overnight with serially diluted serum. Sera used were obtained from Patient 1 during the acute phase (day 5) and after recovery (10 months after onset), and from a patient with *C jejuni*-associated AMAN during the acute phase (positive control) and a healthy human (negative control). The test sections were incubated at 20 °C for 60 minutes with biotin-conjugated anti-human IgM or IgG antibodies (Vector Laboratories, Burlingame, CA) diluted in PBS (1:200). Samples also were incubated first at 4 °C overnight with diluted plasma from the GalC-sensitized demyelinating neuropathy rabbit or with diluted plasma from an AMAN model rabbit sensitized with a bovine brain ganglioside mixture (Cr-4 in the previous report[20]) then at 20 °C for 60 minutes with biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories). The AMAN model rabbit developed tetraparesis. Anti-GM1 IgG antibody was detected in the ELISA of plasma obtained within a week after onset. Axonal degeneration of the sciatic nerve was confirmed pathologically. The samples then were incubated at 20 °C for 30 minutes with the avidin-biotinylated enzyme complex (Vector Laboratories), after which they were incubated for 2 minutes at 20 °C in a solution of 50 m*M* Tris-hydrochloric acid buffer (pH 7.6) containing 0.025% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. The reaction was terminated by washing with PBS, then the slides were treated with 0.05% osmium tetroxide in distilled water for 2 minutes to intensify the 3,3'-diaminobenzidine tetrahydrochloride reaction products.

Search for the GM1 epitope in M pneumoniae. A lipid solution extracted from lyophilized *M pneumoniae* complement fixation antigen (Denka Seiken, Tokyo, Japan), as described elsewhere,[7] was layered and separated on TLC plates. To detect whether *M pneumoniae* had the GM1 epitope, the plates were incubated at 20 °C for 2 hours with the peroxidase-conjugated cholera toxin B-subunit (List Biologic Laboratories, Campbell, Canada; 1:2,000 dilution), which specifically recognizes the GM1-oligosaccharide. Antibody activities in plasmas from the AMAN model rabbit and demyelinative polyneuropathy rabbit also were examined as above.

Patients with GBS subsequent to M pneumoniae *infection.* Retrospective observations were made of patients who had developed GBS after *M pneumoniae* infection and been referred to our neuroimmunologic laboratory between 1996 and 2002 for serum anti-ganglioside antibody tests. They had had preceding respiratory infectious symptoms and elevated anti-*M pneumoniae* antibody (particle agglutination test). Their clinical records were reviewed to clarify the neurologic signs, laboratory findings, and electrophysiologic data. The electrodiagnosis of acute inflammatory demyelinating polyneuropathy (AIDP) or AMAN was made as described elsewhere[12] based on NCS findings during the acute phase. When the AMAN pattern and amplitude reduction of the sensory nerve action potentials (<80% of the lower normal limit) were present, acute motor-sensory axonal neuropathy was diagnosed. Serum samples obtained during the acute phase of neurologic illness were assayed for anti-glycolipid antibodies by an ELISA as described above.

Anti-GalC serology. To clarify the pathophysiologic roles of anti-GalC antibodies, IgM and IgG activities were examined by an ELISA, as above, of sera from four groups: consecutive samples from patients with various neurologic diseases who had been referred to our laboratory for serologic tests over a 2-month period; acute phase sera from GBS patients with a well-confirmed electrodiagnosis who were seen at Chiba University Hospital or affiliated hospitals and at Dokkyo University School of Medicine Hospital; *Mycoplasma*-associated neurologic diseases other than GBS were investigated retrospectively among patients who had been referred to us for serologic tests; sera from patients with clinically diagnosed acute respiratory diseases caused by *M pneumoniae* but who had no neurologic symptoms also were used in the anti-GalC and anti-GM1 antibody assays.

Results. *Anti-glycolipid antibody assays.* Patient 1. The ELISA of serum obtained from Patient 1 on day 5 showed high titers; 4,000 for the anti-GM1 IgM and IgG antibodies and 8,000 for the anti-GalC IgM and IgG antibodies (table 2). Both the anti-asialo-GM1 IgM and IgG antibody titers (2,000), as well as the anti-SLPG IgM antibody titer (500), were increased. Serum obtained 2 days before the onset of neurologic symptoms showed similar antibody activities; high titers of 4,000 for the anti-GM1 IgM, IgG, and anti-GalC IgM antibodies and 8,000 for anti-GalC IgG antibody. By day 144, the IgM and IgG antibody titers to GM1 and to GalC (500 each) had decreased.

Anti-GM1 antibodies were absorbed by GalC (figure 2) at rates of 89% (IgM) and 71% (IgG). Anti-GalC antibodies were absorbed by GM1 at rates of 72% (IgM) and 40% (IgG). None of the antibodies against GM1 and GalC were absorbed by GQ1b, a nonreactive antigen. The IgM from Patient 1 reacted slightly with both GlcCer and LacCer, whereas IgG did not react. Anti-GM1 and anti-GalC IgM antibodies were not absorbed by the glycolipids.

The reactivity of IgM and IgG with GM1 was confirmed on TLC plates (figure 3). IgM reacted with GalC. IgG did not. High anti-GalC IgG antibody activity was detected in the plasma from the rabbit sensitized with GalC.

Immunohistochemical study. IgM obtained on day 5 from Patient 1 selectively immunostained axons of rat ventral roots (figure 4), whereas that obtained from his serum 10 months after onset of neurologic symptoms produced no specific staining. Like the IgM from Patient 1, the IgG from the AMAN model rabbit and the patient with *C jejuni*-related AMAN selectively stained axons, whereas IgG from the GalC-sensitized rabbit did not. The IgG obtained on day 5 from Patient 1 did not produce specific staining, nor did the IgM and IgG from a healthy human.

GM1 epitope in M pneumoniae. The cholera toxin B-subunit stained a band in the lipid extract from *M pneumoniae,* as did the IgG from the AMAN model rabbit (figure 5). Anti-GM1 activities of the reagent and the IgG were confirmed. Neither the IgG nor IgM from the GalC-sensitized rabbit reacted with that band, but they did react with another band in the *M pneumoniae* lipid extract.

Clinical, electrophysiologic, and serologic features of patients with GBS after M pneumoniae *infection.* A preceding *M pneumoniae* infection was confirmed in seven GBS patients, including Patient 1. Their clinical, electrophysiologic, and serologic features are shown in table 2. Clinical symptoms of Patients 1 and 6 fluctuated after the initial therapy, another session being required. Patient 1 fulfilled the electrophysiologic criteria for AMAN, and Patient 3 those for AIDP.[12] NCS findings for Patient 2 showed the isolated F wave absence. Patient 4 had mild MCV delay in the lower limbs, which abnormality did not satisfy the criteria for AIDP. CMAP amplitudes were decreased in the right median and ulnar nerves of Patient 5, but MCV and distal latencies were normal. In Patients 2, 4, and 5, which mechanism, axonal damage or demyelination, was responsible for the abnormality could not be determined. Both anti-GalC IgM and IgG antibodies were present in Patients 1, 2, 4, 6, and 7. Patient 5 had low anti-GalC IgM antibody titer. Anti-GalC and anti-GM1 activities coexisted in Patients 1 (IgM and IgG classes), 2 (IgM), and 6 (IgG).

Anti-GalC serology. Anti-GalC serology is illustrated in (table 3). Serum samples from 129 patients with various neurologic diseases were examined: GBS, 57; Fisher syndrome, 6; Bickerstaff's brainstem encephalitis, 3; acute ophthalmoparesis, 2; chronic inflammatory demyelinating polyneuropathy, 14; multifocal motor neuropathy, 4; and other neurologic disorders, 43 (motor neuron disease, 9; polyneuropathy, 6; acute sensory neuropathy, 4; mononeuritis multiplex, ophthalmoparesis, and brainstem encepha-

Table 2 *Patients with Guillain-Barré syndrome after* Mycoplasma *infection*

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age, y/sex	29/M	29/M	32/M	11/F	2/F	47/M	13/M
Neurologic findings							
Ophthalmoplegia	–	–	–	–	–	–	–
Facial palsy	–	Bilateral	Bilateral	–	–	–	–
Bulbar palsy	–	–	+	–	–	–	–
Limb weakness	+	+	+	+	+	+	+
Hypo- or areflexia	+	+	+	+	+	+	+
Sensory disturbances	–	Hyperesthesia	Hypesthesia	–		Impaired position sense	–
Ataxia	–	–	+		–	Sensory ataxia	–
Autonomic nerve dysfunction	–	Anisocoria	–	Urinary retention, hypotension	–	–	–
CSF							
Cells (/μL)	12	45	4	12	23	3	11
Total protein (mg/dL)	69	61	107	33	16	66	63
Anti-*Mycoplasma* antibody titer	640	2,560	1,280	5,120	5,120	1,024	1,280
Cold agglutinin titer	2,048	64	16	1,024		1,024	64
Nerve conduction study	AMAN	Absent F wave	AIDP	Mild MCV delay	Decreased CMAP amplitude	Normal	
Anti-glycolipid antibody titers IgM antibodies to							
GM1	4,000	16,000	–	–	–	–	–
GalC	8,000	4,000	–	2,000	500	4,000	4,000
GM1b	–	4,000	–	1,000	–	–	–
SLPG	500	4,000		–		–	
Asialo-GM1	2,000	16,000		4,000		–	
Others	–	2,000: GM2, GD1a, GalNAc-GD1a, GT1b, and GQ1b 4,000: GD1b	–	–	–	–	–
IgG antibodies to							
GM1	4,000	–	–	–	–	1,000	–
GalC	8,000	32,000	–	1,000	–	16,000	8,000
GM1b	–	2,000	–	–	–	–	–
SLPG	–	–		–		–	
Asialo-GM1	2,000	4,000		–		2,000	
Others	–	2,000: GD1b	–	–	–	–	–

Blanks: information not available.

AMAN = acute motor axonal neuropathy; AIDP = acute inflammatory demyelinating polyneuropathy; MCV = motor nerve conduction velocity; CMAP = compound muscle action potential; Ig = immunoglobulin; GalC = galactocerebroside; SLPG = sialosyl lactosaminyl paragloboside.

litis, 3 each; polyneuritis cranialis and transverse myelitis, 2 each; cerebral infarction, epilepsy, cervical spondylosis, meningoencephalitis, radiculomyelitis, acute disseminated encephalomyelitis, Creutzfeldt-Jakob disease, acute autonomic and sensory ataxic neuropathy, neuralgic amyotrophy, myopathy, and tetanus, 1 each). Low IgM titer (500) for anti-GalC antibody was found only in the sample from a patient who developed GBS subsequent to *Mycoplasma* infection (Patient 5). Anti-GM1 IgG antibody was positive in nine patients with GBS alone. In two of the nine, *C jejuni* was isolated from stool samples and their sera did not have anti-GalC antibodies. Anti-GM1 IgM antibody was positive in four patients: three with GBS and one with multifocal motor neuropathy.

Serum anti-GalC antibodies of 136 GBS patients were examined: 49 with AIDP, 62 with AMAN, 2 with acute motor-sensory axonal neuropathy, and 23 with unclassified electrophysiology. Twelve of the 23 with unclassified NCS findings had isolated F wave absence. Three of 136 patients had had a preceding *Mycoplasma* infection (Patients 1, 2, and 3). Although anti-GalC antibodies were detected in the sera from Patients 1 (AMAN) and 2 (F wave absence), none of the other samples, including those from all 49 AIDP patients, showed anti-GalC antibody activity.

Five patients had various neurologic manifestations associated with *Mycoplasma* infection. Sera were obtained during the acute phase of neurologic illness: 6 days after the onset of respiratory disease from a patient with encephalitis, 10 days after from one with transverse myelitis, 14 days after from one with meningitis and polyneurop-

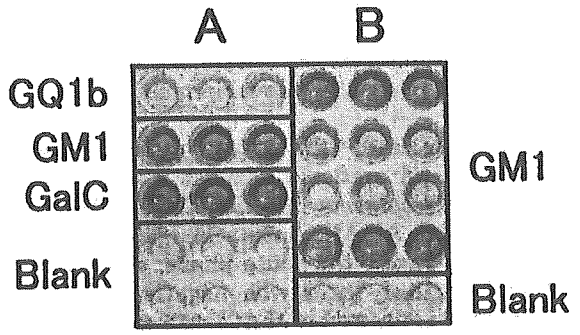

Figure 2 Microtiter plate from the anti-GM1 immunoglobulin M (IgM) antibody absorption study. (A) Serum samples diluted 1:500 were added to the wells, and the plate incubated at 4 °C overnight. Each sample was tested once in triplicate. (B) After incubation, the samples were used as the primary antibodies in the standard ELISA. They show decreased anti-GM1 IgM activity after incubation in wells coated with galactocerebroside, indicative of the cross-reactivity of the anti-GM1 IgM antibody with the glycolipid.

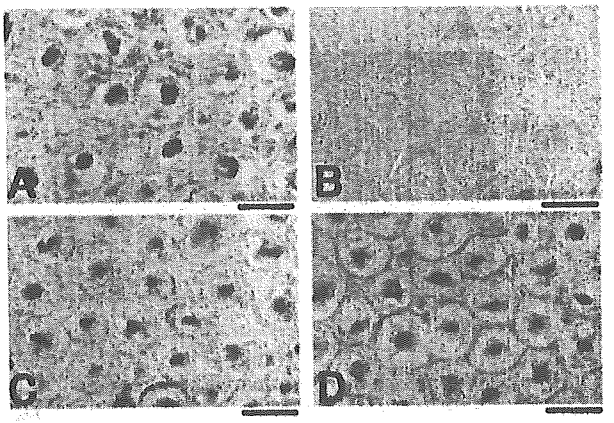

Figure 4. Immunohistochemical study results. Rat ventral roots have reacted with (A) serum obtained on day 5 from Patient 1 (dilution 1:250), and (B) 10 months after onset (dilution 1:100); (C) serum from a patient with acute motor axonal neuropathy (AMAN) subsequent to Campylobacter jejuni *infection (dilution 1:200); and (D) plasma obtained from an AMAN model rabbit sensitized with bovine brain ganglioside mixture within 1 week after onset of limb weakness (dilution 1:500). (A) Axons are selectively immunostained by the immunoglobulin (Ig)M from Patient 1 during the acute phase. (B) There is no selective immunostaining at the axons by the IgM from Patient 1 after recovery. The staining pattern is similar to that of the IgG for (C) the AMAN patient and (D) the AMAN model rabbit. Scale bars = 10 μm.*

athy, 17 days after from one with acute cerebellar ataxia, and 18 days after from one with meningoencephalitis and polyneuropathy. Two had elevated anti-GalC antibody titers during the acute phase: an IgM titer of 1,000 in one patient with acute cerebellar ataxia and 16,000 in one with meningoencephalitis and polyneuropathy. None of the five had anti-GM1 IgM or IgG antibody activity.

Twelve serum samples were examined from patients who had respiratory diseases caused by *M pneumoniae* but were without neurologic symptoms. Samples were obtained within several days of the onset of respiratory symptoms. An anti-GalC IgM antibody titer of 8,000 was detected in one sample, but no sample showed anti-GalC IgG activity. Anti-GM1 IgM and IgG antibodies were negative in all the sera.

Discussion. Patient 1 had tetraparesis with hyporeflexia 2 weeks after developing fever and a dry cough. There was neither cranial nerve nor sensory nerve involvement. NCS findings showed markedly decreased CMAP amplitudes without demyelination and preserved sensory nerve functions: neurologic and electrophysiologic findings compatible with AMAN. The serologic evaluation detected antecedent *M pneumoniae* infection. An ELISA showed both anti-GM1 and anti-GalC antibody present in the acute phase serum. Antibody activities 2 days before onset of neurologic illness were almost the same as those on day 5, indicative that the antibodies were the primary cause of GBS, not secondary phenomena produced by nerve damage, as discussed elsewhere.[21]

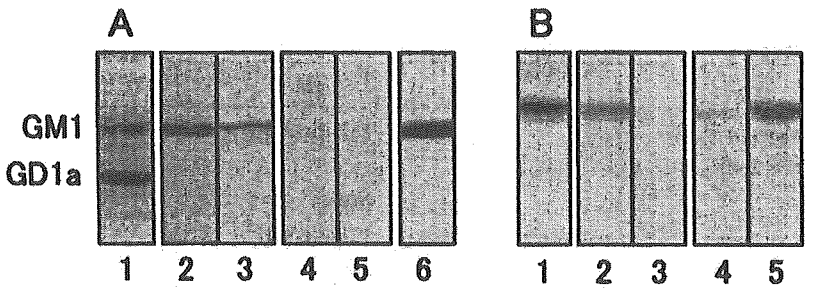

Figure 3. Thin-layer chromatogram with immunostaining. (A) Bovine brain ganglioside mixtures (the fraction enriched with GM1 and GD1a) and (B) galactocerebroside (GalC) were layered on thin-layer chromatography plates, which were developed with chloroform-methanol-0.2% calcium chloride in water (5:4:1). The plates next were stained with orcinol/sulfuric acid for hexose (lane 1), immunoglobulin (Ig)M (lane 2), and IgG (lane 3) from Patient 1 on day 5; IgM (lane 4) and IgG (lane 5) from a GalC-sensitized rabbit within 1 week after the onset of limb weakness; IgG from a patient who developed an acute motor axonal neuropathy (AMAN) after Campylobacter jejuni *enteritis (lane 6). Anti-GM1 IgM and IgG antibodies clearly are present in serum from Patient 1, as well as from the patient with* C jejuni*-related AMAN. Anti-GalC IgM antibody reactivity is present, but no IgG antibody reactivity in the serum from Patient 1. This pattern differs markedly from that of the plasma from the GalC-sensitized rabbit, which shows high anti-GalC IgG antibody activity but no anti-GM1 IgM and IgG antibody activities.*

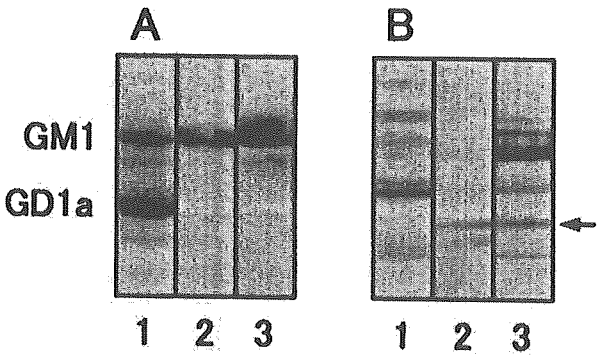

Figure 5. GM1 epitope in Mycoplasma pneumoniae *shown in an immunostained thin-layer chromatogram. (A) Bovine brain ganglioside mixtures (the fraction enriched with GM1 and GD1a) and (B) lipid extract from the lyophilized* Mycoplasma pneumoniae *complement fixation antigen were layered on thin-layer chromatography plates. The plates were developed with chloroform-methanol-0.2% calcium chloride in water (5:4:1), then stained with orcinol/sulfuric acid for hexose (lane 1), the peroxidase-conjugated cholera toxin B-subunit (lane 2), and the immunoglobulin G (IgG) from an acute motor axonal neuropathy model rabbit obtained within 1 week after onset of limb weakness (lane 3). (A) Both the cholera toxin B-subunit and rabbit IgG are bound to GM1. (B) The cholera toxin B-subunit is bound to a band in the lipid extract from* M pneumoniae, *which also reacted with rabbit IgG (arrow).*

Anti-glycolipid antibodies other than anti-GalC and anti-GM1 antibodies have been reported in *Mycoplasma*-associated GBS: anti-SLPG IgM antibody in a patient who showed markedly delayed MCV and temporal dispersion,[22] and in one with anti-GM1b IgG antibody (NCS was not performed).[23] Although anti-SLPG IgM antibody was present in Patient 1, its titer was not as high as the anti-GM1 and anti-GalC antibody titers. TLC with immunostaining confirmed both IgM and IgG activities against GM1. An immunohistochemical study showed that during the acute phase the patient's IgM selectively stained axons, whereas his IgG did not. Moreover, IgM from serum obtained after his recovery did not cause specific staining. The IgG from a GalC-sensitized rabbit did not stain axons. These findings indicate that anti-GM1 IgM antibody is necessary to the development of axonal dysfunction.

In a similar case,[24] the patient developed severe tetraparesis and areflexia without sensory disturbance after *Mycoplasma* infection. NCS results were compatible with AMAN. Anti-GM1 IgM antibody titer was high during the acute phase, whereas anti-GM1 IgG antibody titer was negative. Anti-GalC antibody was not tested in that case report.

Both the cholera toxin B-subunit and IgG antibodies from the AMAN model rabbit stained a band in the *M pneumoniae* lipid extract. Anti-GM1 IgG activity in a patient who developed GBS after a *Mycoplasma* infection was inhibited by preincubation with the *M pneumoniae* reagent.[7] These findings are evidence that the GM1 epitope is present in *M pneumoniae* and indicate that molecular mimicry exists between the bacterium and peripheral nerve tissue. Anti-GM1 IgM antibody may have been induced by the GM1 epitope of *M pneumoniae*, the preceding infectious pathogen, and caused axonal dysfunction in our Patient 1. The chemical structure of the *M pneumoniae* isolated from AMAN patients needs to be determined, as has been done for *C jejuni*.[1]

Anti-GM1 antibodies also have been reported in patients with neurologic manifestations other than GBS associated with *Mycoplasma* infection: in one with chronic polyneuropathy,[25] and in another with meningoencephalitis and cerebellitis.[26] In our series, anti-GM1 antibodies were negative in all five patients who had neurologic illnesses other than GBS preceded by *Mycoplasma* infection. In those patients, the periods between the respiratory disease and serologic examination were similar to those between the prior illness and onset of GBS. Although samples were obtained earlier than those from GBS patients, anti-GM1 antibodies also were negative in all 12 patients who had an acute respiratory disease caused by *M pneumoniae* but no neurologic symptoms.

We confirmed the previous finding that a GalC epitope is present in the *M pneumoniae* lipid ex-

Table 3 *Anti-galactocerebroside antibody assay results*

Patients	IgM	IgG
Consecutive patients with various neurologic conditions (n = 129)	1 (Patient 5)	None
Patients with electrophysiologically verified GBS (n = 136)		
AIDP (n = 49)	None	None
AMAN (n = 62)	1 (Patient 1)	1 (Patient 1)
AMSAN (n = 2)	None	None
Unclassified (n = 23)	1 (Patient 2)	1 (Patient 2)
Patients with neurologic diseases other than GBS (n = 5)	2	None
Respiratory diseases caused by *Mycoplasma* but no neurologic symptoms (n = 12)	1	None

Ig = immunoglobulin; GBS = Guillain-Barré syndrome; AIDP = acute inflammatory demyelinating polyneuropathy; AMAN = acute motor axonal neuropathy; AMSAN = acute motor-sensory axonal neuropathy.

tract[7]; therefore, *M pneumoniae* carries both the GM1 and GalC epitopes. Although GalC is a major myelin component, antibodies from Patient 1 reacted with axons rather than myelin. In that patient, antibody reactivity to GM1 may have been more pathogenic than that to GalC.

As in Patient 1, anti-GalC and anti-GM1 antibodies coexist in some patients with *Mycoplasma*-associated GBS. Electrophysiologic data, however, vary. In our series, Patient 2 had high anti-GM1 as well as anti-GalC IgM antibody titer, but the NCS showed isolated F wave absence, which has been interpreted as demyelinative conduction block in proximal nerve segments when distal CMAP are preserved. During the early phase of GBS associated with anti-ganglioside antibodies, however, the block of F wave generation may be caused by axonal conduction abnormalities in the nerve roots, the mechanism of which may be physiologic conduction block or axonal degeneration, as in AMAN.[27] Which mechanism, demyelination or axonal dysfunction, was responsible for the abnormality could not be determined from NCS data. The latter interpretation, however, seems likely for Patient 2, who had anti-glycolipid antibodies. NCS findings showed no abnormalities in Patient 6 who had anti-GM1 as well as anti-GalC IgG antibodies. Interestingly, positive anti-GM1 antibody activities have been reported in three of four anti-GalC positive GBS patients who had had a *Mycoplasma* infection.[10] No NCS findings were mentioned in that report, but two of the four patients were confirmed to have AIDP electrophysiologically (S. Kusunoki, personal communication). NCS was not performed for the others. Of the 11 anti-GalC-positive patients with *Mycoplasma*-related GBS,[11] three had coexisting anti-GM1 and anti-GalC antibodies (IgM class in two, IgG in one) but there were no NCS findings for those three (C.W. Ang, personal communication).

Similar anti-glycolipid antibody patterns of coexisting anti-GalC and anti-GM1 activities may be the cause of demyelination in some patients and axonal dysfunction in others. This may be because of differences in the fine specificities of the antibodies, as reported for anti-LM1 IgG antibodies.[17] Cross-reactivity of anti-GalC antibody with GM1 has been reported.[28] Similarly, in Patient 1, anti-GalC antibody cross-reacted with GM1 and anti-GM1 antibody with GalC. The reacting epitopes of these antibodies may be the terminal β1-linked galactosyl groups common to GM1 (Gal β1–3 GalNAc β1–4 [NeuAc α2–3] Gal β1–4 Glc β1–1 Cer), and GalC (Gal β1–1 Cer), but GD1b with the same terminal groups was not reactive. These antibodies did not cross-react with GlcCer (Glc β1–1 Cer), LacCer (Gal β1–4 Glc β1–1 Cer), or GM2 (GalNAc β1–4 [NeuAc α2–3] Gal β1–4 Glc β1–1 Cer), and their reactive epitopes remain unknown. In contrast, in Patient 2 no anti-GM1 antibodies were absorbed by GalC, and no anti-GalC antibodies were absorbed by GM1 (data not shown). The cross-reactivity of those autoantibodies was not investigated in an earlier study.[10]

Although anti-GalC antibody is considered a cause of AIDP, we could not confirm it. One patient with anti-GalC activity (Patient 4) showed mild MCV delay but did not satisfy the electrophysiologic criteria for AIDP. Patient 3, for whom the electrodiagnosis was AIDP, did not have anti-GalC antibodies. High anti-GalC antibody titers were detected in Patient 1 with AMAN and in Patient 2 who had F wave absence. None of the 49 patients with electrophysiologically verified AIDP had anti-GalC antibodies.

Our findings confirmed only the association of anti-GalC antibodies with *Mycoplasma* infection. Out of 129 consecutive cases of various neurologic disorders, only one patient with *M pneumoniae*-associated GBS had the anti-GalC IgM antibody (Patient 5). Anti-GalC antibody was negative in all the others, including two who developed anti-GM1 IgG-positive GBS after *C jejuni* enteritis. Six of seven patients who developed GBS after *Mycoplasma* infection had anti-GalC antibodies. Anti-GalC antibodies also were positive in patients who had acute cerebellar ataxia, meningoencephalitis and polyneuropathy (present study), encephalitis,[29] and encephalomyelitis[30] associated with *Mycoplasma* infection. Furthermore, anti-GalC antibodies were detected in 1 of 12 (present study), 6 of 33,[31] 8 of 32,[29] and 11 of 19[11] serum samples from patients who had respiratory diseases caused by *M pneumoniae* infection but no neurologic signs. The number of anti-GalC antibody-positive cases may have been underestimated in our study because the 12 serum samples were obtained within several days of the onset of respiratory disease, whereas the times when samples were obtained were not mentioned in previous reports.[11,29,31]

Our findings suggest that the presence of anti-GalC antibodies is an epiphenomenon related to preceding *M pneumoniae* infection and that these antibodies do not have a pathophysiologic role in the development of GBS and other neurologic illnesses. The pathophysiologic function of anti-GM2 IgM antibody in CMV-related GBS is still controversial because it frequently has been found in GBS patients who did not have a preceding CMV infection and in non-GBS patients who had acute CMV infection.[32] In contrast, patients who had had *C jejuni* enteritis but did not develop a neurologic disorder did not have the anti-GM1 IgG antibody,[33,34] indicative that that antibody is important to the pathophysiologic function in *C jejuni*-associated AMAN.

Patients who develop GBS after *Mycoplasma* infection have diverse clinical, serologic, and electrophysiologic findings. Careful, detailed studies are required to clarify the features of *Mycoplasma*-associated GBS. Our study showed that in certain cases AMAN associated with anti-GM1 antibody may develop after *Mycoplasma* infection. The GM1 epitope was found to have been present in the *M pneumoniae* lipid extract, additional evidence that

the pathogenesis of GBS is based on molecular mimicry.

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Acute facial diplegia and hyperreflexia

A Guillain–Barré syndrome variant

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Abstract—Two patients with acute facial diplegia and hyperreflexia are described. Both patients had serologic evidence of preceding *Campylobacter jejuni* infection and antiganglioside IgG antibodies as well as other laboratory and electrophysiologic findings suggesting Guillain–Barré syndrome (GBS). IV immunoglobulin produced recovery. Hyperreflexia does not necessarily exclude the diagnosis of a GBS variant. Antiganglioside antibodies can help with diagnosis in difficult cases.

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Bilateral simultaneous facial palsy, an uncommon neurologic manifestation, can result from a number of etiologies.[1] A frequent cause is Guillain–Barré syndrome (GBS), and the loss of deep tendon reflexes helps establish this diagnosis.[1] When bifacial weakness is accompanied by hyperreflexia, cerebral diseases such as brainstem encephalitis, pontine glioma, and stroke must be considered. We describe two patients with a misleading neurologic presentation of acute facial diplegia with brisk tendon jerks that was ultimately determined to represent a GBS variant.

Case reports. *Patient 1.* A 34-year-old woman had watery diarrhea for 3 days. Two weeks later, she experienced numbness in both hands (day 1), followed by horizontal diplopia and right facial weakness on day 7. She was admitted on day 9, at which time neurologic examination showed moderate facial diplegia. She could not close both eyes tightly or purse her lips, predominantly on the right side. Taste, lacrimation, and hearing were normal. Abduction was minimally restricted in the right eye. Biceps and patellar reflexes were brisk, and the plantar response was flexor. She reported numbness in both hands, but sensory testing was normal. Brain MRI was unremarkable. Nerve conduction studies of the limbs were normal. Blink reflex testing on day 13 revealed facial nerve involvements (table). Administration of IV immunoglobulin (IVIg; 0.4 g/kg/day) and methylprednisolone (0.5 g/day) for 5 days rapidly ameliorated her distal limb paresthesias. Facial diplegia gradually lessened and resolved by day 58. On day 135, she was free of neurologic symptoms, and deep tendon reflexes were normal.

Patient 2. A 32-year-old woman experienced difficulty in swallowing (day 1), mild weakness of the right hand (day 3), neck weakness (day 4), and facial weakness (day 6). There were no symptoms of a preceding infection. On day 14, examination showed facial diplegia. Tear flow was increased, but taste and hearing were normal. The patient reported difficulty swallowing, but neurologic evaluation of the lower cranial nerves was unremarkable. Strength in the neck muscles was moderately decreased. There was mild weakness in both upper limbs. Grip was 11 kg on the right side and 12 kg on the left side. Deep tendon reflexes were brisk throughout, with flexor plantar responses. CSF protein was elevated with a normal cell count (see the table). Brain MRI was unremarkable. Nerve conduction studies of the limbs were normal on day 15. A motor conduction study of the left facial nerve showed a low amplitude (0.052 mV) with an essentially normal latency (3.6 milliseconds). Blink reflex testing on day 29 showed delayed R1 and R2 latencies (see the table). She received IVIg (0.4 g/kg/day, 5 days) and methylprednisolone pulse therapy (1 g/day, 3 days). Symptoms reached a nadir on day 23, after which the patient began improving. She was almost fully recovered by day 72. One year after onset, there were no neurologic symptoms or findings.

Serologic findings. Serologic assay, as reported elsewhere,[2] confirmed evidence of recent *Campylobacter jejuni* infection in both patients (see the table). Antibodies to cytomegalovirus *Haemophilus influenzae* and *Borrelia Burgdorferi* were negative in both paitents. Serum IgG and IgM antibodies to gangliosides GM2, GM1, GM1b, GD1a, GalNAc-GD1a, GD1b, GT1a, and GQ1b were investigated by ELISA. During the acute phase, high IgG antibody titers to gangliosides, including GT1a, were detected (see the table). Thin-layer chromatography with immunostaining[3] confirmed IgG activity to GT1a in Patient 1 and to GT1a and GD1a in Patient 2 (figure). An absorption test was performed.[4] In Patient 1, anti-GT1a IgG antibody was absorbed by GQ1b and anti-GQ1b IgG was absorbed by GT1a at rates of 45 and 38%, respectively. This indicated cross-reactivity of these antibodies. None of the antibodies against GT1a and GQ1b was absorbed by GM1 or GD1a, which were nonreactive antigens. In contrast, in Patient 2, no anti-GT1a IgG was absorbed by GQ1b or GD1a, and no anti-GD1a IgG was absorbed by GT1a.

Discussion. Both our patients presented with an acute onset of facial diplegia accompanied by other mild symptoms such as diplopia, dysphagia, and neck and limb weakness. The presence of hyperreflexia confused us, and in both patients, cerebral disease was initially suspected. Brain MRI was unremarkable, but electrophysiologic studies indicated peripheral involvement of the facial nerves. Both patients had features suggestive of GBS: watery diarrhea in Patient 1 and CSF albuminocytologic dissociation in Patient 2. Patients with bifacial but no (or minimal) limb weakness, acral paresthesias, and diminished tendon jerks are considered to have a regional variant of GBS called facial diplegia and paresthesias.[5] Hyperreflexia, however, was inconsistent with the diagnosis of GBS.

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Table *Features of two patients with acute facial diplegia and hyperreflexia*

Features	Patient 1	Patient 2
Age, y/sex	34/F	32/F
Ophthalmoplegia	Minimal	No
Facial weakness	Bilateral	Bilateral
Bulbar palsy	No	Minimal
Cervical weakness	MRC 4	MRC 3
Limb weakness	No	Mild in both upper limbs
Deep tendon reflexes	Brisk	Brisk
Sensory disturbance	Paresthesias in both hands	No
Ataxia	No	No
CSF		
Cells, /μL	1	3
Total protein, mg/dL	29	67
Blink reflex, ms, right/left		
R1 (mean ± SD = 10.5 ± 0.8)*	Not evoked/12.4	15.2/14.8
Ipsilateral R2 (30.5 ± 3.4)*	Not evoked/35.2	36.8/41.4
Contralateral R2 (30.5 ± 4.4)*	Not evoked/35.8	40.2/36.0
Serum antibody titers to *C. jejuni*		
IgG (<2,000)*	4,000/1,000	32,000/4,000
IgA (<500)*	2,000/<250	1,000/<250
IgM (<500)*	500/500	<500/<500
Antiganglioside serology		
IgG antibody titers to (<500)*	GT1a: 4,000/<500	GT1a: 16,000/1,000
	GQ1b: 4,000/<500	GD1a: 16,000/2,000

Serologic findings were evaluated with sera obtained during the acute phase/after recovery: day 9/day 58 in Patient 1 and day 14/day 72 in Patient 2. CSF examination was done on day 11 in Patient 1 and on day 25 in Patient 2.

* Normal range.

MRC = Medical Research Council grade; Ipsilateral R2 = R2 components elicited by ipsilateral stimulation; Contralateral R2 = R2 elicited by contralateral stimulation.

Some patients who develop a motor form of GBS after *C. jejuni* enteritis may manifest brisk deep tendon reflexes.[6,7] The mechanism that causes hyperreflexia in these cases is unknown. Hyperexcitability of the motor neurons and dysfunction of the spinal inhibitory interneurons have been proposed.[7]

Antiganglioside serology also supported the diagnosis of a GBS variant. Patient 1 had anti-GT1a and anti-GQ1b IgG antibodies that cross-reacted with each other. This antibody pattern is characteristic of Fisher syndrome[4] and may explain the minimal ophthalmoparesis in this patient. Patient 2 had anti-GT1a and anti-GD1a antibodies without cross-reactivity, minimal bulbar palsy, and neck and mild upper limb weakness. These features are similar to those observed in a previously reported patient.[8] GD1a and GQ1b are both present in human facial nerve.[9] Whether GT1a is present in human facial nerves as well is not known. GBS patients with anti-GT1a antibodies do, however, often develop facial palsy.[3] It is not clear why facial palsy was more prominent than other cranial nerve palsies in our patients. It may represent individual

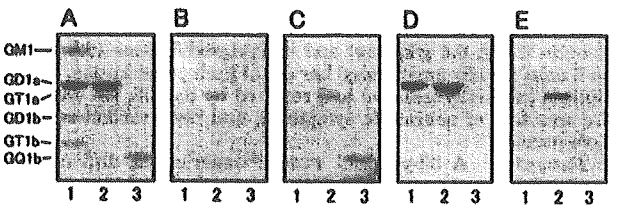

Figure. Thin-layer chromatogram with immunostaining. Lane 1, bovine brain ganglioside mixtures; lane 2, fraction containing GD1a and GT1a, separated from bovine brain ganglioside mixtures by Q-Sepharose column chromatography; lane 3, authentic GQ1b ganglioside. (A) Plate stained with orcinol/sulfuric acid for hexose. (B) Anti-GT1a IgG antibody is detectable in serum obtained from Patient 1 on day 9, whereas anti-GQ1b IgG reactivity is questionable. (C) Both anti-GQ1b and anti-GT1a IgG antibodies are present in serum from a patient with acute ophthalmoparesis. (D) Both anti-GT1a and anti-GD1a IgG antibodies clearly are present in serum obtained from Patient 2 on day 14. (E) Serum from a patient with typical pharyngeal–cervical–brachial weakness showing monospecific IgG antibody to GT1a.

variations in ganglioside distribution. Some *C. jejuni* strains isolated from patients with GBS have lipo-oligosaccharides that bear a GT1a-like structure,[10] and molecular mimicry may have been involved in the development of the facial palsy in our patients.

When acute bifacial weakness occurs, identifying GBS is important because these patients may benefit from IVIg therapy. Hyperreflexia does not exclude GBS or one of its variants. Features such as limb paresthesias, CSF albuminocytologic dissociation, electrophysiologic blink studies, and anti-ganglioside IgG antibodies can be helpful in diagnosing GBS in such cases. Finding evidence of a preceding *C. jejuni* infection can also be very useful.

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CDR3 spectratyping analysis of the T cell receptor repertoire in Guillain–Barré and Fisher syndromes

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Abstract

Several autoimmune and infectious disorders show oligoclonal expansion of particular T cell phenotypes. The extent of T cell involvement in the pathogenesis of Guillain–Barré syndrome (GBS), a post-infectious autoimmune neuropathy, however, is not clear. To identify the pathogenic T cell phenotypes in GBS and Fisher syndrome (FS), variations in T cell receptor use of the Vβ1-24 and Vδ1-5 chain genes were analyzed at complementarity-determining region 3 level in 119 patients with GBS or FS. Overall, Vβ and Vδ spectratypes were expanded more frequently in patients with GBS (Vβ in 77%, Vδ in 53%) or FS (Vβ in 75%, Vδ in 65%) than in the healthy controls (Vβ in 59%, Vδ in 38%). No particular spectratype was significantly associated with GBS or FS. Subgrouping the patients by *Campylobacter jejuni* serology and anti-ganglioside IgG antibodies also failed to detect particular spectratype gene use. The frequency of Vβ5.2 expansion tended to be higher in patients with positive *Haemophilus influenzae* serology (50%) than in the controls (7%), but the difference was not significant. Our findings show that oligoclonal expansion of T cells bearing particular type T cell receptor Vβ and Vδ genes frequently occurs in GBS and FS, suggestive that T cells mediate the development of these neuropathies. The predominant phenotypes vary, even within subgroups of patients with a syndrome of single etiological origin or those with uniform serological features.

Keywords: Guillain–Barré syndrome; T cell receptor; CDR3 spectratyping; Anti-ganglioside antibody; Antecedent infection

1. Introduction

Guillain–Barré syndrome (GBS) originally was considered a neuropathy, in which primarily myelin is disturbed, and was first called acute inflammatory demyelinating polyneuropathy (AIDP). Experimental autoimmune neuritis (EAN) closely resembles AIDP and it can be transferred to naive rats by T cell lines established by repeated stimulation with a myelin component (Linington et al., 1984). These findings suggest that AIDP is a T cell-mediated disorder. In contrast, axonal variants called acute motor axonal neuropathy (AMAN) and acute motor-sensory axonal neuropathy (AMSAN) are now widely recognized (Griffin et al., 1996). The humoral immune response to gangliosides is considered a key to the pathogenesis of AMAN and AMSAN (Willison and Yuki, 2002). The main IgG subclasses of anti-ganglioside antibodies, IgG1 and IgG3, are characteristic of a T cell-dependent antibody response, indicative that T cells also mediate the development of axonal GBS (Guijo et al., 1992; Yuki et al., 1995).

T cell immune responses are initiated by T cell receptor (TCR) recognition of antigen present in the peptide groove of the major histocompatibility complex. Each TCR has two highly variable chains, α and β or γ and δ, and extensive sequence variability is achieved by the rearrangement of scattered gene segments, causing T cells to recognize various antigens (Benoist and Mathis, 1999). Vβ gene use of peripheral blood lymphocytes first was investigated in four patients with AIDP and one with AMSAN (Khalili-Shirazi et al., 1997). In AIDP Vβ15 was found to be frequently used by activated T cells. Later, the percentages of Vδ1+ and Vδ2+ T cells in peripheral blood were examined in eight patients with GBS (Borsellino et al., 2000). Vδ1+ T cells were more frequent in the GBS patients than in those with multiple sclerosis (MS) or the healthy controls. GBS, however, is a heterogeneous disorder caused by various pathogens and anti-ganglioside antibodies (Wil-

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lison and Yuki, 2002). The above studies were done on small populations of patients and, more importantly, did not consider the type of antecedent infection or anti-ganglioside antibodies present.

Identification of pathogenic TCRs may aid in the development of immunotherapy that uses monoclonal antibodies and DNA vaccines to target disease-related TCR (Matsumoto, 2000). We used the complementarity-determining region (CDR) 3 spectratyping method, which is suitable for identifying particular TCR use by peripheral blood lymphocytes (Kim et al., 1998), to investigate the TCR repertoire in a large population of patients with GBS or Fisher syndrome (FS). The GBS-FS population was divided into subgroups based on single etiological origin (*Campylobacter jejuni* or *Haemophilus influenzae*) or anti-ganglioside antibodies present. Pathogenic TCR use in each subgroup, as well as in the entire population then was investigated.

2. Materials and methods

2.1. Patients and healthy subjects

One hundred nineteen patients (77 men, 42 women; median age 41 years [range 10–86 years]) with GBS ($n=83$) or FS ($n=36$), who had been referred to the Neuroimmunological Laboratory of Dokkyo University from university and district general hospitals throughout Japan for anti-ganglioside antibody testing were studied. They fulfilled the clinical criteria for GBS (Asbury and Cornblath, 1990) or FS (Odaka et al., 2001). On receipt of serum samples for antibody testing, we requested the physicians also to send 10 ml of heparinized blood drawn from their patients within 1 month after disease onset. Blood samples taken during the recovery phase were collected when available. Twenty-nine healthy volunteers (13 men, 16 women; median age 36 years [range 22–58 years]) who had no history of common cold or influenzae infection during the previous month served as controls. There was no significant difference in median age between the patients and healthy controls (Mann–Whitney *U*-test), whereas the proportion of males was slightly higher in the patient group ($p=0.049$).

2.2. CDR3 spectratyping

RNA was extracted from peripheral blood mononuclear cells by means of RNAzol B (Baotecx Lab, Houston, USA). cDNA then was synthesized by reverse transcription with ReverTra Ace (TOYOBO, Osaka, Japan). CDR spectratyping was done as described elsewhere (Kim et al., 1998). Briefly, cDNA was amplified in a thermal cycler (Perkin Elmer, Norwalk, USA) by means of primer pairs for TCR. The primers for Vβ1-24 and Vδ1–5 were the same as in previous studies (Choi et al., 1989; Stinissen et al., 1995). Undiluted or diluted PCR products were added to an equal volume of formamide/dye loading buffer and heated at 94 °C for 2 min. Two microliters of each sample was applied to a 6% acrylamide sequencing gel, and the gel run at 30 W for 3 h 30 min at 50 °C. The fluorescence-labeled DNA profile on the gel was recorded directly by a FMBIO fluorescence image analyzer (Hitachi, Yokohama, Japan). Spectratype expansion, evaluated by visual inspection, was based on the following criteria (Maini et al., 1998): The "oligoclonal pattern" of spectratype expansion was an increase in the density and thickness of a band, the other bands remaining normal (distortion of the Gaussian distribution). The "monoclonal" type of spectratype expansion was taken to be a marked increase in the density and thickness of a band with faint or no additional spectratypes (monoclonal pattern).

2.3. Serology

Serum IgG antibodies to GM2, GM1, GM1b, GD1a, GalNAc-GD1a, GD1b, GT1a, GT1b, and GQ1b were measured by an enzyme-linked immunosorbent assay as described elsewhere (Yuki et al., 1997). Serum was considered positive when the antibody titer was 1000 or more. Recent *C. jejuni* or *H. influenzae* infection was assayed serologically as reported elsewhere (Koga et al., 1998a, 2001).

2.4. Statistical analysis

Differences between groups were examined with the χ^2 test or Fisher's exact test when the number of variables expected per cell was less than 5. A difference was considered significant when p was less than 0.05, except in the analysis for ascertaining the cluster increase of a particular spectratype, for which the Bonferroni correction was made based on the number of spectratypes examined. A corrected p value of less than 0.05 was considered significant. The statistical analyses were done with Statcel® software (OMS, Saitama, Japan).

3. Results

3.1. Serology

Positive serology for *C. jejuni* was found for 41 patients with GBS or FS, and for *H. influenzae* eight patients. Ninety-four patients had IgG antibodies against gangliosides: GM2 ($n=2$), GM1 ($n=39$), GM1b ($n=45$), GD1a ($n=29$), GalNAc-GD1a ($n=23$), GD1b ($n=32$), GT1a ($n=35$), GT1b ($n=6$), and GQ1b ($n=41$).

3.2. CDR3 spectratyping analysis

Fig. 1 shows the spectratype profile of a patient with GBS. The normal spectratype pattern (asterisk) has a Gaussian distribution and no spectratype expansion. In

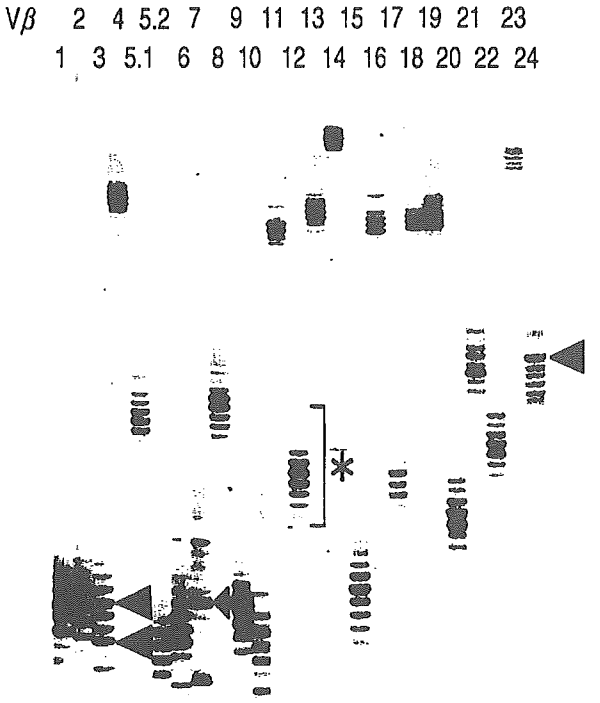

Fig. 1. Complementarity-determining region 3 spectratyping of the T cell receptor of peripheral blood lymphocytes from a patient with Guillain–Barré syndrome. The Vβ7 spectratype (arrow) shows marked monoclonal spectratype expansion. There is additional oligoclonal expansion in the Vβ3 and Vβ24 spectratypes (arrowheads).

sharp contrast, one of the Vβ7 spectratypes is expanded markedly (arrow, Fig. 1). The Vβ3 and Vβ24 spectratypes show moderate oligoclonal expansion (arrowheads).

Expansion of one or more Vβ and Vδ spectratypes was more frequent in patients with GBS or FS than in the healthy controls (Table 1). Expansion frequencies were similar between subgroups divided by diagnosis. *C. jejuni* or *H. influenzae* infection, and anti-ganglioside antibodies. Frequencies of each type of spectratype expansion also were compared statistically between the patients and healthy controls. No particular spectratype was significantly associated with GBS or FS. Vβ7 and Vδ2 expansion, however, tended to be more frequent in the patients (13% and 19%) than in the controls (0% and 4%) (Table 2). The frequency of Vβ7 expansion was almost the same for all the subgroups (Fig. 2). In contrast, Vδ2 predominantly was expanded in the patients with FS (27%: uncorrected p, 0.02; corrected p, 0.11; odds ratio, 8.5; 95% confidence interval [CI], 1.3–54) or with anti-ganglioside IgG antibodies (20%; uncorrected p, 0.049; corrected p, 0.25; odds ratio, 5.8; 95% CI, 0.9–37). Of the antibodies, anti-GM1b (27%), anti-GT1a (27%), and anti-GQ1b (23%) IgG tended to be related to Vδ2 expansion. In patients negative for all the anti-ganglioside IgG antibodies, Vβ15 gene use was fairly frequent (16% versus 3%: uncorrected p, 0.13). Four (50%) of 8 patients

Table 1
Vβ and Vδ expansion frequencies

	Vβ expansion		Vδ expansion	
	Frequency (%)	p value (compared with healthy subjects)	Frequency (%)	p value (compared with healthy subjects)
Healthy subjects	59	–	38	–
GBS and FS	76	0.052	57	0.09
GBS	77	0.055	53	0.2
FS	75	0.16	65	0.04
C. jejuni-related GBS and FS	78	0.08	50	0.33
H. influenzae-related GBS and FS	75	0.34	75	0.08
Anti-ganglioside IgG-positive GBS and FS	78	0.04	60	0.054

GBS, Guillain–Barré syndrome; FS, Fisher syndrome.

Table 2
Vβ and Vδ spectratype expansion frequencies in patients with Guillain–Barré syndrome or Fisher syndrome

	Patients with GBS or FS	Healthy subjects	Uncorrected P	Corrected P
Vβ	% (n = 119)	% (n = 29)		
1	8	0	0.1	
2	5	10	0.9	
3	12	10	0.6	
4	3	0	0.4	
5.1	3	3	0.7	
5.2	18	7	0.1	
6	11	14	0.8	
7[a]	13	0	0.02	0.61
8	7	7	0.7	
9	12	17	0.9	
10	4	7	0.9	
11	4	3	0.7	
12	7	7	0.7	
13	5	0	0.3	
14	11	7	0.4	
15	8	3	0.4	
16	5	10	0.9	
17	1	0	0.8	
18	2	3	0.9	
19	3	0	0.4	
20	1	3	0.4	
21	3	3	0.7	
22	4	10	1.0	
23	10	3	0.2	
24	10	3	0.2	
Vδ	% (n = 113)	% (n = 24)		
1	18	8	0.2	
2	19	4	0.054	
3	34	25	0.4	
4	3	0	0.6	
5	3	0	0.6	

GBS, Guillain–Barré syndrome; FS, Fisher syndrome.
[a] Odds ratio, 9.4; 95% confidence interval, 1.2–76.7.

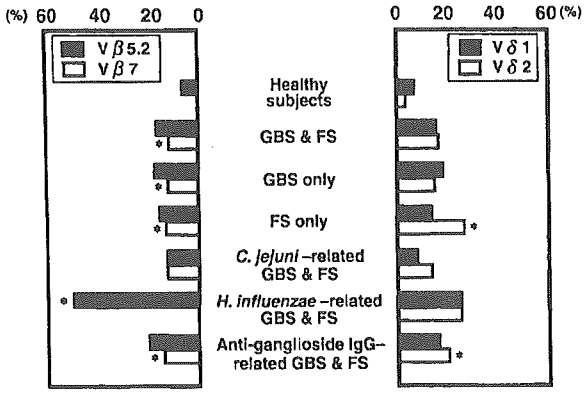

Fig. 2. Vβ5.2/7 and Vδ1/2 expansion frequencies. GBS, Guillain–Barré syndrome; FS, Fisher syndrome. *Uncorrected $p<0.05$, but corrected $p>0.05$.

with positive *H. influenzae* serology had Vβ5.2 expansion, a frequency higher than that for the healthy controls (7%), but it did not reach significance (uncorrected p, 0.01; corrected p, 0.32; odds ratio, 13.5; 95% CI, 2.4–77) (Fig. 2).

3.3. Longitudinal study

Follow-up blood samples were available 6 months after onset for 15 patients who showed Vβ spectratype expansion during the acute phase of illness. Vβ spectratype expansion had disappeared ($n=4$) or decreased ($n=4$) by the follow-up period in eight of them. The expanded spectratypes differed from patient to patient and were not associated with any particular type of antecedent infection. Vβ spectratype expansions in the other seven patients remained unchanged ($n=4$) or were slightly increased ($n=3$) in the follow-up period.

Vδ spectratype analysis was done longitudinally in five patients who showed Vδ spectratype expansion during the acute phase of illness. In two patients, Vδ2 expansion had disappeared by the recovery phase, whereas the Vδ3 spectratype was newly expanded. The other three patients had similar spectratype expansions during the acute and recovery phases of illness.

4. Discussion

The development of several autoimmune and infectious diseases often is closely associated with a particular type of peripheral blood T cell. For example, there is a marked bias toward oligoclonality of CD8+ T cells that express Vβ3 TCR in patients with rheumatoid arthritis (Hingorani et al., 1996), and T cells bearing Vδ1 are expanded in patients infected by human immunodeficiency virus (Autran et al., 1989). Although pathological and immunological studies suggest that T cells mediate the development of GBS as well (Cornblath et al., 1987; Hartung et al., 1990; Guijo et al., 1992; Sharief et al., 1993; Yuki et al., 1995), there is no consensus on which T cell phenotypes are related to GBS pathogenesis. The TCR Vβ1-20 and Vδ1/2 spectratypes were analyzed previously in small populations of GBS patients (Khalili-Shirazi et al., 1997; Borsellino et al., 2000). GBS, however, is a heterogeneous disorder of different etiological origins, therefore for this type of analysis it is essential to have a large population of patients with uniform clinical or immunological manifestations.

Our comprehensive study of a large population, for the first time including FS patients, showed that various TCR Vβ and Vδ gene products more often were used by peripheral blood lymphocytes of patients with GBS or FS than by those of healthy persons. Vβ expansion tended to disappear during the follow-up period. In contrast, no spectratype was significantly associated with GBS or FS. Moreover, no particular TCR spectratype was related to patients with a serological evidence of prior infection by *C. jejuni* or *H. influenzae*. Although there is a possibility that finding of abnormalities within GBS/FS group may merely reflect the high incidence of preceding infection, we suggest that the TCR Vβ and Vδ genes probably are involved in the development of GBS and FS and that these predominant spectratype expansions vary with the individual, even within subgroups having single etiological origin or uniform serological features.

αβ T cells are a major subpopulation of human peripheral blood lymphocytes. Myelin protein is a possible autoantigen in MS, as well as in experimental autoimmune encephalomyelitis, and particular Vβ gene uses are reported to be associated with the development of these diseases (Burns et al., 1989; Wucherpfennig et al., 1990; Kotzin et al., 1991). Vβ gene use also has been investigated in EAN induced by sensitization with P2 protein, but which TCR phenotype is used by the disease-inducing T cells is not clear. Vβ15 gene use was reported to be enhanced in circulating activated T cells from four patients with AIDP but not in cells from a patient with axonal GBS or those from healthy persons (Khalili-Shirazi et al., 1997). Because no electrophysiological data were available in our study, it was impossible to evaluate the association of TCR phenotypes and AIDP. Findings for patients negative for anti-ganglioside IgG antibodies, however, may reflect the features of AIDP patients because most patients with AIDP did not have such antibodies (Ogawara et al., 2000). We found that Vβ15 gene use was relatively rare in patients without IgG antibodies, and failed to obtain the evidence that this T cell phenotype is pathogenic in AIDP patients.

In contrast to αβ T cells, γδ T cells account for less than 6% of the total peripheral T cells. They recognize nonpeptide antigens, whereas αβ T cells do not (Tanaka et al., 1995). During the last decade, the immune response against nonpeptide antigens such as gangliosides and lipooligosaccharide came to be considered a key to the pathogenesis of GBS subsequent to *C. jejuni* enteritis (Willison and Yuki, 2002; Hartung et al., 2002). A preferential expansion of γδ T cells from healthy humans was seen after stimulation by

non-proteinaceous compounds of *C. jejuni* in vitro and this phenomenon was mediated via the γδ TCR (van Rhijn et al., 2003). Accordingly, γδ T cells may have a pathogenic role in the development of that form of GBS. In fact, a T cell line established from a sural nerve specimen from a patient with GBS subsequent to *C. jejuni* enteritis, consisted mainly of γδ T cells and preferentially expressed Vγ8/δ1 (Ben-Smith et al., 1996; Cooper et al., 2000). The percentage of γδ T cells expressing Vδ1 TCR is reported to be higher in the peripheral blood of patients with GBS than in the blood of those with MS and the healthy controls (Borsellino et al., 2000). Furthermore, the number of γδ T cells from *C. jejuni*-related GBS patients was increased on stimulation by the *C. jejuni* antigen (Ben-Smith et al., 1997). These observations suggest that the recognition of *C. jejuni* by Vδ1+ T cells is critical for the development of GBS subsequent to *C. jejuni* enteritis.

Our study could not confirm there is an association of Vδ1+ T cells with GBS subsequent to *C. jejuni* enteritis, but the Vδ1 gene tended to be used more frequently in all the GBS and FS patients than in the controls. The absence of a relationship between GBS and Vδ1 expansion in this study does not seem to be due to the sensitivity of the assay used because previously we found that oligoclonality in patients with MS is efficiently detected by this assay (Matsumoto et al., 2003). It was reported that particular human leukocyte antigen (HLA) types were associated with GBS and FS after *C. jejuni* enteritis (Rees et al., 1995; Koga et al., 1998b), although no HLA types were related to GBS or FS as a whole (Adams et al., 1977; Stewart et al., 1978; Latovitzki et al., 1979; Kaslow et al., 1984; Winer et al., 1988; Hillert et al., 1991; Chiba et al., 1995). Determination of HLA types is unnecessary to examine the possible association between *C. jejuni*-related GBS/FS and particular γδ spectratype, because γδ T cells can recognize antigens without need for processing and presentation by a major histocompatibility complex-like molecule (Schild et al., 1994). Although our findings do not rule out the possibility that Vδ1+ T cells in nerve tissue and the gut have a pathogenic role in some cases of GBS subsequent to *C. jejuni* enteritis, the clonal expansion of γδ T cells does not seem to be characteristic of that type of GBS.

H. influenzae has emerged as a causative pathogen in GBS and FS (Mori et al., 2000; Koga et al., 2001). Molecular mimicry between the bacterial lipooligosaccharide and human nerve tissue very likely functions in the development of GBS and FS subsequent to *H. influenzae* infection, as it does in GBS subsequent to *C. jejuni* enteritis (Willison and Yuki, 2002). If the oligoclonal expansion of Vβ5.2+ T cells is related to GBS and FS after *H. influenzae* infection, it is surprising because T cells bearing the TCR β chain do not recognize nonpeptide antigens. Whether frequent expansion of the Vβ5.2 gene seen in GBS and FS subsequent to *H. influenzae* infection reflects only the infection or the pathogeneses of these autoimmune neuropathies must now be clarified.

In conclusion, our comprehensive study showed that oligoclonal expansion of T cells bearing TCR Vβ and Vδ genes frequently occurs in GBS and FS, suggestive that T cells mediate the development of these neuropathies. Our results conflict with the previous ones that particular TCR types were related to GBS. The predominant phenotypes vary, even within subgroups of patients with a syndrome of single etiological origin or those with uniform serological features.

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Acute Motor Axonal Neuropathy Rabbit Model: Immune Attack on Nerve Root Axons

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Macrophages in the periaxonal space and surrounding intact myelin sheath are the most prominent pathological feature of acute motor axonal neuropathy (AMAN). We describe this characteristic in nerve roots from paralyzed rabbits immunized with bovine brain ganglioside or GM1. IgG was deposited on nerve root axons. Distal nerve conduction was preserved, and late F wave components were absent during the acute phase. Initial lesions were located mainly on nerve root axons, as in human AMAN. This study thus provides supportive evidence that the rabbits constitute a model of AMAN.

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Until recently, Guillain–Barré syndrome has been considered an acute inflammatory demyelinating polyneuropathy. Collaborative Chinese–American studies have established the existence of two forms of primary axonal Guillain–Barré syndrome, acute motor axonal neuropathy (AMAN) and acute motor-sensory axonal neuropathy.[1,2] The pathology of AMAN is a noninflammatory, axonal degeneration of the motor nerves with little or no demyelination.[1,2] Macrophages within the periaxonal space surrounded by an intact myelin sheath provide supportive evidence of a primary, immune-mediated attack on the axons in AMAN.[2] AMAN often is associated with anti-GM1 IgG antibody.[3,4] We previously produced a model of AMAN by sensitizing rabbits with a bovine brain ganglioside (BBG) mixture or GM1, but we did not show periaxonal macrophages.[5] Now, we report their presence in GM1- or BBG-immunized rabbits. The distribution of initial lesions in our model also was investigated.

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Materials and Methods

Immunization Procedure

Male Japanese white rabbits (Kbs:JW) were obtained from Oriental Bioservice Kanto (Ibaraki, Japan). A 0.5mg portion of GM1 (Sigma, St. Louis, MO), 2.5mg of BBG (Cronassial; Fidia, Padova, Italy), or 1mg of galactocerebroside (GalC; Sigma) was injected subcutaneously to the back at 3-week intervals until limb weakness developed or maximal sensitization occurred four times as described elsewhere.[5] Control rabbits were injected under the same protocol with the same inoculums but without glycolipids. The end point was 16 weeks from the beginning of sensitization. None of the rabbits reported previously[5] were included. This research was approved by the Animal Care and Use Committee, Dokkyo University School of Medicine (approval number 99-234). Rabbits were treated according to the Guidelines for the Care and Use of Laboratory Animals of Dokkyo University School of Medicine.

Enzyme-Linked Immunosorbent Assay

Anti-GM1 and anti-GalC IgG antibodies were tested in plasma obtained within 1 week after symptom onset as reported elsewhere.[5]

Pathological and Immunohistochemical Studies

Lumbar spinal nerve root, cauda equina, and sciatic nerve specimens were evaluated pathologically as described previously.[5] For electron microscopy, ultrathin sections were cut from tissue fragments embedded in Epon 812 resin, stained with uranyl acetate and lead citrate, and then examined in a Hitachi-7100 electron microscope (Hitachi, Tokyo, Japan). Specimens were also stained with peroxidase-conjugated protein G (Sigma) as reported elsewhere.[5]

Electrophysiological Studies

A nerve conduction study (NCS) was done as described elsewhere.[6] Before inoculation and every 2 weeks thereafter, conduction in motor fibers connected to plantar muscles was examined under general anesthesia: an intramuscular injection of ketamine hydrochloride (35mg/kg; Sankyo, Tokyo, Japan) and xylazine hydrochloride (5mg/kg; Sankyo), being given and repeated as needed. Thigh temperature was kept between 37 and 38°C by a hot carpet when necessary. Evoked compound muscle action potentials (CMAPs) were displayed on a MS-25 electromyograph (Medelec, Surrey, UK). A needle electromyogram was recorded from the left anterior tibial muscle in resting position. The patellar tendon reflex was evaluated during anesthesia.

Results

Induction of Paralysis

Five of the seven GM1-sensitized rabbits and all six BBG-immunized rabbits developed flaccid limb paresis. All the paralyzed rabbits had anti-GM1 IgG antibody

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Table. Immunohistochemical and Electrophysiological Studies of AMAN Rabbits

Rabbits	Cr-14	Cr-15	Cr-16	Cr-17	Cr-18	Cr-19	Gm-1	Gm-2	Gm-3
Immunogen	BBG	BBG	BBG	BBG	BBG	BBG	GM1	GM1	GM1
Inoculation times	3	3	2	3	3	2	4	2	3
Onset of limb weakness (day)	50	51	25	50	58	36	72	42	55
Poorest functional grade	3	4	4	3	2	4	1	4	3
Areflexia		+	+	+			−	+	+
Anti-GMI IgG antibody titer	32,000	128,000	4,000	32,000	64,000	128,000	16,000	128,000	64,000
Days from onset to death	9	16	4	48	19	3	66	3	54
Number of fibers with IgG deposit on the axon (/100 fibers)									
Spinal nerve root	7	5	Not available	Not available	2	10			
Cauda equina	16	5	17	6	9	5			
Sciatic nerve	2	3	1	0	0	2			
Tibial nerve	0	0	0	0	0	0			
Nerve conduction study results after onset									
Days from onset to study		16	4	8			14	2	4
DL (milliseconds) (2.15 ± 0.17[a])		2.46	2.42	2.38			2.20	2.66	2.10
MCV (m/sec) (52.4 ± 3.5[a])		50.0	50.5	53.2			55.2	52.9	62.5
Distal CMAP amplitude (mV) (11.8 ± 3.4[a])		12.0	17.8	8.2			11.6	12.4	6.4
CMAP amplitude proximal/distal ratio (0.53 ± 0.11[a])		0.6	0.56	0.68			0.77	0.49	0.73
Distal CMAP duration (millisecond) (1.56 ± 0.15[a])		1.77	1.78	1.45			1.67	1.30	1.40
CMAP duration proximal/distal ratio (1.20 ± 0.20[a])		1.13	1.31	1.19			1.27	1.18	1.10
Minimal F latency (10.5 ± 0.5[a])		11.0	10.9	10.8			10.3	10.3	10.6
Late F wave components		No change	No change	Absent			No change	Absent	Absent
Denervation potentials		+	-	+			-	+	+
Wallerian-like degeneration in the sciatic nerve	Moderate	Mild	Not found	Moderate	Mild	Not found	Not found	Mild	Mild

Functional grade: 0, normal; 1, weakness of the hind limbs; 2, mild weakness of the four limbs, able to walk; 3, moderate weakness of the four limbs, unable to walk; 4, severe weakness of the four limbs which were spread out; 5, dead. Blanks: examination not performed. Numbers of fibers with IgG deposits on their axons were counted on cross-sections of each sample. Distal and proximal CMAPs were recorded, respectively, by stimulating at the ankle and sciatic notch. MCV between the knee and ankle was measured. Amplitude was measured between the base line and negative CMAP peak. Duration was from the onset to the positive CMAP peak. In the healthy rabbits, the mean shortest F latency was 10.5 milliseconds (SD = 0.5), and F wave durations were relatively long (mean ± SD = 7.35 ± 1.74 milliseconds). Late F components are defined as the parts of F waves later than 14 milliseconds. Wallerian-like degeneration in the sciatic nerve: mild, < 10% of degenerated fibers; moderate, 10–40%; severe, > 40% on Toluidine blue-safranine stained cross-sections. Because the electrophysiological study was performed on the left lower limb, the right sciatic nerve was evaluated pathologically to avoid the possibility of artifacts derived from the needle electrode.

[a]Mean ± SD were measured from nerve conduction study data before beginning inoculation (n = 34).
AMAN = acute motor axonal neuropathy; BBG = bovine brain ganglioside; DL = distal latency; MCV = motor conduction velocity; CMAP = compound muscle action potential.

(Table). Seven of eight GalC-immunized rabbits also developed flaccid limb paresis, and all had anti-GalC IgG antibody. None of the seven control rabbits showed limb weakness.

Pathological and Immunohistochemical Studies

Wallerian-like degeneration occurred in sciatic nerves from the GM1- or BBG-immunized rabbits (see Table). Unlike the sciatic nerves, the cauda equina and spinal nerve root specimens from the paralyzed rabbits (Gm-2, Cr-14, Cr-16, and Cr-19) frequently showed macrophage infiltration in the periaxonal space (Fig 1A–E). The surrounding myelin sheaths appeared almost normal. This finding was not present in paralyzed GalC-immunized rabbits.

Six BBG-immunized rabbits were prepared for im-

Fig 1. (A–E) Macrophages in nerve fibers. Cross-sections of the cauda equina from a GM1-immunized rabbit (Gm-2). (A–C) Toluidine blue-safranine stain. Macrophages are present in the nerve fibers. Demyelination and remyelination are rare, and there are no inflammatory cells in the endoneurium. (D) Electron micrograph of the nerve fiber with macrophage infiltration shown in A. A macrophage (m) occupies the periaxonal space, and the axon has disappeared. (E) Another example of a nerve fiber with macrophage infiltration. Macrophage (m) processes surround the atrophic axon (a). The surrounding myelin sheath appears almost normal. Scale bars = 10μm. (F–K) IgG deposits on axons. Specimens from a bovine brain ganglioside–immunized rabbit (Cr-19) stained with serially diluted peroxidase-conjugated protein G. (F, G) Adjacent cross-sections (20μm thick) of ventral roots (dilution of protein G = 1μg/ml). (F) Some axons are strongly stained (arrow, arrowhead). *(G) Staining intensity gradually decreases* (arrow). *(H) High-power magnification of the nerve fiber* (arrowhead in G). *The axon is stained diffusely* (arrowhead). *(I) Cross-section of the cauda equina (dilution of protein G = 2μg/ml). Selective staining is seen along the axonal membrane* (arrow). *(J, K) Longitudinal sections of the cauda equina (dilution of protein G = 0.5μg/ml). (J) The axon is selectively stained for approximately 50μm, the intensity gradually decreasing on both sides* (arrow). *(K) Ranvier nodes are stained selectively* (arrowheads). *Scale bars = 10μm.*

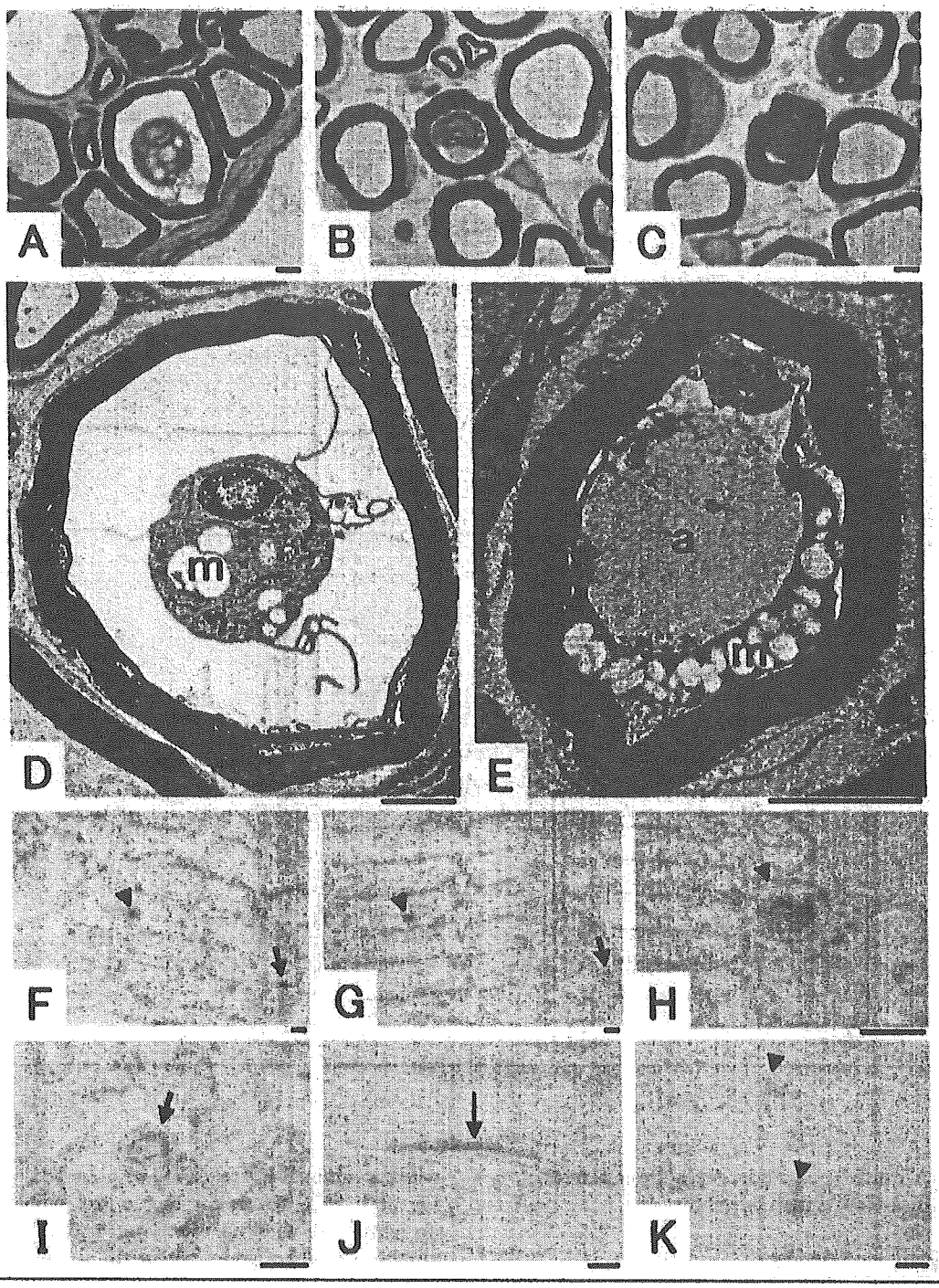

Figure 1

munohistochemical study (see Table). Protein G positively stained some axons, evidence of IgG deposits in or around the axons (see Fig 1F–J). This occurred mainly in the nerve roots or cauda equina and in a few sciatic nerves, but never in the tibial nerves (see Table). Ranvier nodes were specifically stained in longitudinal sections of the cauda equina from a BBG-immunized rabbit (see Fig 1K). No selectively stained axons were

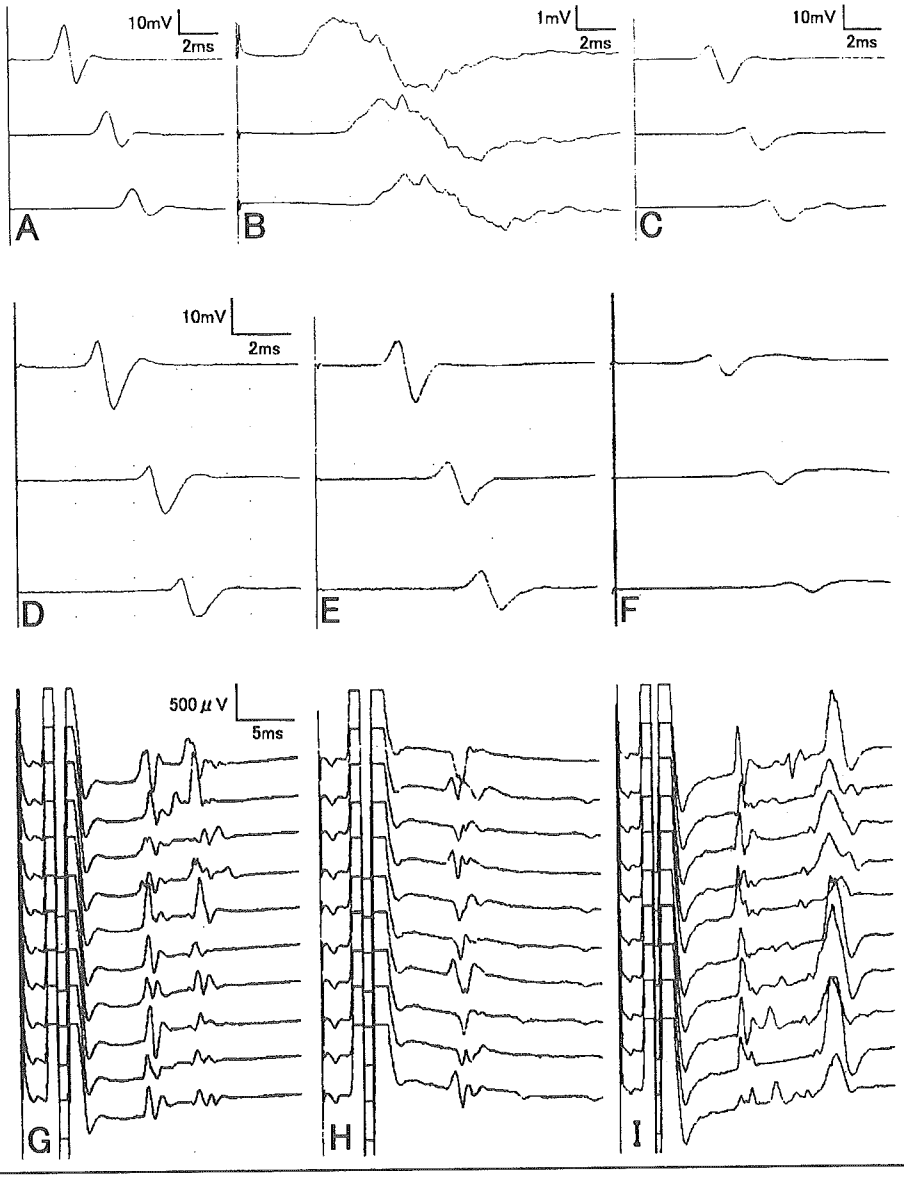

Figure 2

present in cauda equina specimens from a normal, a control, or a GalC-sensitized rabbit.

Electrophysiological Studies

Evaluations of three rabbits inoculated with BBG, seven with GM1, eight with GalC, and seven control animals were made. As reported previously,[7] GalC-immunized rabbits had typical demyelinative findings (Fig 2B). The Table shows available data for the paralyzed GM1- and BBG-immunized rabbits. Changes in NCS data were minimal during the acute phase. Late F wave components were absent in three rabbits, whereas minimal F latencies were preserved (see Table, Fig 2H). NCS results for the control rabbits showed no remarkable abnormalities. Four of the six paralyzed rabbits had denervation potentials in needle electromyography.

We followed the clinical course of one rabbit (Gm-3). There were no obvious changes in NCS data in the acute phase (see Table, Fig 2E). Late F wave components were absent (see Fig 2H). Amelioration began 8 days after weakness onset. Late F wave components reappeared with slightly prolonged latencies 14 days after onset (see Fig 2I). In the needle electromyography, de-

nervation potentials were detectable 18 days after onset. Limb muscle power gradually improved, but body weight loss and muscle atrophy continued. CMAP amplitudes were decreased 6 weeks after onset (see Fig 2F).

Discussion

Griffin and colleagues have shown that the pathological substrate of AMAN is periaxonal macrophages.[1,2] We now report this similar pathology in nerve roots from GM1- or BBG-immunized rabbits. In our previous study, the sciatic nerve specimens examined did not show this pathology.[5] Retrospectively, we found it in the cauda equina of a BBG-immunized rabbit (Cr-10) in that study. Immunohistochemical studies of the early stage of AMAN show IgG and the complement activation product C3d frequently bound to the nodal axolemma or present in the periaxonal space of myelinated internodes of motor nerve fibers.[8] In our AMAN model, IgG was deposited on axons, internodal axolemmas, and Ranvier nodes. Like the periaxonal macrophages, IgG deposits on axons primarily were in nerve roots. This distribution corresponds well to the lesions in AMAN patients,[2] which are postulated to occur because nerve roots have a blood-nerve barrier deficiency.[9] In humans, Wallerian-like degeneration occurs in the peripheral nerves in severe cases.[1,2] Wallerian-like degeneration was not found in sciatic nerve specimens in rabbits killed 3 (Cr-19) or 4 (Cr-16) days after onset, whereas it was frequent in AMAN rabbits killed in the advanced phase.

NCS results support this distribution of initial lesions. In the AMAN rabbits, distal motor nerve conduction was preserved in the acute phase. In three paralyzed rabbits, a component of the F wave was absent. We believe these potentials are part of the F wave: they are present on supramaximal stimulation, their latencies are shorter on stimulation at proximal sites other than the ankle, and the waveform varies. Similarly, the isolated absence of F waves has been reported during the acute phase of human AMAN.[10] These findings indicate nerve conduction failure at proximal sites. CMAP amplitude reduction, later detected in one rabbit (Gm-3), may indicate subsequent Wallerian-like degeneration. Several points, however, differ from F wave findings in human AMAN.[10] Latencies of the late F wave components reelicited in the recovery phase were slightly prolonged in Gm-3. This electrophysiological finding may indicate demyelination, remyelination, or a widened-paranodes, consistent with the pathology of the nerve root specimens. In the paralyzed rabbits, minimal F wave latencies were normal. This suggests that some fast-conducting motor fibers may be spared throughout their entire length.

This study thus provides supportive evidence that our GM1- or BBG-immunized rabbits constitute a model of AMAN.

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Fig 2. (A–F) Distal nerve conduction study results. Stainless steel needles were inserted close to the left sciatic nerve and its tibial branch at the ankle, knee, and sciatic notch to stimulate the nerves. At each level, a needle placed close to the nerve served as the cathode and a remote subcutaneous one as the anode. Recording from plantar muscles was through subcutaneous needles; one placed transversely over the muscle bellies in the sole of the foot, the other at a distance. Compound muscle action potentials (CMAPs) were recorded after supramaximal stimulation. The top, middle, and bottom traces are for stimulus to the left sciatic nerve and its tibial branch at the three levels ankle, knee, and sciatic notch. (A–C) Serial nerve conduction studies of a galactocerebroside-sensitized rabbit. (A) CMAPs recorded before sensitization. Distal CMAP amplitude is 13.0mV, distal latency (DL) 2.10 milliseconds, and motor conduction velocity (MCV) 48.0m/sec. (B) CMAPs recorded 2 weeks after the onset of limb weakness (nadir of the symptoms). Note that the amplitude scale is 1mV/division. CMAPs show remarkable temporal dispersion, and their amplitudes are markedly decreased. Distal CMAP amplitude is 1.5mV, DL is prolonged to 3.40 milliseconds, and MCV is decreased to 37.5m/sec. (C) CMAPs recorded 14 weeks after the onset of limb weakness. Muscle power has recovered almost to normal. CMAP amplitude has increased with the resolution of temporal dispersion (7.0mV). DL is still prolonged (3.50 milliseconds). MCV has recovered to 51.0m/sec. (D–F) Serial nerve conduction studies of a GM1-immunized rabbit (Gm-3). (D) CMAPs recorded 14 days before the onset of limb weakness. Distal CMAP amplitude is 6.8mV, DL 2.10 milliseconds, and MCV 51.1m/sec. (E) CMAPs recorded 4 days after the onset of limb weakness. CMAP amplitude (6.4mV) and DL (2.10 milliseconds) show no marked changes. MCV is 62.5m/sec. (F) CMAPs recorded 6 weeks after the onset of limb weakness. Muscle power has gradually returned, but limb weakness and muscle atrophy are still present. CMAP amplitude has decreased to 2.5mV, DL 2.30 milliseconds, and MCV 52.3m/sec. (G–I) Serial F wave recordings from a GM1-immunized rabbit (Gm-3). F waves were recorded after ankle stimulation. Ten consecutive recordings (minimum) were obtained after supramaximal stimulation delivered at the frequency of 1Hz. (G) F waves before sensitization. (H) F waves recorded 4 days after onset of limb weakness. No late F wave components have been elicited, whereas minimal F wave latencies are preserved. (I) F waves recorded 2 weeks after onset of limb weakness. Clinical symptoms have begun to be ameliorated. Late F wave components again are recorded, but their latencies are slightly delayed.