOS and hypothesized that ganglioside mimicry is involved in the development of FS after *H. influenzae* infection as well,[8] but whether such epitope is present in FS-related strains has yet to be determined.

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the May 10 issue to find the title link for this article.

From the Department of Neurology (Drs. Koga, Yuki, and Hirata) and Institute for Medical Science (S. Koike), Dokkyo University School of Medicine, Tochigi, Department of Microbiology (Dr. Takahashi), Tokyo Metropolitan Institute of Public Health, and Department of Biochemistry II (Dr. Furukawa), Nagoya University School of Medicine, Aichi, Japan; and Institute for Biological Sciences (Drs. Gilbert and Li), National Research Council Canada, Ottawa, Ontario, Canada.

Supported in part by grants-in-aid from the Ichiro Kanehara Foundation, the Kanae Foundation for Life and Socio-Medical Science, and the Japan Intractable Diseases Research Foundation; by a grant for Scientific Research (B) (KAKENHI 14370210 to N.Y.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a Research Grant for Neuroimmunological Diseases from the Ministry of Health, Labour, and Welfare of Japan; a Health Sciences Research Grant (Research on Psychiatric and Neurological Diseases and Mental Health) from the Ministry of Health, Labour, and Welfare of Japan; and a grant from the Human Frontier Science Program (RGP0038/2003-C).

Received October 26, 2004. Accepted January 12, 2005.

Address correspondence and reprint requests to Dr. M. Koga, Department of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan; e-mail: kogamrk@dokkyomed.ac.jp

We conducted a prospective case-control study of antecedent infectious serology in FS and then chemically determined the terminal oligosaccharide structure of the LOS on isolates obtained from an FS patient. By immunizing complex ganglioside-lacking knockout mice with the bacterial LOS carrying a GT1a epitope, we could clone mAb with reactivities against GQ1b and GT1a for use in examining whether the GQ1b-like LOS on the isolate is associated with FS.

Methods. *Patients.* We receive many requests from physicians throughout Japan to test for serum antiganglioside antibodies in patients with various neurologic disorders. On receipt of serum samples from FS patients, we have requested the primary physicians to send hospital control (HC) serum from sex- and age-matched (±5 years) patients without an autoimmune disease who did not have a history of recent enteritis but were in hospital at that time. If no patients fulfilled these criteria, we accepted as HCs sex- and age-matched (±5 years) healthy persons working at the hospital. We also collected sera from family members if possible. Between February 2000 and November 2003, we received 367 serum samples from FS patients, of which 73 paired samples from FS patients (men/women, 45/28; median age 32) and HC subjects (45/28; age 33) were available. Twenty control (8/12; age 49) samples from 18 families also were available. During the same period, we received 1,814 serum samples from patients with GBS, of which 73 (men/women, 45/28; median age 49) were selected randomly to be the disease controls. FS and GBS diagnoses were based on published clinical criteria.[13,14] Diagnosis of FS also was made for patients who initially presented with ophthalmoplegia, ataxia, and areflexia and then developed generalized muscle weakness. Although most had not been our patients, we collected their cases prospectively and asked the physicians whether diagnostic criteria had been fulfilled. One of the authors reviewed the medical records to verify the diagnoses.

Infectious serology. Recent *C. jejuni* infection was detected by an ELISA, as reported previously,[7] with altered criteria for seropositivity to increase its sensitivity and specificity (see appendix E-1 on the *Neurology* Web site at www.neurology.org). Under this condition, 43 of 47 GBS patients from whom *C. jejuni* had been isolated were judged positive within 4 weeks of GBS onset, whereas only 2 of 73 HC subjects with no history of recent enteritis were. Our assay therefore had a sensitivity of 91%, a specificity of 97%, and an efficiency of 95%. Evidence of recent *H. influenzae* infection was assayed serologically as reported elsewhere.[8] Infections by cytomegalovirus (CMV), Epstein–Barr virus (EBV), and *Mycoplasma pneumoniae* were also tested because a case-control study showed that they are related to the GBS development.[15] Serum IgM anti-CMV antibody and IgM anti-EBV capsid antigen antibody were tested using commercially available ELISA kits, Cytomegalo IgM(II)-EIA "SEIKEN" (Denka Seiken, Tokyo, Japan), and ETI-EBV-M reverse (DiaSorin, Stillwater, MN), according to the manufacturers' instructions. Serum anti-*M. pneumoniae* antibody was detected by the particle agglutination test (Serodia-Mycon II test kit; Fujirebio, Tokyo, Japan) after heating sera to 56 °C to inactivate complement.

Antiganglioside antibodies. We measured serum anti-GQ1b IgG/IgM/IgA and IgG antibodies against GT1a, GM1, and GD1a by ELISAs as described elsewhere.[7,16] Serum was considered positive when the optical density was ≥0.1 at a dilution of 1:500 for the IgG and 1:100 for the IgM and IgA antibodies. The IgG subclasses of anti-GQ1b antibody were examined in an ELISA with peroxidase-conjugated mouse anti-human γ1-, γ2-, γ3-, and γ4-chain-specific mAbs (Southern Biotechnology Associates, Birmingham, AL) as the secondary antibodies, as reported elsewhere.[17]

Analysis of O-deacylated LOSs. A *C. jejuni* strain, CF93-6, isolated from a patient with FS,[10] was used. Overnight growth of the strain on an agar plate was done as described previously,[18] except that we used 60 μg/mL proteinase K, 200 μg/mL RNAse A, and 100 μg/mL DNAse I. The *O*-deacylated LOS sample was analyzed by capillary electrophoresis–electrospray ionization–mass spectrometry (CE-ESI-MS), as described elsewhere.[19]

Generation of anti-GQ1b mAbs. Mice lacking the functional gene for β-1,4-*N*-acetylgalactosaminyltransferase (GM2/GD2 synthase; EC 2.4.1.92) were raised and their genotypes determined as described elsewhere.[20] They expressed no complex gangliosides, including neither GQ1b nor GT1a and therefore are an immune naive host and show strong IgG response to ganglioside-like LOS, whereas wild mice do not.[21] LOS was extracted from two *C. jejuni* strains (CF93-6 and CF90-26 [a GM1 epitope-bearing isolate from a GBS patient][22]) by the hot phenol-water technique,[23] after which the aqueous layer was dialyzed and centrifuged at 105,000 *g* for 16 hours. The mice were immunized intraperitoneally four times at 2-week intervals with 100 μg of LOS or 10 mg of a heat-killed lysate of *C. jejuni* dissolved in 50 μL of 2 mg/mL keyhole lympet hemocyanin solution (Sigma, St Louis, MO) that had been mixed with 50 μL of complete Freund adjuvant. Three days after final immunization with 50 μg of LOS in 50 μL of phosphate-buffered saline, mAbs were obtained as described elsewhere.[24] 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, Dokkyo University School of Medicine.

Detection of ganglioside-like LOSs. The presence of the GQ1b epitope was examined in *C. jejuni* and *H. influenzae* strains isolated from patients with FS (20 *C. jejuni* and 10 *H. influenzae*), GBS (70 *C. jejuni* and 4 *H. influenzae*), uncomplicated enteritis (65 *C. jejuni*), or a respiratory infection (10 *H. influenzae*). Anti-GQ1b IgG antibody was positive in patients from whom *C. jejuni* (FS, 18/20 [90%]; GBS, 3/70[4%]) or *H. influenzae* (FS, 10/10 [100%]; GBS, 0/4 [0%]) had been isolated. All the *H. influenzae* strains used were nontypable. Most of FS/GBS-related *C. jejuni* strains were isolated by one of the authors,[25] and all *H. influenzae* strains were obtained from hospitals throughout Japan. Ganglioside epitopes (GQ1b, GM1, and GD1a) were examined by thin-layer chromatography with immunostaining (*C. jejuni*) and ELISA (*H. influenzae*), as shown in appendix E-2.

Statistical analysis. Differences in the infectious serology frequencies of FS and HC were tested with the McNemar test, and frequency differences between groups were compared by the Fisher exact test. Differences in medians were examined by the Mann–Whitney *U* test. All calculations were done with SPSS 12.0J software (SPSS, Chicago, IL). A difference was considered significant when the two-sided *p* value was <0.05.

Results. *Infectious serology.* Recent infectious agents were identified in 24 (33%) of the patients with FS, serologic evidence of recent *C. jejuni* (21%) and *H. influenzae* (8%) infections being more common than in the HCs, whereas frequencies of the other agents did not differ (table 1). As compared with the patients with GBS, the frequency of antecedent *C. jejuni* infection was lower and that of *H. influenzae* infection higher in patients with FS, but the differences were not significant. One family member, the 80-year-old husband of a *C. jejuni*-negative patient with FS, who had no history of recent infectious symptoms, was seropositive for *C. jejuni*. No family members were positive for *H. influenzae*. Positive serology for CMV infection was found for 3 of 20 family members, of whom 2 (mother and daughter) had coughs and nasal discharges at the time of sampling. Positive serology for more than one infection was found for only three (4%) of the FS patients: *C. jejuni* and *H. influenzae*, *C. jejuni* and CMV, and *C. jejuni* and EBV.

C. jejuni*-related FS.* Men predominated in the FS patients with *C. jejuni* infection (men/women, 11/4) as in patients without this infection (34/24). Teenagers and young adults (age <30) were proportionally higher in patients with *C. jejuni*-related FS (53%) than in the other patient groups (24%) ($p = 0.06$), but the median age did not differ significantly (28 vs 37 years old; $p = 0.19$). Patients with *C. jejuni* infection more often had a history of antecedent gastrointestinal symptoms (60 vs 35%; $p =$

Table 1 *Infectious serology*

					Two-tailed p value		
	FS	HC	Family control	GBS	FS vs HC	FS vs family	FS vs GBS
n	73	73	20	73			
Campylobacter jejuni	15 (21)	2 (3)	1 (5)	23 (32)	<0.001*	NS	NS
Haemophilus influenzae	6 (8)	0	0	2 (3)	0.04†	NS	NS
Mycoplasma pneumoniae	3 (4)	4 (5)	1 (5)	4 (5)	NS	NS	NS
Cytomegalovirus	2 (3)	0	3 (15)	2 (3)	NS	NS	NS
Epstein–Barr virus	1 (1)	4 (5)	0	1 (1)	NS	NS	NS

Values in parentheses are percentages.

* Odds ratio, 9.1; 95% CI, 2.5–34.0.
† Odds ratio, 14.2; 95% CI, 1.1–15.8.

FS = Fisher syndrome; HC = hospital control; GBS = Guillain–Barré syndrome; NS = not significant ($p > 0.05$).

0.03). The neurologic features of facial palsy (27%), bulbar palsy (27%), limb weakness (13%), sensory disturbance (33%), and autonomic disturbance (7%) did not differ markedly among the groups. Anti-GQ1b and anti-GT1a IgG antibodies were present in all the patients with *C. jejuni* infection, more frequently than in the other patients (p = 0.06 and 0.02), and anti-GM1 and anti-GD1a IgG antibodies also were detected more often (p = 0.01 and 0.11) (figure 1). The anti-GQ1b antibody IgG subclass distribution was similar to that for patients without this infection (table 2). IgA and IgM anti-GQ1b antibodies were more frequent in patients with *C. jejuni* infection (80 and 73%) than in those without it (55 and 51%), but the differences did not reach significance (p = 0.14 and 0.16).

H. influenzae*-related FS.* The median age of the six patients with *H. influenzae*-related FS was 54 years (range 14 to 86 years), and four were women. Upper respiratory tract infection preceded FS onset in four (67%) and gastrointestinal symptoms in one (17%). Bulbar palsy tended to be more frequent in patients with *H. influenzae* infection (50%) than in those without it (18%) (p = 0.10), but the other neurologic features such as facial palsy (33%), limb weakness (17%), sensory disturbance (50%), and autonomic disturbance (0%) did not differ markedly. Five (83%) patients had anti-GQ1b and anti-GT1a IgG antibodies, but none had the anti-GM1 IgG antibody (see figure 1). The anti-GQ1b antibody IgG subclass distribution was similar to that found for the other patients (see table 2). A higher percentage of patients with *H. influenzae* infection had the IgM class of anti-GQ1b antibody (83 vs 54%; p = 0.22), but IgA antibody frequency did not differ between the two groups (67 vs 60%; p = 1.0).

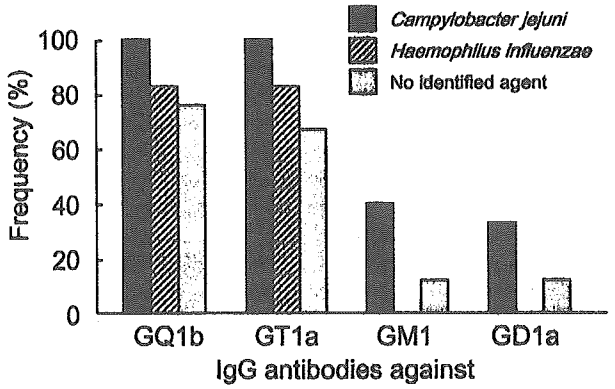


Figure 1. Frequency of positive antiganglioside IgG antibody in patients with Fisher syndrome. Black columns = Fisher syndrome after Campylobacter jejuni *infection (n = 15); hatched columns = Fisher syndrome after* Haemophilus influenzae *infection (n = 6); white columns = Fisher syndrome without identified agents (n = 49).*

FS with no identified infection. None of the five infections examined was found in 49 of the FS patients. Their median age was 35 years (range 4 to 81 years), and 30 were men. An antecedent upper respiratory tract infection was more frequent in this (88%) than the other (63%) group (p = 0.03), whereas gastrointestinal symptom frequency did not differ (29 vs 42%; p = 0.30). The neurologic features of facial palsy (16%), bulbar palsy (18%), limb weakness (16%), sensory disturbance (49%), and autonomic disturbance (8%) did not differ markedly between the groups. Antiganglioside IgG antibodies were less common in patients without identified infectious agents than in those with them (anti-GQ1b, p = 0.12; anti-GT1a, p = 0.04; anti-GM1, p = 0.19; anti-GD1a, p = 0.19) (see figure 1). The anti-GQ1b antibody IgG subclass distribution was similar to that found for the other FS patients (see table 2). Patients in this group less commonly had IgA (53 vs

Table 2 *IgG subclass classification of anti-GQ1b antibodies in patients with Fisher syndrome*

	Fisher syndrome		
	C. jejuni related	*H. influenzae* related	No identified agents
n	15	5	37
IgG subclass			
IgG1	14 (93)	5 (100)	35 (95)
IgG2	0	0	1 (3)
IgG3	7 (47)	3 (60)	22 (59)
IgG4	0	0	0

Values in parentheses are percentages.

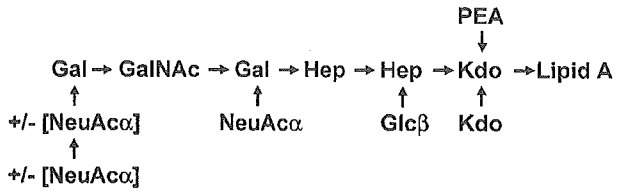


Figure 2. Proposed lipo-oligosaccharide (LOS) outer core structures based on capillary electrophoresis–electrospray ionization–mass spectrometry analysis of an O-deacylated LOS sample from Campylobacter jejuni *CF93-6. Gal = galactose; NeuAc = N-acetylneuraminic acid; GalNAc = N-acetylgalactosamine; Hep = L-glycero-D-manno-heptose; Kdo = 3-deoxy-D-manno-2-octulosonic acid; PEA = phosphoethanolamine; Glc = glucose.*

75%; $p = 0.08$) and IgM (49 vs 71%; $p = 0.09$), and anti-GQ1b antibodies were less common than in patients with identified infections.

LOS structures of C. jejuni *CF93-6.* Because an LOS sample from *C. jejuni* CF93-6 was used to immunize mice to obtain mAbs against specific gangliosides (see Methods and Results), we performed various analyses to better define the ganglioside mimics present in the outer cores of that sample. CE-ESI-MS analysis of the *O*-deacylated LOS sample from *C. jejuni* CF93-6 yielded various masses, in which the major species was $[M\text{-}4H]^{4-}$ = (3,937 Da). Other variants, shown in table E-1, are due to lipid A variations as well as to the presence or absence of the terminal sialic acid. The presence of one terminal sialic acid produces a GD1a mimic, whereas the presence of two yields a GT1a mimic (figure 2). The presence of two sialic acids as a chain (diNeu5Ac) in some of the variants was confirmed by CE-MS-MS and looking for the precursor ion at 581 Da (data not shown).

mAb characteristics. A clone (FS1) with anti-GQ1b IgG activity (IgG2bκ) was obtained from a mouse inoculated with *C. jejuni* (CF93-6) LOS, and two clones (FS2 and FS3, IgG2bκ) were obtained from mice inoculated with the *C. jejuni* heat-killed lysate. An ELISA showed that FS1 reacted with GD3 as well as with GQ1b and GT1a and that FS2 and FS3 specifically reacted with GQ1b and GT1a (table 3). Two clones were obtained from a mouse inoculated with *C. jejuni* (CF90-26) LOS. One (GB2), as reported elsewhere,[24] reacted strongly with GM1 and weakly with GD1a. The other (GB1) reacted strongly with GD1a but did not react with GM1.

Ganglioside-like LOS. FS1 was used to detect the GQ1b epitope on *C. jejuni* LOS because FS2 and FS3 had not been cloned at that time. Although we used two reagents (mAb and the patient's serum) in the detection of the ganglioside epitope, overall results were the same, except for some differences that probably were due to reagent sensitivities (table 4). The GQ1b epitope was more frequent in the FS-related *C. jejuni* strains than the GBS- and enteritis-related strains. In contrast, GM1 and GD1a epitope frequencies did not differ between the FS and enteritis strains. GBS-related strains more often had GM1- and GD1a-like LOSs than did the FS and enteritis ones.

Ganglioside epitopes on the *H. influenzae* LOS were examined quantitatively in an ELISA with FS3, GB2, and GB1 mAbs. The cut-off value for the presence of a ganglioside epitope was defined as an optical density of 0.1 based on results found for strains from patients with uncomplicated upper respiratory tract infections. Four (40%) of the 10 FS strains had a GQ1b-like LOS, whereas none of the GBS and uncomplicated strains did (figure 3). Of the four strains bearing a GQ1b epitope, three had neither the GM1 nor the GD1a epitope and one had the GM1 epitope. GM1 reactivities were present in the three (30%) FS-related strains, one of which also had GD1a reactivity. Unexpectedly, none of the four GBS-related strains was judged to have GM1 and GD1a epitopes.

Table 3 *Reactivity of monoclonal antibodies*

Clone	Antiganglioside reactivity		
FS1	GQ1b (++)	GD3 (++)	GT1a (+)
FS2	GQ1b (+++)	GT1a (++)	
FS3	GQ1b (+++)	GT1a (+++)	
GB1	GD1a (+++)	GalNAc-GD1a (+)	GT1b (+)
GB2	GM1 (+++)	GD1a (+)	

All clones were obtained by inoculating the mice lacking the complex gangliosides with *C. jejuni* (CF93-6; GT1a/GD1a/GM1 mimics) lipo-oligosaccharide (LOS) (FS1) or heat-killed lysate (FS2 and FS3) or *C. jejuni* (CF90-26; GM1/GD1a mimics) LOS (GB1 and GB2). IgG antibody activities against GM1, GM1b, GM2, GD1a, GalNAc-GD1a, GD1b, GD2, GD3, GT1a, GT1b, and GQ1b were tested in ELISAs. Results are expressed as relative activity at the appropriate dilution rate for each reagent. Only gangliosides bound (optical density [OD] of >0.5) by the reagents are shown. (+++) = OD of >2.5; (++) = OD 1.5–2.5; (+) = OD 0.5–1.5.

Discussion. Our study showed that *C. jejuni* and *H. influenzae* are causal agents of FS. No antecedent pathogens were identified in 67% of the FS patients studied, and no difference in the frequencies of CMV, EBV, and *M. pneumoniae* infections was found between FS and HC, whereas all were associated with GBS.[15] Although we changed the definition of serologic evidence of antecedent *C. jejuni* infection, its frequency in FS was similar to that reported earlier (18%).[7] It is noteworthy that most of the FS patients without identified infections had histories of antecedent upper respiratory infectious symptoms, indicative that respiratory infectious pathogens should be investigated as causal agents of FS. However, our results might merely reflect the low sensitivity of the infectious serology assays used. In spite of numerous infections reported as preceding FS onset,[26] there are only a few clues as to the major antecedent agents. β-Hemolytic streptococcal infection is an attractive candidate[27] because it may be followed by acute rheumatic fever and acute glomerulonephritis.[28] One serologic study, however, has reported that this antecedent infection is not common in FS.[29]

We showed that FS-related *C. jejuni* strains had a GQ1b/GT1a-like LOS more often than GBS- and enteritis-related ones, confirmation of the results of a small study showing that all four FS-related *C. jejuni* strains had a GQ1b-like LOS.[30] Moreover, for the first time, we determined that anti-GQ1b/GT1a mAb binds to LOSs from some FS-related *H. influen-*

Table 4 *Frequency of ganglioside epitopes on* C. jejuni *lipo-oligosaccharide*

				Two-tailed p value	
	FS	GBS	Enteritis	FS vs GBS	FS vs enteritis
n	20	70	65		
Resorcinol* reactive	16 (80)	66 (94)	47 (72)	NS	NS
GQ1b epitope					
FS1†	10 (50)	5 (7)	13 (20)	<0.001	0.02
S7577	14 (70)	11 (16)	17 (26)	<0.001	0.001
GM1 epitope					
GB2	4 (20)	52 (74)	25 (38)	<0.001	NS
S6960‡	4 (20)	49 (70)	22 (34)	<0.001	NS
GD1a epitope					
GB1	4 (20)	40 (57)	13 (20)	0.005	NS
S5174§	2 (10)	42 (60)	12 (18)	<0.001	NS

Values in parentheses are percentages.

* Reagent for staining sialic acid.
† Anti-GQ1b IgG-positive serum from a patient with FS.
‡§ Anti-GM1 ‡ and anti-GD1a § IgG-positive sera from patients with GBS.

NS = not significant ($p > 0.05$); FS = Fisher syndrome; GBS = Guillain–Barré syndrome.

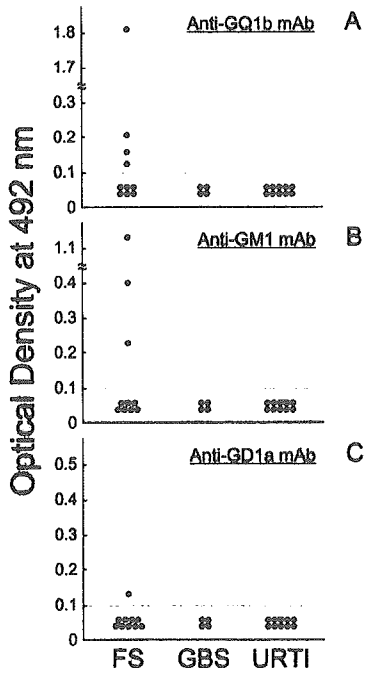


Figure 3. Ganglioside epitopes on the Haemophilus influenzae *lipo-oligosaccharide. Plots of individual antibody activities against ganglioside-like lipo-oligosaccharides on* Haemophilus influenzae *strains isolated from patients with Fisher syndrome (FS; n = 10), Guillain–Barré syndrome (GBS; n = 4), and uncomplicated upper respiratory tract infections (URTI; n = 10). Anti-GQ1b (FS3) (A), anti-GM1 (GB2) (B), and anti-GD1a (GB1) (C) were the monoclonal antibodies (mAbs).*

zae strains. These findings indicate a common pathogenesis of molecular mimicry in the development of *C. jejuni* and *H. influenzae*-related FS. The IgG subclass of anti-GQ1b antibody was almost always IgG1, IgG3, or both, as reported elsewhere,[31] IgG1 predominating irrespective of the antecedent infection identified. IgG2 was reported to be the main subclass of anti-GQ1b antibody in FS patients with an antecedent gastrointestinal infection (*C. jejuni* had been isolated from three of five patients),[32] whereas other investigators reported IgG1 and IgG3 in an FS patient from whom *C. jejuni* had been isolated.[33] Our and the other findings[32] apparently conflict. The reason is not clear. Our results agree with those of other studies as to the frequent presence of anti-GM1 IgG1 antibody in GBS cases in which there was *C. jejuni* serology.[17,33,34] We therefore believe that the IgG2 subclass of anti-GQ1b antibody is not associated with FS that develops subsequent to *C. jejuni* infection. Because the IgG subclass of antibody response is related closely to the types of antigens targeted and to T-cell dependency,[35] the similar IgG subclassifications of the anti-GQ1b antibody present in FS after *C. jejuni*, *H. influenzae*, or unidentified infections suggest that the mechanism of autoantibody production, probably mediated by ganglioside-like molecules on the infectious agent, is common in FS populations other than *C. jejuni*- and *H. influenzae*-related ones.

Ganglioside epitopes were not detected in some *C. jejuni* strains isolated from FS patients, but this does not indicate that other mechanisms than the molecular mimicry principle are applied to the antiganglioside antibody production in these patients. Ganglioside-like structure on *C. jejuni* LOS would

disappear during repeated culture owing to the phase variation in homopolymeric G tract in LOS biosynthesis genes.[36] Another possibility is that FS patients were infected by several *C. jejuni* strains and the "causative" strain bearing ganglioside-like LOS had not been occasionally isolated. The presence of a GM1-mimicking epitope on the *C. jejuni* LOS[9,22,37] and the development of acute motor axonal neuropathy with anti-GM1 antibody after inoculation of rabbits with the GM1-like LOS[24] strongly suggest that anti-GM1 IgG antibody production is mediated by GM1 mimicry of *C. jejuni* LOS. To show that the principle of molecular mimicry provides a common pathogenesis of FS and GBS, it is necessary to establish the FS animal model by immunizing GQ1b-bearing LOSs of *C. jejuni* and *H. influenzae*.

Our serologic findings and the detection of GQ1b-like LOS in FS-related *H. influenzae* strains provide strong support for a relationship between FS and *H. influenzae*. This bacterium is classified as having capsulated (serotypes a to f) and uncapsulated (nontypable) strains. We determined the serotypes of the FS- and GBS-related *H. influenzae* strains and found all were nontypable. We cannot say that all the isolates were related to the development of FS and GBS because this bacterium is a major pathogen of respiratory infection, and patients with FS or GBS sometimes contract pneumonia after neuropathic onset, possibly owing to bulbar palsy. Our results, however, do indicate that uncapsulated strains are important in the development of *H. influenzae*-related FS and GBS, but whether *H. influenzae* is a major causative agent of GBS has still to be determined.[8,15,38,39] In our study, there was serologic evidence of this infection in only 3% of the GBS patients, a frequency similar to findings of previous studies.[8,15,39] Because all the studies, including the current one, used only serologic methods to test for antecedent *H. influenzae* infection, the seropositive frequency may have been underestimated owing to the low sensitivity of the assay.[8] A standardized, highly sensitive serologic method and a culture survey are needed to establish the frequency of *H. influenzae*-related GBS.

The LOSs of *C. jejuni* and *H. influenzae* vary considerably in the oligosaccharide structures on their outer cores, and previous studies showed that both bacteria commonly have sialylated LOSs.[40] Because ganglioside classification is based on the sialylation type, sialylation of a bacterial LOS may be the key to the development of FS and GBS after *C. jejuni* or *H. influenzae* infection. Three genes (*cst-I*, *-II*, and *-III*) in *C. jejuni*[41,42] and three genes (*lic3A*, *siaA*, *lsgB*) in *H. influenzae*[43,44] have been cloned for the sialylation enzyme. Whether the presence of the *cst-II* gene is a risk factor for developing GBS/FS after *C. jejuni* enteritis is not clear,[45,46] but it seems to be essential for the biosynthesis of a GQ1b-like LOS and therefore is closely related to the anti-GQ1b antibody in FS.[45] Variation in the LOS outer core could, however, be created not only by diverse gene contents.[36,47] We sequenced the genes that encode the glycosyltransferases involved in synthesis of the outer core of the LOS in *C. jejuni* CF93-6 (GenBank accession no. AY644679). The DNA sequence is 99% identical (6,041 of 6,047 bp) to the corresponding region in *C. jejuni* OH4384 (GenBank accession no. AF130984), which had been isolated from a patient with GBS who showed ophthalmoplegia[9,48] and expresses GT1a-like LOS similar to that of CF93-6.[9] The amino acid sequences of these glycosyltransferases involved in the addition of the *N*-acetylgalactosamine residue (CgtA), terminal galactose residue (CgtB), and sialic acid residues (Cst-II) are 100% identical for *C. jejuni* CF93-6 and OH4384. This suggests that gene alleles also are critical for the biosynthesis of variable ganglioside mimics. To determine the critical factor in the development and characterization of FS after *C. jejuni* or *H. influenzae* infection, the presence and polymorphism of sialyltransferase-encoding genes need to be investigated.

Acknowledgment

The authors thank Denis Brochu, Scott Houliston, Frank St. Michael, and Evgeny Vinogradov for the lipo-oligosaccharide analyses, Marie-France Karwaski and Sonia Leclerc for help with the DNA sequencing, and Maki Okazaki for support with serologic assays.

References

1. Fisher M. An unusual variant of acute idiopathic polyneuritis: syndrome of ophthalmoplegia, ataxia and areflexia. N Engl J Med 1956; 255:57–65.
2. Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain 2002;125:2591–2625.
3. Chiba A, Kusunoki S, Shimizu T, Kanazawa I. Serum IgG antibody to ganglioside GQ1b is a possible marker of Miller Fisher syndrome. Ann Neurol 1992;31:677–679.
4. Willison HJ, Veitch J, Paterson G, Kennedy PGE. Miller Fisher syndrome is associated with serum antibodies to GQ1b ganglioside. J Neurol Neurosurg Psychiatry 1993;56:204–206.
5. Yuki N, Sato S, Tsuji S, et al. Frequent presence of anti-GQ1b antibody in Fisher's syndrome. Neurology 1993;43:414–417.
6. Susuki K, Yuki N, Hirata K. Fine specificity of anti-GQ1b IgG and clinical features. J Neurol Sci 2001;185:5–9.
7. Koga M, Yuki N, Takahashi M, et al. Close association of IgA antiganglioside antibodies with antecedent *Campylobacter jejuni* infection in Guillain–Barré and Fisher's syndromes. J Neuroimmunol 1998;81: 138–143.
8. Koga M, Yuki N, Tai T, Hirata K. Miller Fisher syndrome and *Haemophilus influenzae* infection. Neurology 2001;57:686–691.
9. Aspinall GO, McDonald AG, Pang H, et al. Lipopolysaccharides of *Campylobacter jejuni* serotype O:19 structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with Guillain–Barré syndrome. Biochemistry 1994;33:241–249.
10. Yuki N, Taki T, Takahashi M, et al. Molecular mimicry between GQ1b ganglioside and lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Fisher's syndrome. Ann Neurol 1994;36:791–793.
11. Salloway S, Mermel LA, Seamans M, et al. Miller-Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD3. Infect Immun 1996;64:2945–2949.
12. Goodyear CS, O'Hanlon GM, Plomp JJ, et al. Monoclonal antibodies raised against Guillain–Barré syndrome-associated *Campylobacter jejuni* lipopolysaccharides react with neuronal gangliosides and paralyze muscle–nerve preparations. J Clin Invest 1999;104:697–708.
13. Asbury AK, Cornblath DR. Assessment of current diagnostic criteria for Guillain–Barré syndrome. Ann Neurol 1990;27(suppl):S21–S24.
14. Odaka M, Yuki N, Hirata K. Anti-GQ1b IgG antibody syndrome: clinical and immunological range. J Neurol Neurosurg Psychiatry 2001;70: 50–55.
15. Jacobs BC, Rothbarth PH, van der Meché FGA, et al. The spectrum of antecedent infections in Guillain–Barré syndrome: a case-control study. Neurology 1998;51:1110–1115.
16. Yuki N, Tagawa Y, Irie F, et al. Close association of Guillain–Barré syndrome with antibodies to minor monosialogangliosides GM1b and GM1α. J Neuroimmunol 1997;74:30–34.

17. Koga M, Yuki N, Hirata K, et al. Anti-GM1 antibody IgG subclass: a clinical recovery predictor in Guillain–Barré syndrome. Neurology 2003;60:1514–1518.
18. Szymanski CM, St Michael F, Jarrell HC, et al. Detection of conserved *N*-linked glycans and phase-variable lipooligosaccharides and capsules for *Campylobacter* cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. J Biol Chem 2003;278:24509–24520.
19. St Michael F, Szymanski CM, Li J, et al. The structures of the lipooligosaccharide and capsule polysaccharide of *Campylobacter jejuni* genome sequenced strain NCTC 11168. Eur J Biochem 2002;269:5119–5136.
20. Takamiya K, Yamamoto A, Furukawa K, et al. Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system. Proc Natl Acad Sci USA 1996; 93:10662–10667.
21. Bowes T, Wagner ER, Boffey J, et al. Tolerance to self gangliosides is the major factor restricting the antibody response to lipopolysaccharide core oligosaccharides in *Campylobacter jejuni* strains associated with Guillain–Barré syndrome. Infect Immun 2002;70:5008–5018.
22. Yuki N, Taki T, Inagaki F, et al. A bacterium lipopolysaccharide that elicits Guillain–Barré syndrome has a GM1 ganglioside-like structure. J Exp Med 1993;178:1771–1775.
23. Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol–water and further applications of the procedure. Methods Carbohydr Chem 1965;5:83–91.
24. Yuki N, Susuki K, Koga M, et al. Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipo-oligosaccharide causes Guillain–Barré syndrome. Proc Natl Acad Sci USA 2004;101: 11404–11409.
25. Takahashi M, Koga M, Yokoyama K, Yuki N. Epidemiology of *Campylobacter jejuni*-isolated Guillain–Barré and Fisher syndromes in Japan. J Clin Microbiol 2005;43:335–339.
26. Takano H, Yuki N. Fisher's syndrome associated with chickenpox and anti-GQ1b antibody. J Neurol 1995;242:255–256.
27. Munsat TL, Barnes JE. Relation of multiple cranial nerve dysfunction to the Guillain–Barré syndrome. J Neurol Neurosurg Psychiatry 1965; 28:115–120.
28. Stollerman GH. Rheumatic fever. Lancet 1997;349:935–942.
29. Yuki N, Hirata K. Fisher's syndrome and group A streptococcal infection. J Neurol Sci 1998;160:64–66.
30. Ang CW, Laman JD, Willison HJ, et al. Structure of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity and clinical features of Guillain–Barré and Miller Fisher patients. Infect Immun 2002;70:1202–1208.
31. Willison HJ, Veitch J. Immunoglobulin subclass distribution and binding characteristics of anti-GQ1b antibodies in Miller Fisher syndrome. J Neuroimmunol 1994;50:159–165.
32. Schwerer B, Neisser A, Bernheimer H. Distinct immunoglobulin class and immunoglobulin G subclass patterns against ganglioside GQ1b in Miller Fisher syndrome following different types of infection. Infect Immun 1999;67:2414–2420.
33. Yuki N, Ichihashi Y, Taki T. Subclass of IgG antibody to GM1 epitope-bearing lipopolysaccharide of *Campylobacter jejuni* in patients with Guillain–Barré syndrome. J Neuroimmunol 1995;60:161–164.
34. Deisenhammer F, Keir G, Pfausler B, Thompson EJ. Affinity of anti-GM1 antibodies in Guillain–Barré syndrome patients. J Neuroimmunol 1996;66:85–93.
35. Papadea C, Check IJ. Human immunoglobulin G and immunoglobulin G subclass: biochemical, genetic, and clinical aspects. Crit Rev Clin Lab Sci 1989;27:27–58.
36. Gilbert M, Karwaski M-F, Bernatchez S, et al. The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*: biosynthesis of sialylated ganglioside mimics in the core oligosaccharide J Biol Chem 2002;277:327–337.
37. Prendergast MM, Lastovica AJ, Moran AP. Lipopolysaccharides from *Campylobacter jejuni* O:41 strains associated with Guillain–Barré syndrome exhibit mimicry of GM1 ganglioside. Infect Immun 1998;66: 3649–3655.
38. Mori M, Kuwabara S, Miyake M, et al. *Haemophilus influenzae* infection and Guillain–Barré syndrome. Brain 2000;123:2171–2178.
39. Ju YY, Womersley H, Pritchard J, et al. *Haemophilus influenzae* as a possible cause of Guillain–Barré syndrome. J Neuroimmunol 2004;149: 160–166.
40. Moran AP, Prendergast MM, Appelmelk BJ. Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. FEMS Immunol Med Microbiol 1996;16:105–115.
41. Gilbert M, Brisson J-R, Karwaski M-F, et al. Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384: identification of the glycosyltransferase genes, enzymatic synthesis of model compounds, and characterization of nanomole amounts by 600-MHz ^{1}H and ^{13}C NMR analysis. J Biol Chem 2000;275:3896–3906.
42. Guerry P, Ewing CP, Hickey TE, et al. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. Infect Immun 2000;68:6656–6662.
43. Hood DW, Cox AD, Gilbert M, et al. Identification of a lipopolysaccharide α-2,3-sialyltransferase from *Haemophilus influenzae*. Mol Microbiol 2001;39:341–350.
44. Jones PA, Samuels NM, Phillips NJ, et al. *Haemophilus influenzae* type b strain A2 has multiple sialyltransferases involved in lipooligosaccharide sialylation. J Biol Chem 2002;277:14598–14611.
45. van Belkum A, van den Braak N, Godschalk P, et al. *Campylobacter jejuni* gene associated with immune-mediated neuropathy. Nat Med 2001;7:752–753.
46. Nachamkin I, Liu J, Li M, et al. *Campylobacter jejuni* from patients with Guillain–Barré syndrome preferentially expresses a GD1a-like epitope. Infect Immun 2002;70:5299–5303.
47. Hood DW, Richards JC, Moxon ER. *Haemophilus influenzae* lipopolysaccharide. Biochem Soc Trans 1999;27:493–498.
48. Yuki N, Tsujino Y. Familial Guillain–Barré syndrome subsequent to *Campylobacter jejuni* enteritis. J Pediatr 1995;126:162.

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 335–339
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.335–339.2005

Vol. 43, No. 1

Epidemiology of *Campylobacter jejuni* Isolated from Patients with Guillain-Barré and Fisher Syndromes in Japan

Masaki Takahashi,[1] Michiaki Koga,[2] Keiko Yokoyama,[1] and Nobuhiro Yuki[2]*

Department of Microbiology, Tokyo Metropolitan Institute of Public Health, Tokyo,[1] and Department of Neurology, Dokkyo University School of Medicine, Tochigi,[2] Japan

Received 7 May 2004/Returned for modification 17 August 2004/Accepted 8 September 2004

***Campylobacter jejuni* isolation is the standard for the diagnosis of this type of bacterial infection, but there have been no epidemiological studies of a large number of *C. jejuni* isolates from patients with Guillain-Barré syndrome (GBS) and Fisher syndrome (FS). For 13 years, stool specimens from GBS/FS patients have been sent from 378 hospitals throughout Japan to the Tokyo Metropolitan Institute of Public Health. A total of 113 strains (11%) were isolated from the stool specimens from 1,049 patients. The isolation rate did not differ by region. The rates were 22% for 449 patients with a history of diarrhea and 2% for the others. An additional 18 isolates were provided by various hospitals. There was no noticeable seasonal distribution in the onset of *C. jejuni* isolated from patients with GBS/FS. The male/female ratios were 1.7:1 for GBS and 2.2:1 for FS. The patient age range showed a peak in 10- to 30-year-old subjects who had GBS and in 10- to 20-year-old subjects who had FS. The predominance of young adults and male patients who had *C. jejuni*-associated GBS/FS may be related to the preponderance of young adults and male patients who had *C. jejuni* enteritis. The median interval from diarrhea onset to neurologic symptom onset was 10 days for GBS/FS. Penner's *C. jejuni* serotype HS:19 was more frequently present in GBS (67%) than in enteritis (6%) patients. HS:2 was more frequent in FS (41%) than in enteritis (14%) patients. These findings suggest that certain *C. jejuni* strains specifically trigger GBS and that others specifically trigger FS.**

Guillain-Barré syndrome (GBS), the prototype of postinfectious autoimmune diseases, is characterized by acute onset of limb weakness and loss of tendon reflexes. Since the near elimination of poliomyelitis worldwide, GBS is the most frequent cause of acute flaccid paralysis, the mean annual incidence being 1.3 cases per 100,000 population (11). Fisher syndrome (FS) is characterized by acute onset of ophthalmoplegia, ataxia, and areflexia. It is considered a variant of GBS because some who present with FS progress to GBS. The annual incidence rate is estimated as 0.09 per 100,000 population (4). Of the identified microorganisms, the gram-negative bacterium *Campylobacter jejuni*, a leading cause of acute gastroenteritis, is the most frequent antecedent pathogen in GBS/FS (12). A comprehensive Japanese study showed 31% of 201 patients with GBS and 18% of 65 with FS seropositive for recent *C. jejuni* infection (16).

Serological studies are important for understanding the epidemiology of *C. jejuni*-associated GBS/FS, but there are no recommended methodologies as to the antigens to be used or the standards of judgment. A comparative study made in Japan and The Netherlands on the presence of anti-*C. jejuni* antibody in GBS showed that serological assay systems vary considerably between laboratories (15). *C. jejuni* isolation is the standard diagnosis for bacterial infection and should be used to assess the epidemiology of *C. jejuni*-associated GBS/FS. The latent period between preceding intestinal infection and the onset of GBS/FS, however, often exceeds the excretion period of viable *C. jejuni* cells in stools. Consequently, there have been no epidemiological studies done on a large number of *C. jejuni* isolates from patients. We analyzed the epidemiological features of more than 100 patients with GBS/FS from whom *C. jejuni* had been isolated and investigated the presence of Penner's serotypes in the isolates.

* Corresponding author. Mailing address: Department of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan. E-mail: yuki@dokkyomed.ac.jp.

MATERIALS AND METHODS

Stools of GBS and FS patients. Stool specimens were sent from 378 hospitals throughout Japan to the Tokyo Metropolitan Institute of Public Health for *C. jejuni* isolation between December 1990 and November 2003. There was only one stool sampling per patient. The stools were collected in transport medium (SEEDSWAB no.1; Eiken, Tokyo Japan) immediately after patient admission. In addition to these isolates, 18 isolates from GBS (n = 15) and FS (n = 3) patients that had been provided by other hospitals were analyzed simultaneously. One of the authors (M.K.) reviewed the following information obtained from each primary physician: antecedent illness, initial symptoms, neurological signs during the illness, and clinical course. Rediagnoses were made based on the clinical criteria for GBS and FS (1, 23).

Bacterial strains. GBS/FS-related strains that had been isolated by one of the authors (M.T.) or provided by other hospitals were used. A total of 554 strains isolated from other patients with enteritis with no neurological complications in Tokyo Metropolitan Hospitals (Komagome and Bokutoh) were the controls to test whether particular serotypes were increased in GBS/FS-related strains.

Stool culture for *C. jejuni*. Each stool specimen was plated on three plates of solid selective media (i.e., CCDA, CM739, and SR155E; Oxoid, Ltd., Basingstoke, United Kingdom) without enrichment culture. The CCDA medium was incubated at 37°C for 72 h under microaerophilic conditions (5% O_2, 7.5% CO_2, 7.5% H_2, and 80% N_2) in an TE-HER CAMPYLO-INCUBATOR (Hirasawa, Tokyo, Japan). Suspicious colonies initially received a morphological check with a phase-contrast microscope. If an organism showed the typical curved, spiral morphology of *Campylobacter*, it was regarded as a type of *C. jejuni* or *C. coli* and replated on blood agar to obtain pure cultures. The identity of *C. jejuni* was confirmed by both biochemical tests and multiplex PCR techniques for assay for *C. jejuni* and *C. coli*. The biochemical tests used oxidase, hippurate hydrolysis, indoxyl acetate hydrolysis, and susceptibility to disks containing cephalothin (30 μg) and nalidixic acid (30 μg). Species-specific multiplex PCR were done with combined *C. jejuni*- and *C. coli*-specific primer sets (18, 29).

Serotyping. Penner serotyping of *C. jejuni* was carried out by using a *Campylobacter* antisera Seiken Set (Denka Seiken, Tokyo Japan) according to the manufacturer's protocol. The antisera in the commercial set were composed of

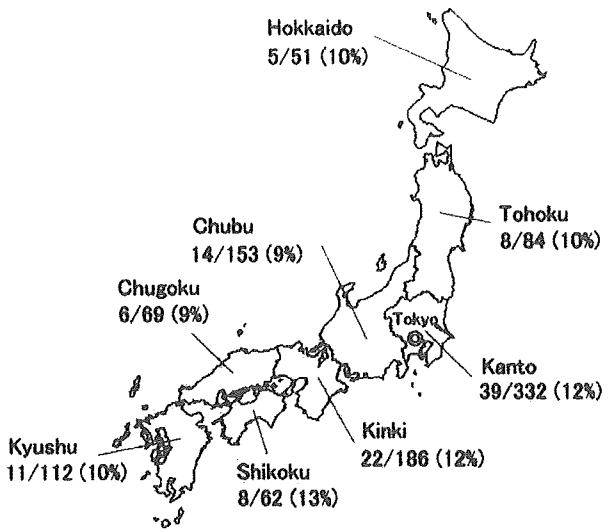


FIG. 1. Isolation rate of *C. jejuni* by district in Japan.

25 groups of antisera as follows: group A, HS:1/44; group B, HS:2; group C, HS:3; group D, HS:4/13/16/43/50; group E, HS:5; group F, HS:6/7; group G, HS:8; group I, HS:10; group J, HS:11; group K, HS:12; group L, HS:15; group N, HS:18; group O, HS:19; group P, HS:21; group R, HS:23/36/53; group S, HS:27; group U, HS:31; group V, HS:32; group Y, HS:37; group Z, HS:38; group Z_2, HS:41; group Z_4, HS:45; group Z_5, HS:52; group Z_6, HS:55; and group Z_7, HS:57.

Anti-ganglioside antibody assay. Serum samples obtained during the first 4 weeks after onset, before immune treatment, were frozen and stored at −80°C until used. An enzyme-linked immunosorbent assay, performed as reported elsewhere (33), was used to measure immunoglobulin G (IgG) antibodies to GM1, GD1a, and GQ1b in serum. Serum was considered positive when the titer was ≥500.

Statistics. Differences in frequency between groups were compared by using the Fisher exact test with SPSS 12.0J software (SPSS Inc., Chicago, Ill.). A difference was considered significant when the two-sided P value was <0.05.

RESULTS

Culture results. We obtained stool specimens from 1,049 patients with GBS (n = 763) or FS (n = 286). The median interval between neuropathy onset and stool sampling was 13 days (range, 1 to 150 days). Gastrointestinal symptoms occurred in 449 (43%) patients; upper respiratory symptoms occurred in 503 (48%) patients. A total of 113 *C. jejuni* strains (GBS, 87; FS, 26) (11%) were isolated from the 1,049 patients, although at least 11 patients had been treated with antibiotics. The combined isolation rate, 11% (GBS, 11%; FS, 9%), did not differ by region (Fig. 1). The rate was 22% for the 449 patients with a history of diarrhea and 2% for the the remaining patients (Table 1). Most *C. jejuni*-positive specimens were obtained within 2 weeks of neuropathy onset, some more than 1 month later (Fig. 2). Except for the of two siblings with GBS reported elsewhere (35), all cases were sporadic. *C. coli* (n = 2), *C. curvus* (n = 2), *Campylobacter* spp. (n = 12), and *Arcobacter* sp. (n = 1) were isolated from the *C. jejuni*-negative patients.

Characteristics of *C. jejuni*-positive patients. The seasonal distribution (Fig. 3) is lower in October and November. *C. jejuni*-associated GBS showed a peak in 10- to 30-year-olds (Fig. 4). *C. jejuni*-associated FS demonstrated a peak in 10- to 20-year-olds. Males predominated for both GBS (male/female ratio, 1.7:1) and FS (2.2:1).

Antecedent symptoms in culture-positive patients were reported in 95% of 102 GBS patients and in 93% of 29 FS patients. The median latent period between antecedent symptoms and neuropathy onset was 10 days for both groups; within 2 weeks in most cases (Fig. 5). Gastrointestinal symptoms were most frequent in cases of GBS (90%) and FS (79%), often being accompanied by fever. Six (5%) patients with GBS/FS had a history of upper respiratory tract infectious symptoms only, and three (2%) had experienced only preceding fever. Anti-ganglioside IgG antibodies in serum were tested during the acute phase of illness in 120 of 131 patients (Table 2). Most patients had some antibodies; anti-GM1 and anti-GD1a IgG antibodies were frequent in GBS and anti-GQ1b IgG antibody in FS.

Serogroups of *C. jejuni* isolates. HS:19 was more frequently isolated from GBS patients than from enteritis patients. The HS:2 and HS:4 complexes were isolated more frequently from FS patients than from enteritis patients. None of the GBS and FS isolates were HS:41.

DISCUSSION

Assessing the frequency of *C. jejuni* infection in GBS/FS patients is difficult since it depends on such varied factors as antecedent symptoms, the duration of convalescent excretion of the bacterium, and the kinds of antibiotics administered. Thus, few surveillance studies have included large numbers of patients. Kuroki et al. (17) reported that 30% of 46 GBS patients in the Kinki district of Japan had positive stool cultures. In a subsequent study, these authors isolated 13 strains (17%) of *C. jejuni* from 76 patients (9). In contrast, 8% of 103 patients had positive cultures in England (25), and 9% of 138 patients had positive cultures in The Netherlands (28). The isolation rate was 11% in the present study, which does not differ significantly from European values.

The latent period between intestinal infection and the onset of GBS/FS often exceeds the duration of convalescence excretion of the bacterium. GBS/FS patients therefore frequently have negative stool cultures. Kuroki's group (9) reported that all 13 positive stool specimens were obtained within 10 days after onset of GBS symptoms, and 9 of the 13 specimens were obtained within 5 days. We could isolate *C. jejuni* from stool specimens obtained more than 1 month after the onset of GBS (the 35th day) or FS (the 40th day). This finding is not excep-

TABLE 1. Isolation rate of *C. jejuni* and antecedent illness in patients with GBS and FS

Antecedent symptoms[a]	No. of stool specimens (%) from patients with:			
	GBS		FS	
	Total	*C. jejuni* positive	Total	*C. jejuni* positive
GI only	255	62 (24)	49	17 (35)
GI plus URTI	107	16 (15)	38	4 (11)
URTI only	209	2 (1)	149	4 (3)
Fever only	42	3 (7)	10	0
Others	25	0	5	0
None	121	3 (2)	31	1 (3)
Total[b]	759	86 (11)	282	26 (9)

[a] GI, gastrointestinal infection; URTI, upper respiratory tract infection.
[b] Eight cases were excluded because detailed information was not available.

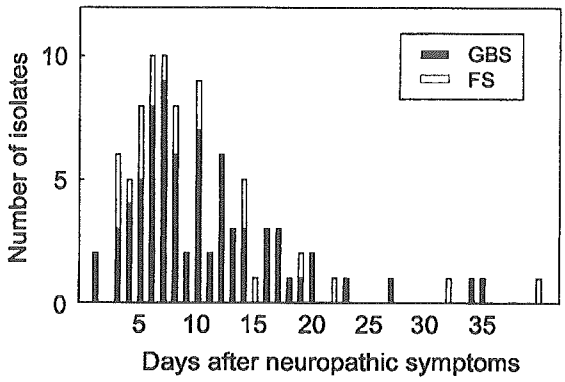


FIG. 2. Interval from the onset of neuropathic symptoms to the day stool specimens were obtained from *C. jejuni*-positive patients. Bars: ■, GBS; □, FS.

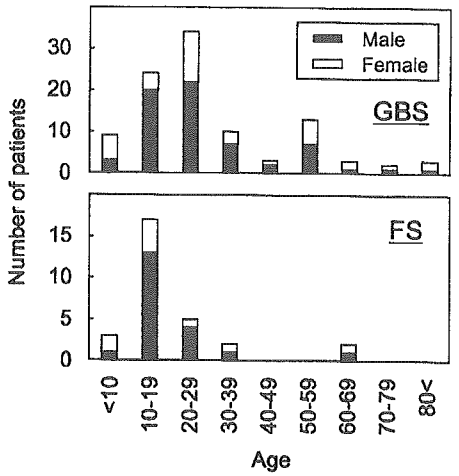


FIG. 4. Age distribution of *C. jejuni*-positive GBS and FS patients. Bars: ■, males; □, females.

tional because in some cases the excretion of *C. jejuni* in stools has continued for 6 to 9 weeks (14, 30).

There is no clear seasonal distribution of GBS in Western countries (11), whereas in northern China GBS peaks in late summer, which may be due to the high frequency of *C. jejuni* infection (10). In a previous study done in the Kinki district of Japan, the seasonal distribution of *C. jejuni*-associated GBS peaked in March, an autumn occurrence was relatively infrequent, and there was no seasonal difference found for the group of GBS patients without *C. jejuni* infection (9). We also detected no seasonal predominance. Autumn occurrences were relatively infrequent. Although the seasonality of *C. jejuni* enteritis in Japan is less pronounced than in other developed countries, which show a seasonal distribution with a well-defined summer peak (26), the incidence of *C. jejuni* enteritis tends to be higher from April to July (21).

In North America and Europe, a bimodal age distribution has been reported for GBS, with peaks at 20 to 40 years and 60 to 70 years (2, 13). Males are more commonly affected by GBS than females (~1.25:1) (11). In England the male/female ratio was 3.5:1 in the *C. jejuni*-seropositive group, whereas it was 1.5:1 in the *C. jejuni*-seronegative group (25). *C. jejuni*-associated GBS in northern China occurs mainly among children and young adults (10), the male/female ratio being 1.2:1 in *C. jejuni*-seropositive GBS patients and 0.8:1 in *C. jejuni*-seronegative patients. In the Kinki district of Japan, the age distribution showed a peak between 10 and 30 years (9). The male/female ratio was 2.4:1 for GBS patients who had had *C. jejuni* infection and 2.2:1 for the other GBS patients. In the present study, the age distribution showed a peak between 10 and 30 years for GBS and between 10 and 20 years for FS. A majority of male patients had both GBS and FS, the respective male/female ratios being 1.7:1 and 2.2:1. In English patients suffering from gastrointestinal symptoms, the highest *C. jejuni* isolation rates were for young adults, males predominating in the 15 to 24 years (1.7:1) and 45 to 54 years (1.6:1) age groups (26). The dominance of young adult and male patients among those with *C. jejuni* infection may therefore partly explain the findings for *C. jejuni*-associated GBS/FS. In England, the median interval between the onset of diarrhea and neuropathic symptoms was 9 days (range, 2 to 20) for 19 GBS patients (25). In our study, the median interval was 10 days (range, 1 to 24) for 115 GBS/FS patients.

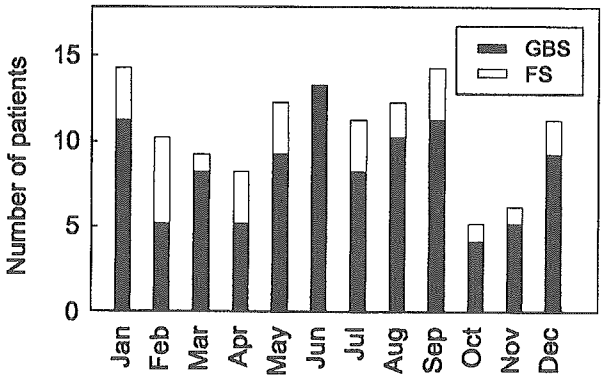


FIG. 3. Seasonal distributions of *C. jejuni* isolates in GBS and FS patients. Bars: ■, GBS; □, FS.

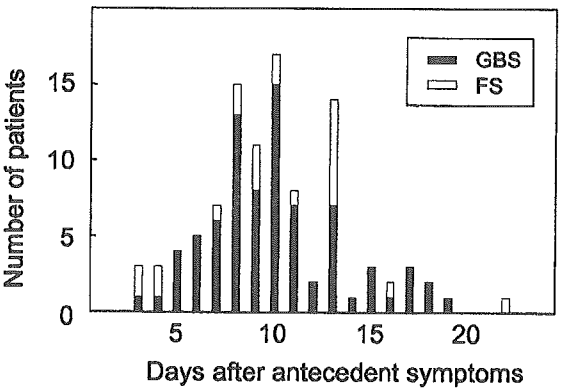


FIG. 5. Interval from diarrhea onset to the onset of neuropathic symptoms. Bars: ■, GBS; □, FS.

TABLE 2. Anti-ganglioside antibodies in patients with GBS and FS from whom *C. jejuni* was isolated

IgG antibody	No. of patients (%) with:	
	GBS (n = 92)	FS (n = 28)
Anti-GM1	68 (74)	4 (14)
Anti-GD1a	45 (49)	5 (18)
Anti-GQ1b	3 (3)	23 (82)

Penner's serotyping system is a useful phenotyping technique for *C. jejuni* and *C. coli* (24). The system can divide *C. jejuni* and *C. coli* into about 60 types. *C. jejuni* has been isolated from patients' stools at GBS onset. A large number of Penner serotypes have been reported in the literature: HS:1, HS:2, HS:4, HS:5, HS:10, HS:13/65, HS:16, HS:19, HS:23, HS:35, HS:37, HS:41, HS:44, and HS:64 (5), but Kuroki's group showed that 75% of 16 isolates from GBS patients were HS:19 and that 1 of 2 isolates from FS patients was HS:2 (22). Elsewhere we reported that in Japan HS:19 strains are significantly over-represented in GBS patients, accounting for 52% of 31 isolates from GBS patients but only 5% of 215 control isolates (34). HS:2 was found to be over-represented in FS patients (present in 71% of the patients versus 38% of the controls), but only 7 isolates from FS patients were studied. In South Africa, 6 of 9 GBS isolates were HS:41; this serotype accounts for only 0.1% of enteritis strains (8). In contrast, in The Netherlands and Belgium only two isolates from 12 GBS patients were HS:19, and none were HS:41 (5). Only one isolate from four FS patients was HS:2 (5). Our study confirms that GBS is significantly associated with HS:19 and that statistically FS is associated with HS:2. None of our *C. jejuni* isolates from GBS patients were HS:41. These findings are a strong indication that certain *C. jejuni* strains trigger the development of GBS, others the development of FS.

HS:19 strains, whether isolated from GBS patients or enteritis patients without neurological involvement, were related clonally (7, 19, 22), whereas clonality was lacking for other serotypes associated with GBS (5, 6). The hypothesis that a "bad bug causes GBS/FS" cannot be dismissed. The methods used may simply lack the power to detect particular *C. jejuni* strain determinants related to GBS/FS. There is cumulative evidence of molecular mimicry between human gangliosides and that the *C. jejuni* lipo-oligosaccharide (LOS) causes GBS/FS (31). Anti-GM1 and anti-GD1a IgG antibodies have been detected in patients who developed GBS subsequent to *C. jejuni* enteritis. Sensitization with a GM1-like LOS, as well as with GM1 ganglioside, has produced an animal model of GBS (32, 36). Analysis of the genes involved in the synthesis of ganglioside-like LOS may be of great importance for clarifying which *C. jejuni* strains involved in the pathogenesis of GBS/FS. The presence of LOS synthesis genes (*cst-II*, *cgtA*, and *cgtB*) is associated statistically with GBS-related isolates (20). GQ1b ganglioside is a target molecule for IgG antibody in FS (3), and all *C. jejuni* strains studied that bore the GQ1b epitope had *cst-II*, an essential gene for production of GQ1b-like LOS (27). Whether these genes are present in our GBS/FS isolates needs to be investigated, as well as whether their presence is more frequent than in the enteritis controls.

TABLE 3. Penner serogroup of isolates from GBS and FS and uncomplicated enteritis

Penner serogroup	Serotype(s)	No. of *C. jejuni* isolates (%) from:		
		GBS (n = 102)[a]	FS (n = 29)	Enteritis (n = 554)
A	1, 44	5 (5)	0	37 (7)
B	2	6 (6)	12 (41)[c]	80 (14)
C	3	1 (1)	1 (3)	21 (4)
D	4, 13, 16, 43, 50	8 (8)	11 (38)[d]	69 (12)
E	5	2 (2)	0	6 (1)
F	6, 7	1 (1)	1 (3)	10 (2)
G	8	3 (3)	0	21 (4)
O	19	68 (67)[b]	1 (3)	34 (6)
U	31	0	1 (3)	1 (0.2)
Y	37	3 (3)	0	33 (6)
Z	38	0	1 (3)	2 (0.4)
Untypeable		8 (8)	1 (3)	141 (25)

[a] Two strains were serotyped as A/G or O/Y serogroups.
[b] $P < 0.001$ (compared to the enteritis isolates; odds ratio [OR], 30.6; 95% confidence interval [CI], 17.9 to 52.4).
[c] $P < 0.001$ (compared to the enteritis isolates; OR, 4.2; 95% CI, 1.9 to 9.1).
[d] $P < 0.001$ (compared to the enteritis isolates; OR, 4.3; 95% CI, 1.9 to 9.5).

ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid from the Ichiro Kanehara Foundation, by the Kanae Foundation for Life & Socio-Medical Science, by the Japan Intractable Diseases Research Foundation, by a grant for Scientific Research (B) (KAKENHI 14370210) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by a Research Grant for Neuroimmunological Diseases from the Ministry of Health, Labour, and Welfare of Japan, by a Health Sciences Research Grant (Research on Psychiatric and Neurological Diseases and Mental Health) from the Ministry of Health, Labour, and Welfare of Japan, and by a grant from the Human Frontier Science Program (RGP 38/2003).

We thank the Divisions of Clinical Microbiology of the Tokyo Metropolitan Hospitals (Komagome and Bokutoh) for the isolation of the sporadic enteritis strains.

REFERENCES

1. **Asbury, A. K., and D. R. Cornblath.** 1990. Assessment of current diagnostic criteria for Guillain-Barré syndrome. Ann. Neurol. **27**(Suppl.):S21–S24.
2. **Boucquey, D., C. J. M. Sindic, M. Lamy, M. Delmée, J. P. Tomasi, and E. C. Laterre.** 1991. Clinical and serological studies in a series of 45 patients with Guillain-Barré syndrome. J. Neurol. Sci. **104**:56–63.
3. **Chiba, A., S. Kusunoki, T. Shimizu, and I. Kanazawa.** 1992. Serum IgG antibody to ganglioside GQ1b is a possible marker of Miller Fisher syndrome. Ann. Neurol. **31**:677–679.
4. **Emilia-Romagna Study Group on Clinical and Epidemiological Problems in Neurology.** 1998. Guillain-Barré syndrome variants in Emilia-Romagna, Italy, 1992–3: incidence, clinical features, and prognosis. J. Neurol. Neurosurg. Psychiatry **65**:218–224.
5. **Endtz, H. P., C. W. Ang, N. van Den Braak, B. Duim, A. Rigter, L. J. Price, D. L. Woodward, F. G. Rodgers, W. M. Johnson, J. A. Wagenaar, B. C. Jacobs, H. A. Verbrugh, and A. van Belkum.** 2000. Molecular characterization of *Campylobacter jejuni* from patients with Guillain-Barré and Miller-Fisher syndrome [sic]. J. Clin. Microbiol. **38**:2297–2301.
6. **Engberg, J., I. Nachamkin, V. Fussing, G. M. McKhann, J. W. Griffin, J. C. Piffaretti, E. M. Nielsen, and P. Gerner-Smidt.** 2001. Absence of clonality of *Campylobacter jejuni* in serotypes other than HS:19 associated with Guillain-Barré syndrome and gastroenteritis. J. Infect. Dis. **184**:215–220.
7. **Fujimoto, S., B. M. Allos, N. Misawa, C. M. Patton, and M. J. Blaser.** 1997. Restriction fragment length polymorphism analysis and random amplified polymorphic DNA analysis of *Campylobacter jejuni* strains isolated from patients with Guillain-Barré syndrome. J. Infect. Dis. **176**:1105–1108.
8. **Goddard, E. A., A. J. Lastovica, and A. C. Argent.** 1997. *Campylobacter* O:41 isolation in Guillain-Barré syndrome. Arch. Dis. Child. **76**:526–528.
9. **Hao, Q., T. Saida, S. Kuroki, M. Nushimura, M. Nukina, H. Obayashi, and K. Saida.** 1998. Antibodies to gangliosides and galactocerebroside in patients with Guillain-Barré syndrome with preceding Campylobacter jejuni [sic] and other identified infections. J. Neuroimmunol. **81**:116–126.

10. **Ho, T. W., B. Mishu, C. Y. Li, C. Y. Gao, D. R. Cornblath, J. W. Griffin, A. K. Asbury, M. J. Blaser, and G. M. McKhann.** 1995. Guillain-Barré syndrome in northern China: relationship to *Campylobacter jejuni* infection and anti-glycolipid antibodies. Brain **118**:597–605.
11. **Hughes, R. A. C., and J. H. Rees.** 1997. Clinical and epidemiologic features of Guillain-Barré syndrome. J. Infect. Dis. **176**(Suppl. 2):S92–S98.
12. **Jacobs, B. C., P. H. Rothbarth, F. G. A. van der Meché, P. Herbrink, P. I. M. Schmitz, M. A. de Klerk, and P. A. van Doorn.** 1998. The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. Neurology **51**:1110–1115.
13. **Kaplan, J. E., L. B. Schonberger, E. S. Hurwitz, and P. Katona.** 1983. Guillain-Barré syndrome in the United States, 1978–1981: additional observations from the national surveillance system. Neurology **33**:633–637.
14. **Kapperud, G., J. Lassen, S. M. Ostroff, and S. Aasen.** 1992. Clinical features of sporadic *Campylobacter* infections in Norway. Scand. J. Infect. Dis. **24**: 741–749.
15. **Koga, M., C. W. Ang, N. Yuki, B. C. Jacobs, P. Herbrink, F. G. A. van der Meché, K. Hirata, and P. A. van Doorn.** 2001. Comparative study of preceding *Campylobacter jejuni* infection in Guillain-Barré syndrome in Japan and The Netherlands. J. Neurol. Neurosurg. Psychiatry **70**:693–695.
16. **Koga, M., N. Yuki, M. Takahashi, K. Saito, and K. Hirata.** 1998. Close association of IgA anti-ganglioside with antecedent *Campylobacter jejuni* in Guillain-Barré syndrome. J. Neuroimmunol. **81**:138–143.
17. **Kuroki, S., T. Saida, M. Nukina, T. Haruta, M. Yoshioka, Y. Kobayashi, and H. Nakanishi.** 1993. *Campylobacter jejuni* strains from patients with Guillain-Barré syndrome belong mostly to Penner serogroup 19 and contain β-*N*-acetylglucosamine residues. Ann. Neurol. **33**:243–247.
18. **Linton, D., A. J. Lawson, R. J. Owen, and J. Stanley.** 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J. Clin. Microbiol. **35**: 2568–2572.
19. **Nachamkin, I., J. Engberg, M. Gutacker, R. J. Meinersman, C. Y. Li, P. Arzate, E. Teeple, V. Fussing, T. W. Ho, A. K. Asbury, J. W. Griffin, G. M. McKhann, and J. C. Piffaretti.** 2001. Molecular population genetic analysis of *Campylobacter jejuni* HS:19 associated with Guillain-Barré syndrome and gastroenteritis. J. Infect. Dis. **184**:221–226.
20. **Nachamkin, I., J. Liu, M. Li, H. Ung, A. P. Moran, M. M. Prendergast, and K. Sheikh.** 2002. *Campylobacter jejuni* from patients with Guillain-Barré syndrome preferentially expresses a GD_{1a}-like epitope. Infect. Immun. **70**: 5299–5303.
21. **National Institute of Infectious Diseases and Infectious Diseases Control Division, Ministry of Health and Welfare of Japan.** 1999. *Campylobacter* enteritis 1995–1998. Infect. Agents Surv. Rep. **20**:107–108. (In Japanese.)
22. **Nishimura, M., M. Nukina, S. Kuroki, H. Obayashi, M. Ohta, J. J. Ma, T. Saida, and T. Uchiyama.** 1997. Characterization of *Campylobacter jejuni* isolated from patients with Guillain-Barré syndrome. J. Neurol. Sci. **153**:91–99.
23. **Odaka, M., N. Yuki, and K. Hirata.** 2001. Anti-GQ1b IgG antibody syndrome: clinical and immunological range. J. Neurol. Neurosurg. Psychiatry **70**:50–55.
24. **Penner, J. L., and J. N. Hennessy.** 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. J. Clin. Microbiol. **12**:732–737.
25. **Rees, J. H., S. E. Soudain, N. A. Gregson, and R. A. C. Hughes.** 1995. *Campylobacter jejuni* infection and Guillain-Barré syndrome. N. Engl. J. Med. **333**:1374–1379.
26. **Skirrow, M. B.** 1987. A demographic survey of campylobacter, salmonella, and shigella infections in England: a public health laboratory service survey. Epidemiol. Infect. **99**:647–657.
27. **van Belkum, A., N. van den Brrak, P. Godschalk, W. Ang, B. Jacobs, M. Gilbert, W. Wakarchuk, H. Verbrugh, and H. Endtz.** 2001. A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. Nat. Med. **7**:752–753.
28. **van Koningsveld, R., P. A. van Doorn, P. I. M. Schmitz, C. W. Ang, and F. G. A. van der Meché.** 2000. Mild forms of Guillain-Barré syndrome in an epidemiologic survey in The Netherlands. Neurology **54**:620–625.
29. **Winter, D. K., A. E. O'Leary, and M. F. Slavik.** 1997. Rapid PCR with nested primers for direct detection of *Campylobacter jejuni* in chicken washes. Mol. Cell. Probes **11**:267–271.
30. **Wright, E.P.** 1982. Duration of excretion period of *Campylobacter jejuni* in human subjects, p. 294–298. *In* D. G. Newell (ed.), *Campylobacter*. MTP Press, Lancaster, United Kingdom.
31. **Yuki, N.** 2001. Infectious origins of, and molecular mimicry in Guillain-Barré and Fisher syndromes. Lancet Infect. Dis. **1**:29–37.
32. **Yuki, N., K. Susuki, M. Koga, Y. Nishimoto, M. Odaka, K. Hirata, K. Taguchi, T. Miyatake, K. Furukawa, T. Kobata, and M. Yamada.** 2004. Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barré syndrome. Proc. Natl. Acad. Sci. USA **101**:11404–11409.
33. **Yuki, N., Y. Tagawa, F. Irie, Y. Hirabayashi, and S. Handa.** 1997. Close association of Guillain-Barré syndrome with antibodies to minor monosialogangliosides GM1b and GM1α. J. Neuroimmunol. **74**:30–34.
34. **Yuki, N., M. Takahashi, Y. Tagawa, K. Kashiwase, K. Tadokoro, and K. Saito.** 1997. Association of *Campylobacter jejuni* serotype with antiganglioside antibody in Guillain-Barré syndrome and Fisher' s syndrome. Ann. Neurol. **42**:28–33.
35. **Yuki, N., and Y. Tsujino.** 1995. Familial Guillain-Barré syndrome subsequent to *Campylobacter jejuni* enteritis. J. Pediatr. **126**:162.
36. **Yuki, N., M. Yamada, M. Koga, M. Odaka, K. Susuki, Y. Tagawa, S. Ueda, T. Kasama, A. Ohnishi, S. Hayashi, H. Takahashi, M. Kamijo, and K. Hirata.** 2001. Animal model of axonal Guillain-Barré syndrome induced by sensitization with GM1 ganglioside. Ann. Neurol. **49**:712–720.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neuroscience Letters 368 (2004) 63–67
Neuroscience Letters
www.elsevier.com/locate/neulet

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

Keiichiro Susuki*, Yukihiro Nishimoto, Michiaki Koga, Takahide Nagashima, Izumi Mori, Koichi Hirata, Nobuhiro Yuki

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

Received 19 May 2004; received in revised form 19 June 2004; accepted 22 June 2004

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.

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

* Corresponding author. Tel.: +81 282 86 1111ext.2578; fax: +81 282 86 5884.
E-mail address: ksusuki@dokkyomed.ac.jp (K. Susuki).

0304-3940/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2004.06.059

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™; 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™) 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 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,*

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.

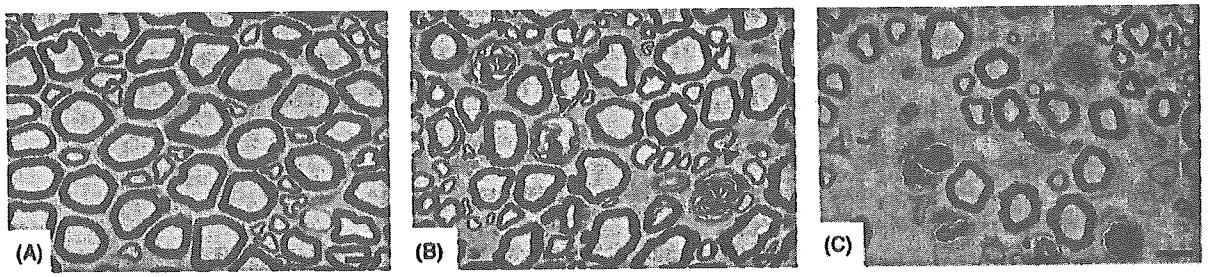


Fig. 1. 0.5% toluidine blue-stained transverse sections. (A) Sciatic nerve from a normal rabbit. (B) Sciatic nerve from rabbit Bg-12 (*Group 4* in Table 2; immunized to male NZW rabbit). Myelin ovoid produced by Wallerian-like degeneration of a myelinated fiber is occasionally noted (arrow heads), whereas 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.

Acknowledgments

The authors thank Dr. K. Yamaguchi (Institute of Medical Science, Dokkyo University School of Medicine) for the support of pathological evaluations; Ms. Y. Ueno and C. Yanaka for their technical assistance. K. Susuki is a Research Resident of the Japan Health Science Foundation. This research was supported by grants for Research on Specific Diseases from the Japan Health Sciences Foundation and for Scientific Research (B) (KAKENHI 14370210 to N.Y.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Health Sciences Research Grant (Research on Psychiatric and Neurological Diseases and Mental Health) from the Ministry of Health, Labour, and Welfare of Japan, and Human Frontier Science Program (RGP 38/2003).

References

[1] J.R. Broderson, A retrospective review of lesions associated with the use of Freund's adjuvant, Lab. Anim. Sci. 39 (1989) 400–405.
[2] I. Illa, N. Ortiz, E. Gallard, C. Juarez, J.M. Grau, M.C. Dalakas, Acute axonal Guillain-Barré syndrome with IgG antibodies against motor axons following parenteral gangliosides, Ann. Neurol. 38 (1995) 218–224.