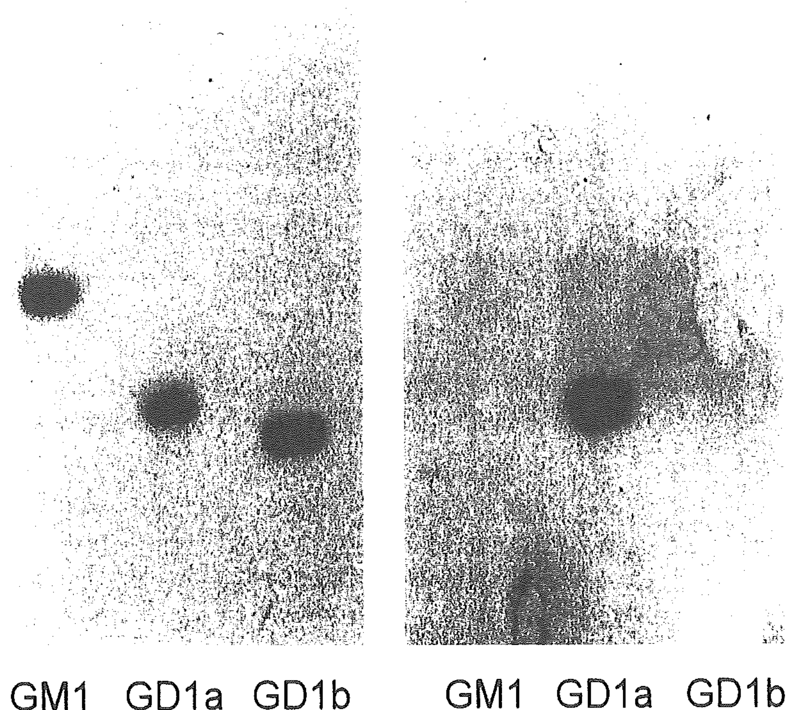

Figure. Thin-layer chromatogram immunostaining was performed as described previously.[3] (Left) Stained with orcinol to visualize 1 μg each of GM1, GD1a, and GD1b. (Right) Immunostained with the IgG of the patient's serum, diluted 1:40.

pathogenicity of GD1a has been proven in animal models.[5] Because our two brothers live at the same residence, they should have similar genetic and environmental backgrounds. These findings suggest that factors besides anti-ganglioside antibodies may have contributed to the development of GBS.

To identify such factors, we examined known molecules associated with GBS, such as soluble intercellular adhesion molecule-1 (sICAM-1), soluble interleukin-2 receptor, interferon-γ, and tumor necrosis factor-α.[1,6] We found that the serum level of sICAM-1 was high (395 ng/mL [normal 115 to 306 ng/mL) in the brother with GBS and, unexpectedly, much higher (695 ng/mL) in the asymptomatic brother. Both brothers had normal levels of other factors, and the level of sICAM-1 was decreased later. An association of high sICAM-1 levels with a good outcome has been reported previously; serum levels of sICAM-1 are frequently elevated in cytomegalovirus- and Epstein–Barr virus–associated GBS, both of which generally have a good outcome.[1] In contrast, sICAM-1 is not significantly elevated in post–*C. jejuni* GBS associated with a poor outcome.[1] sICAM-1, cleaved from ICAM-1 on the endothelial cell surface, may bind to leukocyte integrins and inhibit leukocyte adhesion to the endothelium, thereby blocking the inflammatory process.[6] These findings suggest that sICAM-1 may be one factor preventing the development of GBS. The mechanism for the transient elevation of sICAM-1 was unclear; however, this elevation probably occurred during the diarrhea. Therefore, the diarrhea itself or the treatment of the diarrhea may have contributed to the sICAM-1 induction. In fact, only the elder brother had received IV fosfomycin, an antibiotic, whereas the younger brother and four other post–*C. jejuni* GBS patients in our clinic had taken only oral antidiarrheals. Although a future large-scale study is required, the IV fosfomycin could possibly induce sICAM-1 or have a direct action to modulate immunoreactivity, as it is reported to have transforming growth factor-β–like activities besides antibacterial action.[7]

Multiple mechanisms may be involved in the pathogenesis of GBS. It is important to clarify the mechanism underlying post–*C. jejuni* GBS because >30% of patients who receive current treatments have a poor outcome, including death or inability to walk unaided.[1] Further studies of factors related to the GBS onset in patients with *C. jejuni* enteritis may provide clues to treatment or prevention.

Acknowledgment

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LETTER TO THE EDITOR

CHRONIC SENSORY ATAXIC NEUROPATHY WITH POLYCLONAL IgM REACTIVITY TO VARIOUS DISIALOSYL GANGLIOSIDES

Monoclonal immunoglobulin M (IgM M-protein) binding to a broad spectrum of gangliosides with disialosyl residue, such as GD1b, GD3, GT1b, and GQ1b, has been reported exclusively in cases of chronic sensory ataxic neuropathy (CSAN). The GD1b may be the causative antibody-binding site of CSAN. The present case should therefore be of interest.

A 53-year-old man developed numbness in his hands and feet in January 1998. His symptoms gradually worsened and he was admitted to our hospital in April 1999. On admission, physical examination revealed no abnormalities. On neurological examination, he had normal cranial nerve function and limb power. All deep tendon reflexes were absent. His vibratory and position senses were severely impaired to the knees and elbows. Touch, temperature, and pinprick sensations were mildly disturbed in a glove–stocking distribution. Coordination was clumsy in all limbs because of sensory loss. He had gait ataxia with Romberg's sign. Autonomic function was intact.

Routine laboratory tests were normal except for an elevated serum level of rheumatoid factor (1,310 IU/ml; normal value, <12 IU/ml). Serum concentrations of IgM and other immunoglobulins were within the normal range. Serum immunoelectrophoresis did not show monoclonal gammopathy. Autoantibodies such as anti-DNA, anti-SS-A, SS-B, and anti-Hu were not detected. The titer of cold agglutinin was not increased. There was no cerebrospinal fluid (CSF) pleocytosis, and CSF protein level was 74 mg/dl (IgG 10.7%; normal, 2–5%). Although motor nerve conduction velocity was normal in all four limbs, sensory nerve action potentials (SNAP) could not be recorded. His electromyogram and electroencephalogram were normal. His serum was assayed for antibodies to glycolipids by enzyme-linked immunosorbent assay (ELISA) as previously described.[3] Antigens used in ELISA were GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, GQ1b, GA1, and galactocerebroside (Gal-C). He was found to have extremely high IgM antibody titers against GM2, GM3, GD1a, GD1b, GD3, GT1b, and GQ1b, but no IgG antibody activities against any of the gangliosides.

A clinical diagnosis of chronic sensory ataxic neuropathy was made. In July 1999, IVIg therapy, 400 mg/kg daily for 5 days, was administered. His sensory impairment began to improve 3 days after the first treatment. Two weeks later, he began to walk steadily without ataxia. He remained well for several months, but after 8 months his numbness and sensory ataxia relapsed. Further IVIg therapy improved his condition again (Fig. 1). The serum concentration of rheumatoid factor (RF) decreased thereafter, but gradually increased again as symptoms worsened. In order to prevent a relapse, 30 mg of prednisolone was added after the fourth relapse, but had to be tapered to 10 mg because of diabetes mellitus. SNAPs remained undetectable after IVIg therapy.

This patient was diagnosed with chronic sensory ataxic neuropathy, and electrophysiological findings suggested that large sensory myelinated fibers or dorsal root ganglion neurons were involved. He also had serum polyclonal IgM antibody activities against a variety of gangliosides with disialosyl residue. It has previously been reported that IgM M-protein binding to a broad spectrum of gangliosides with disialosyl residue, such as GD1b, GD3, GT1b, and GQ1b, may be associated with sensory ataxic neuropathy.[2,4,5,7] Our patient, however, also had markedly elevated RF titer and was without IgM M-protein. The relation between RF and CSAN is unclear. His antibody titers of IgM against GM2, GM3, and GD1a, which are without disialosyl residue, were as high as those against gangliosides with disialosyl epitope. In serum that had been preincubated with either GD1a or GD3, all the IgM activities against the gangliosides described above were significantly reduced (data not shown). Thus, the same IgM may bind to both gangliosides with and without disialosyl residue. Eight of the 18 anti-disialosyl IgM antibodies associated with sensory ataxic neuropathy cross-react with GD1a and GM3.[8] In addition, GM2 was recognized by the IgM of our patient. In contrast, his serum IgM did not recognize GM1, which has a monosialosyl residue binding

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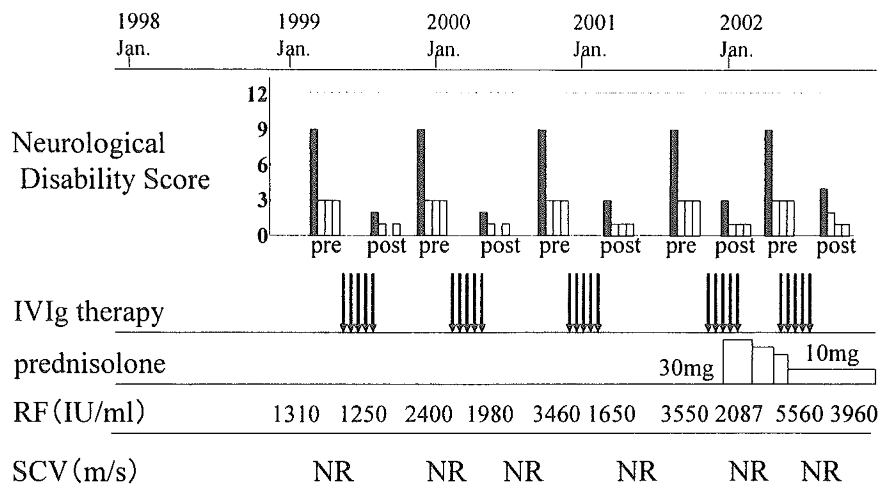

FIGURE 1. The clinical course and therapy of the present case. The neurological disability score (0–12), indicated by filled columns, was assessed by summation of sensory impairment score (0–4), disequilibrium score (0–4), and activity of daily life score (0–4), indicated by respective open columns. Each score was evaluated by the modified method of Dyck et al.[1] Scoring: 0, no deficit; 1, mild deficit; 2, moderate deficit; 3, severe deficit; 4, complete absence of function or severest deficit. The neurological disability score was assessed before treatment (pre) and 2 weeks after IVIg (post). NR, not recordable; RF, rheumatoid factor; SCV, sensory nerve conduction velocity.

to galactose in the second position of the gangliotetraose structure. Glycolipids without sialic acid, such as galactocerebroside and GA1, were also not recognized. The IgM of the present patient may bind to the monosialosyl residue in the terminal, or the next to the terminal, position of the backbone structure as well as to the disialosyl residue. The reaction with the sialic acid in the second position of the gangliotetraose structure of GM1 may be hindered by Gal-GalNAc residue.

Although various treatments including prednisolone, plasmapheresis, or cyclophosphamide have been administered to patients with CSAN with antibody activities against gangliosides,[2,4] beneficial treatments are still undetermined. In our patient, IVIg therapy consistently resulted in almost complete clearance of sensory ataxia, although additional IVIg at intervals of 7 to 11 months was required to maintain the improvement. Takeuchi et al.[6] also reported that the effect of IVIg lasted for 4 to 8 months in patients with idiopathic CSAN.

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CME

GalNAc-GD1a in human peripheral nerve

Target sites of anti-ganglioside antibody

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Abstract—*Background:* The authors previously reported that immunoglobulin G (IgG) antibody to the ganglioside N-acetylgalactosaminyl GD1a (GalNAc-GD1a) is associated with the pure motor variant of Guillain-Barré syndrome (GBS). Elucidation of the localization of GalNAc-GD1a in human peripheral nerve tissue may lead to understanding of the pathogenetic role of anti-GalNAc-GD1a antibody in GBS. *Methods:* IgG anti-GalNAc-GD1a-monospecific antibody was purified from anti-GalNAc-GD1a antibody-positive rabbit sera through an affinity column. Anti-neurofilament-200 monoclonal and anti-HNK-1 monoclonal antibodies were used as the markers for axon and myelin. Immunohistochemical study using double fluorescence labeling technique was conducted in human ventral roots (VR), dorsal roots (DR), intramuscular nerves, and sural nerves. Human teased ventral fibers also were studied. *Results:* Anti-GalNAc-GD1a antibody immunostained an inner part of compact myelin and additionally a periaxonal-axolemma-related portion in the VR, small-diameter DR fibers, and IM nerves. In sural nerves, small fibers were selectively stained. In VR, the staining was localized in the paranodal region. *Conclusion:* Anti-GalNAc-GD1a antibodies in patients' sera may bind to those regions in the VR and IM nerves where GalNAc-GD1a is localized, and may function in the pathogenesis of pure motor type GBS. Further investigation is needed to explain the discrepancy between the immunolocalization of GalNAc-GD1a in sensory nerves and the absence of sensory disturbance in patients with GBS with IgG anti-GalNAc-GD1a antibodies.

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Gangliosides are concentrated on the surfaces of neurons and function in such cell surface-mediated events as receptor function, cell-cell recognition processes, and membrane-mediated transfer of information.[1-3] Anti-ganglioside antibodies, frequently present in acute phase sera from patients with Guillain-Barré syndrome (GBS), may function in the development of GBS by induction of both changes in nerve cell function and destruction of the axon or myelin through antibody-mediated immunoresponses.[4,5] We elsewhere reported that the minor ganglioside N-acetylgalactosaminyl GD1a (GalNAc-GD1a) is a target molecule for serum antibody in some patients with GBS.[6] Moreover, we showed that the immunoglobulin G (IgG) antibody monospecific to GalNAc-GD1a is closely associated with the pure motor variant of GBS characterized by distal-dominant weakness and infrequent cranial nerve involvement.[7] Others have found the same clinical features in patients with anti-GalNAc-GD1a antibody.[8,9] How anti-GalNAc-GD1a antibody contributes to development of the pure motor variant of GBS, however, has yet to be clarified. Determination of the localization of GalNAc-GD1a in human peripheral nerve tissue is the key to understanding the pathogenetic role of anti-GalNAc-GD1a antibody. The localization of GalNAc-GD1a has never been identified in human nerve tissue. We prepared monospecific anti-GalNAc-GD1a antibody and examined its localization by the double fluorescence labeling immunohistochemical technique.

Methods. *Immunization procedure.* Immunizations with GalNac-GD1a were done by the method described elsewhere,[10] with slight modifications. GalNAc-GD1a was purified from bovine brain ganglioside purchased from Sigma (St. Louis, MO), as described elsewhere.[6] Five hundred micrograms of GalNAc-GD1a was dissolved in phosphate-buffered saline (PBS) containing 0.5 mL of keyhole limpet hemocyanin (KLH; 2 mg/mL, purchased from Sigma). A 0.5-mL portion of Freund complete adjuvant supplemented with heat-killed *Mycobacterium tuberculosis* (10 mg/mL), H37Ra, was added, and the mixture emulsified. A 1-mL sample of the emulsion was injected intracutaneously at multiple sites on the hind feet of three adult, female, Japanese white rabbits. Intracutaneous booster injections of the same emulsion were given 3 weeks later at multiple sites on the back. After another 3 weeks, the animals were given an IV booster injection of 100 μg GalNAc-GD1a in saline. Under the same protocol, three control rabbits were given the same inoculum without GalNAc-GD1a. Serum samples were taken by ear vein puncture at 1- or 2-week intervals. The rabbits were checked for clinical symptoms and weighed once or twice a week.

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Anti-glycolipid antibody examined by an ELISA. The reactivity of the serum antibody with glycolipid antigens (GalNAc-GD1a, GM1, GM2, GD1a, GD1b, and GT1b) was determined by an ELISA, as previously reported.[10] GalNAc-GD1a was prepared in our laboratory as described above.[6] The other gangliosides were purchased from Sigma. Serum diluted 1:100 with 1% bovine serum albumin in PBS was added to wells coated with 0.2 μg of antigen. Uncoated wells served as controls. As the secondary antibody, peroxidase-conjugated antibody to rabbit Ig (H&L chain specific; Cappel, West Chester, PA; diluted 1:500), rabbit IgM (μ chain specific; Cappel; 1:500), or rabbit IgG (γ chain specific; Zymed, South San Francisco, CA; 1:500) was added to each well. A color reaction was obtained by incubation with 200 μL of orthophenylene-diamine dihydrochloride (40 mg/dL phosphate citrate buffer, pH 5.0) at room temperature for 2 minutes. The reaction was stopped by the addition of 8N H2SO4, and the optical density (OD, 492 nm) read with an ELISA reader (Bio-Rad, Hercules, CA). OD values were corrected by subtracting the OD of the similarly processed control well. Each anti-GalNAc-GD1a-positive serum sample was diluted serially from 1:100 to 1:102400, and an ELISA done. Antibody titer was expressed as the maximal dilution factor that gave a corrected OD of more than 0.1.

Histologic analysis. All three rabbits immunized with GalNAc-GD1a and one control rabbit were killed 6 months after the first sensitization, and pathologic examinations performed. The lumbar spinal cord, sciatic nerve, ventral roots (VR), and dorsal roots (DR) were excised from each rabbit and prepared as described previously.[10] Semi-thin sections (1.0 μm) were cut from the Epon block, then stained with toluidine blue.

Purification of anti-GalNAc-GD1a antibody in an affinity column. The conjugate of octyl-sepharose and GalNAc-GD1a was prepared as described previously.[11] Octyl-Sepharose 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The gel was washed several times with methanol/water (1:1, by volume) containing 0.1 M KCl (solution A) and equilibrated with the same solution. A 0.5 mg portion of the purified GalNAc-GD1a was dissolved in 2 mL of solution A. The GalNAc-GD1a-coated gel obtained was packed in a small column (bed volume 0.5 mL) and subjected to the affinity chromatography.

Two hundred microliters of anti-GalNAc-GD1a-positive rabbit serum from a GalNAc-GD1a-immunized rabbit was layered on the affinity column. After incubation for 1 hour at room temperature, the column was washed with 500 μL of PBS. The PBS solution eluted through the column was again layered on the column. After incubation for 30 minutes, the PBS was collected from the column and the procedure repeated. After the second PBS collection, the column was washed with 2.5 mL of PBS, then the anti-GalNAc-GD1a antibody was eluted by a 3.0 M NaSCN-PBS solution. After overnight dialysis with PBS at 4 °C, the eluate was collected. A few microliters of PBS was added to the eluate so that it might add up to just 4 mL. The eluate was diluted serially from 1:20 to 1:1600 and studied for antibody activity to GalNAc-GD1a, GM1, GM2, GD1a, GD1b, GM1b, and GT1b by an ELISA as described above. Furthermore, the eluate (diluted 1:100) was studied for antibody activity to GalNAc-GD1a, GM1, GM2, GD1a, GD1b, and GA1 with thin-layer chromatogram immunostaining, as described elsewhere.[10] Anti-rabbit IgG antibody (γ chain specific; Zymed; 1:200) was used as the secondary antibody. Immunoabsorption study on the eluate was conducted with GalNAc-GD1a or GM1 antigen as described previously.[12] Quantitative analysis of the rabbit IgG in the eluate was done with a Rabbit IgG ELISA Quantitative kit (BETHYL Laboratories, Inc., Montgomery, TX) according to the manufacturer's instructions.

Tissue preparation for teased fibers. Human DR and VR were obtained at autopsy from a patient with no neurologic disorder. The roots were fixed in 2% paraformaldehyde for 1 hour on ice. The human nerves were washed in PBS and incubated in collagenase type IV in PBS (1 mg/mL) for 30 minutes at room temperature. After multiple washes in ice cold PBS, the nerves were desheathed and teased into small bundles of fibers under a stereomicroscope. These teased fibers were immunostained as described below.

Immunohistochemical study. An immunohistochemical study was done by the double fluorescence labeling technique to determine the localization of GalNAc-GD1a in human nerve tissue. Human VR, DR, and sural nerves from five adult patients with no neurologic disorder were obtained within 24 hours of death. Biceps muscle, obtained at muscle biopsy from an adult patient with polymyositis, was used for the intramuscular (IM) nerve study. Specimens were stored at −70 °C until used. Slides on which frozen 10-μm sections of human nerve tissue were mounted were fixed with acetone and then incubated for 30 minutes with 10% goat serum in PBS. An avidin-biotin blocking kit (Vector laboratories, Burlingame, CA) was used to block any nonspecific reaction, after which the avidin-biotin was washed out with PBS. Each slide was incubated overnight at 4 °C with rabbit anti-GalNAc-GD1a antibody (diluted 1:20 with 10% goat serum in PBS) purified as described above; mouse monoclonal IgG anti-200kD neurofilament (NF200) antibody (diluted to 1:40; Sigma); or mouse monoclonal IgM anti-CD57 (HNK-1) antibody (diluted to 1:20; Pharmingen International, San Diego, CA). Monoclonal anti-HNK-1 antibody was used as the marker for myelin. The HNK-1 epitope is present in P0 glycoprotein, peripheral myelin protein-22, myelin-associated glycoprotein, sulfate-3-glucuronyl paragloboside (SGPG), and sulfate-3-glucuronyl lactosaminyl paragloboside (SGLPG, a higher homologue of SGPG).[13] Normal mouse IgG1 (Chemicon International, Temecula, CA), IgM (Chemicon International), and normal rabbit IgG (Inter-cell Technologies, Hopewell, NJ) were the controls (diluted 1:50, 1:100, and 1:4000 with 10% goat serum in PBS). After being washed with PBS, each slide was incubated for 90 minutes with biotinylated anti-rabbit IgG (H+L) (Vector Laboratories; diluted 1:400 with PBS) and Texas Red–conjugated antimouse immunoglobulin (Amersham Pharmacia Biotech, Centennial Avenue, NJ; diluted 1:50 with PBS). After 5 more minutes of incubation with fluorescence-conjugated streptavidin (Sigma; diluted 1:100 with PBS), each slide was mounted with the medium Vectorshield (Vector Laboratories). Double fluorescence microscopy was done with a Zeiss Axioskop microscope (Jena, Germany) and a charge-coupled device camera. Individual images were converted to the PICT format and merged as pseudo-color RGB images by Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Cryosections were treated with chloroform: methanol 1:1 (by volume) for 30 seconds at room temperature to extract glycolipid and then immunostained as described above.

Results. *Assessments of rabbits immunized with GalNAc-GD1a and of purified anti-GalNAc-GD1a antibody.* IgG and IgM anti-GalNAc-GD1a antibody titers were increased in sera from the two rabbits immunized with GalNAc-GD1a. Serum samples from one rabbit (rabbit A) had only anti-GalNAc-GD1a antibody (titers 1:1600, IgG and IgM, both), whereas those from the other rabbit (rabbit B) had IgG antibody against only GalNAc-GD1a (titer 1:102,400) and IgM antibodies against GalNAc-GD1a (1:25,600) and GM1 (1:12,800). No rabbits immunized with GalNAc-GD1a showed any clinical symptoms or pathologic changes in their nerve tissues. The rabbit B serum was used for the purification of anti-GalNAc-GD1a antibody in an affinity column. The purified solution gave a 1:800 IgG anti-GalNAc-GD1a antibody titer in the ELISA, but had no IgG antibody activity to any of the other test gangliosides. Overlay thin-layer chromatogram immunostaining also confirmed that the purified solution had IgG antibody reactivity only with GalNAc-GD1a (figure 1). Immunoabsorption test showed that the purified IgG antibody was absorbed with GalNAc-GD1a, but not with GM1 (data not shown). This purified solution was diluted 1:20 with 10% goat serum in PBS (IgG concentration: 1.1 μg/mL) and used for an immunohistochemical study.

Localization of GalNAc-GD1a in human nerve tissue. Anti-GalNAc-GD1a antibody immunostained an inner part of compact myelin and additionally a periaxonal-axolemma-related region in the VR, particularly in the nodes of Ranvier and paranodal region (figure 2). In cross section, staining by the anti-GalNAc-GD1a antibody in the VR encircled staining by the anti-NF200 antibody in the region

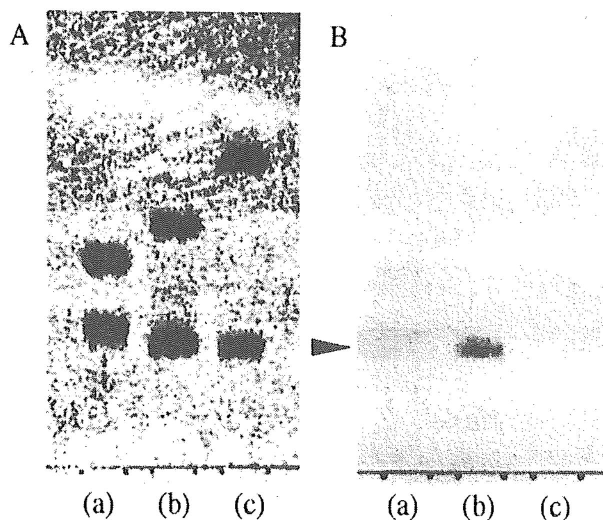

Figure 1. (A) Thin-layer chromatographic pattern of test gangliosides. Each ganglioside (2.0 μg) was developed in solvent system, chloroform/methanol/water (50/40/10, by volume). GM1 (upper) and GD1a (lower) were developed in lane (a), GM2 (upper) and GalNAc-GD1a (lower) in lane (b), and GA1 (upper) and GD1b (lower) in lane (c). Each lane was stained with orcinol reagent. Thin-layer chromatogram immunostaining with purified immunoglobulin G anti-GalNAc-GD1a antibody (diluted 1:100) is shown in (B). Only GalNAc-GD1a was stained in lane (b) with the purified antibody (arrowhead).

adjacent to the axolemma and overlapped in the anti-HNK-1 antibody staining. In teased ventral fibers, immunostaining by anti-GalNAc-GD1a antibody was observed in the nodal and paranodal regions inside of anti-HNK-1 antibody staining (figure 3). In the DR, anti-GalNAc-GD1a antibody staining was present selectively in the periaxonal

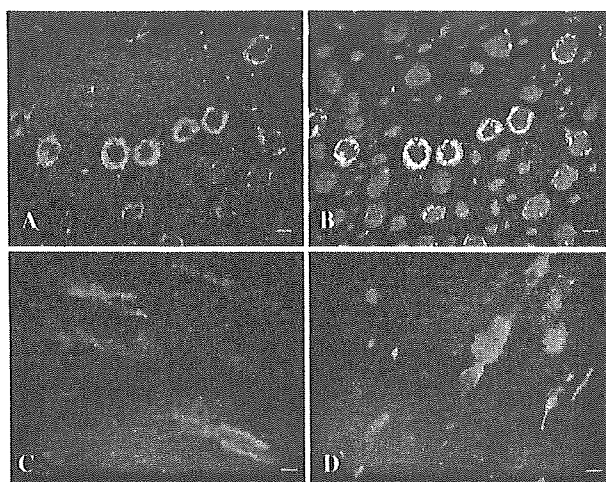

Figure 2. (A, B) Cross sections of human ventral roots (VR). (A) Immunostaining by anti-GalNAc-GD1a antibody (fluorescein isothiocyanate [FITC], green). (B) Double fluorescence labeling by anti-GalNAc-GD1a antibody (green) and anti-neurofilament-200 antibody (Texas red, red). (C, D) Longitudinal sections of human VR. Immunostaining by anti-GalNAc-GD1a antibody (FITC, green). Cross section, D shows a more oblique aspect. (Bar = 10 μm.)

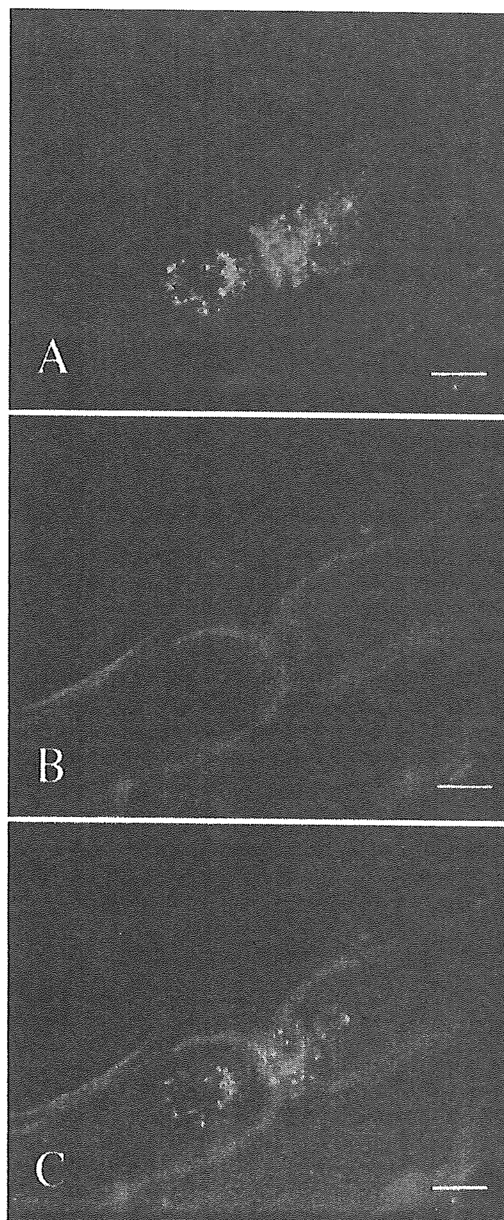

Figure 3. Teased fiber preparations of human ventral fibers. (A) Immunostaining by anti-GalNAc-GD1a antibody (fluorescein isothiocyanate [FITC], green). (B) Immunostaining by anti-HNK-1 antibody (Texas red, red). (C) Double fluorescence labeling by anti-GalNAc-GD1a antibody (green) and anti-HNK-1 antibody (red). (Bar = 10 μm.)

region on small-diameter fibers (figure 4), but overlapping with anti-HNK-1 antibody staining was not confirmed there. No significant staining was observed in teased dorsal fibers. In the IM nerves, anti-GalNAc-GD1a antibody immunostained almost all the nerve fibers, encircling the staining by anti-NF200 antibody (figure 5, A and B). Anti-GalNAc-GD1a staining was observed inside of anti-HNK-1 staining and partially overlapped with the latter (figure 5, C, D, and E). It might be around axon-myelin interface. In the sural nerves, anti-GalNAc-GD1a antibody stained some small fibers. Because of artifactual changes, staining of periaxonal region in the small fibers was not clearly confirmed (figure 6). Prior treatment with chloroform:

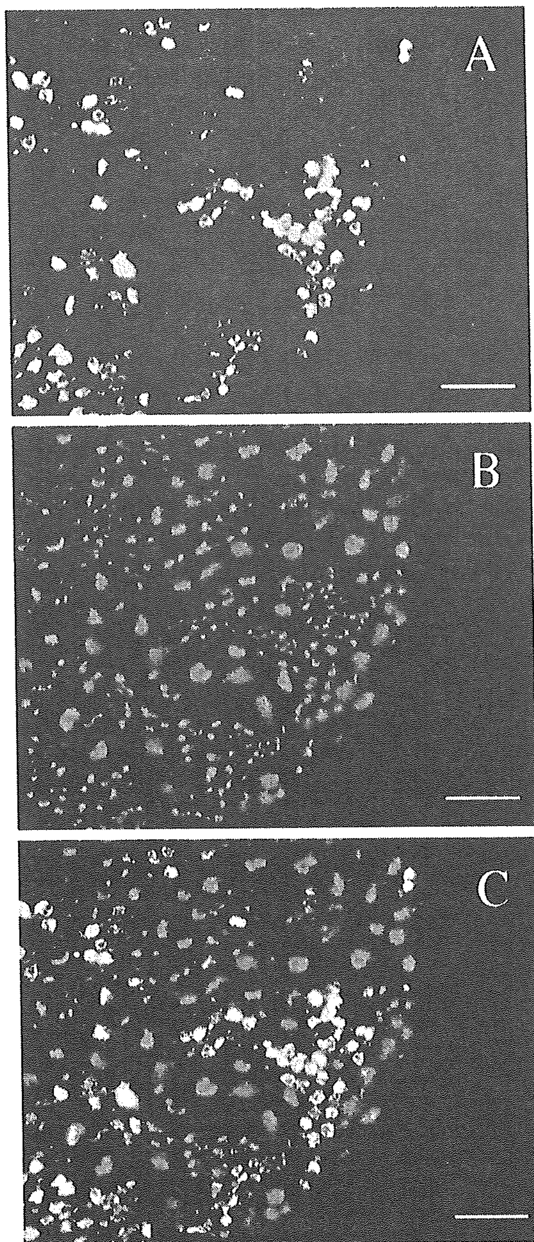

Figure 4. Cross sections of human dorsal roots (DR). (A) Immunostaining by anti-GalNAc-GD1a antibody (fluorescein isothiocyanate [FITC], green). (B) Immunostaining by anti-neurofilament-200 antibody (Texas red, red). (C) Double fluorescence labeling by anti-GalNAc-GD1a antibody (green) and anti-neurofilament-200 antibody (red). Periaxonal regions of some DR fibers are immunostained with anti-GalNAc-GD1a antibody. (Bar = 50 μm.)

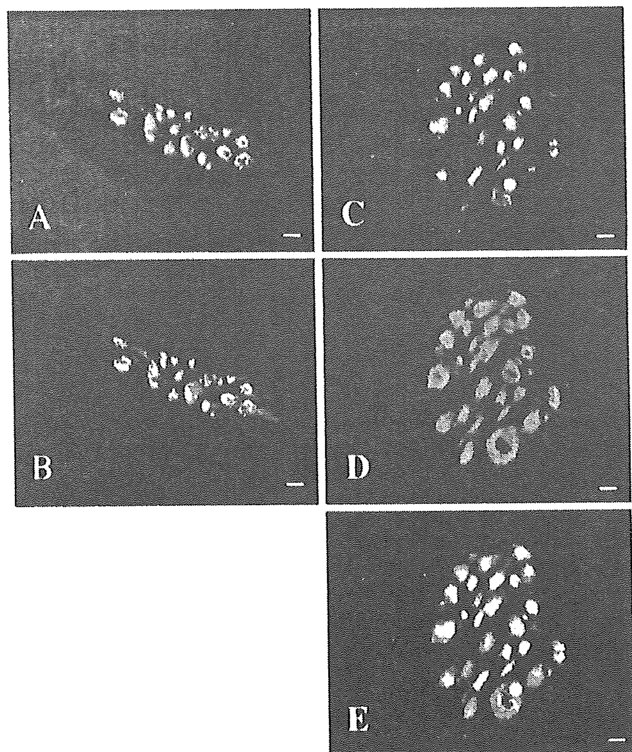

Figure 5. (A, B) Cross sections of human IM nerve. (A) Immunostaining by anti-GalNAc-GD1a antibody (fluorescein isothiocyanate [FITC], green). (B) Double fluorescence labeling by anti-GalNAc-GD1a antibody (green) and anti-neurofilament-200 antibody (Texas red, red). (C, D, E) Cross sections of human IM nerve. (C) Immunostaining by anti-GalNAc-GD1a antibody (FITC, green). (D) Immunostaining by anti-HNK-1 antibody (Texas red, red). (E) Double fluorescence labeling by anti-GalNAc-GD1a antibody (green) and anti-HNK-1 antibody (Texas red, red). (Bar = 10 μm.)

methanol 1:1 eliminated anti-GalNAc-GD1a antibody staining in each tissue, whereas anti-neurofilament and anti-HNK-1 antibody staining was preserved.

Discussion. GalNAc-GD1a is a minor ganglioside in human brain and peripheral nerves,[14,15] but its localization in human nerve tissue has yet to be determined. The purified anti-GalNAc-GD1a antibody used in this study, diluted more than 1:20, was considered monospecific to GalNAc-GD1a. In this study, however, we did not investigate antibody to a ganglioside, GalNAc-GM1b, which shares [GalNAcβ1–4 (NeuAcα2–3)Gal-GalNAcβ1-] with GalNAc-GD1a. Yuki et al. pointed out that IgG anti-GalNAc-GD1a antibodies from patients with GBS often have reactivity to GalNAc-GM1b.[16] Therefore, a part of IgG in the purified anti-GalNAc-GD1a antibody solution might react with an epitope, [GalNAcβ1–4 (NeuAcα2–3)Gal-GalNAcβ1-], shared by GalNAc-GD1a and GalNAc-GM1b. Elimination of immunostaining by prior treatment with chloroform: methanol 1:1 indicates that anti-GalNAc-GD1a antibody recognizes a glycolipid rather than a glycoprotein. This immunostaining by anti-GalNAc-GD1a antibody therefore may indicate the location of GalNAc-GD1a in nerve tissues.

The findings indicate that GalNAc-GD1a is located on the periaxonal-axolemma-related region or an inner part of compact myelin (including paranodal loops). But the two possibilities cannot be distinguished. GalNAc-GD1a was present in almost all the IM nerve fibers, and appeared to be localized in the paranodal regions of the VR. Immunostaining in teased ventral fibers also suggests the localization of GalNAc-GD1a on the nodal and paranodal regions.

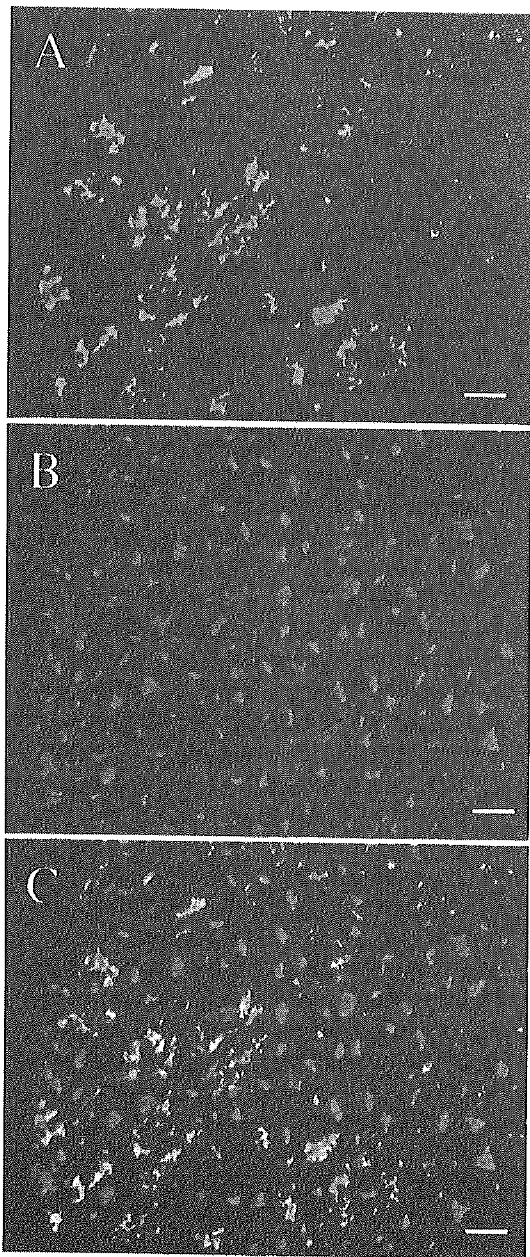

Figure 6. Cross sections of human sural nerve. (A) Immunostaining by anti-GalNAc-GD1a antibody (fluorescein isothiocyanate [FITC], green). (B) Immunostaining by anti-neurofilament 200 antibody (Texas red, red). (C) Double fluorescence labeling by anti-GalNAc-GD1a antibody (green) and anti-neurofilament-200 antibody (red). (Bar = 20 μm.)

The difference of the immunostaining between the IM nerve fibers and VR may reflect the difference of distribution of GalNAc-GD1a between the two sites. Whether IgG anti-GalNAc-GD1a antibody is associated with primary axonal damage or demyelination in the pathogenesis of GBS is under discussion.[7-9] Very low compound muscle action potentials and normal distal latencies are common in patients with GBS with IgG anti-GalNAc-GD1a antibodies,[6,7] which is explained by the localization of GalNAc-GD1a on the axolemmas of IM nerves. Different from A and B in figure 5, showing periaxonal staining of anti-GalNAc-GD1a antibody, the whole axon seems to be stained by anti-GalNAc-GD1a antibody in some of the nerve fibers in C and E. Such staining may be due to the low resolution or the artifactual changes associated with fixation condition and cryostat cutting of autopsy specimens. Although an immunohistochemical investigation that uses electron microscopy would be useful, the optimum conditions for such an investigation of glycolipid antigens have not been established. Further study is needed to clarify in detail the distribution of GalNAc-GD1a in the peripheral nerves.

IgG antibodies monospecific to GalNAc-GD1a are closely associated with the pure motor type of GBS that has few sensory signs and symptoms[7-9] and with chronic motor axonal neuropathy.[17] Our investigation showed that GalNAc-GD1a is localized in both the motor and sensory nerves. The reason for the discrepancy between the immunolocalization of GalNAc-GD1a in sensory nerves and the absence of sensory disturbance in patients with GBS with IgG anti-GalNAc-GD1a antibodies is not known. Several factors may explain the discrepancy. First, geometric and conformational properties of GalNAc-GD1a in the sensory nerves may differ from those in motor nerves. Analysis of gangliosides from human motor and sensory nerves has shown that the ceramide compositions of sensory nerve gangliosides distinctly differ from those of the motor nerves.[18] Phospholipid interaction with membrane proteins, which can modify ganglioside conformation,[19,20] may differ in the motor and sensory nerves. Secondly, specificity of IgG anti-GalNAc-GD1a antibody of patients with GBS might be different from that of the rabbit antibody used in the current study. IgG anti-GalNAc-GD1a antibody in patients with GBS is supposedly generated against glycoconjugates in infective organisms of the antecedent infections. On the other hand, the rabbit IgG anti-GalNAc-GD1a antibody in our study is obtained from rabbit sera after inoculation of authentic GalNAc-GD1a. It is therefore possible that the anti-GalNAc-GD1a antibody of patients with GBS binds only to epitopes of GalNAc-GD1a on motor nerves and that the rabbit antibody binds to GalNAc-GD1a on both motor and sensory nerves. As we reported previously,[12] IgM antibodies that react with GalNAc-GD1a and GM2 are associated with motor and sensory nerve damages, whereas IgM antibodies monospecific for GalNAc-GD1a can cause damage of the motor nerves only. Only anti-GalNAc-GD1a antibodies that cross-react with GM2 could possibly gain access to the antigen on the sensory nerves.

Interestingly, our results are partially consistent with a recent report of localization of major gangliosides.[21] The report shows that antibody specific to GD1a binds preferentially to motor nerve fibers and, additionally, to small, unmyelinated fibers in human dorsal roots. The staining pattern is similar to our results in human ventral and dorsal roots, although the purified anti-GalNAc-GD1a antibody in our

study does not cross-react with GD1a. Why IgG antibody to GD1a or GalNAc-GD1a associated with acute motor axonal variant of GBS recognizes unmyelinated fibers or Remak bundles in the human dorsal roots is a problem to be solved.

Differential tissue preparation and localization techniques may yield different results.[22] We conducted immunohistochemical studies with different fixation techniques in the current study. Immunostaining in 2% paraformaldehyde fixation was much weaker than staining in acetone fixation (data not shown). Staining in nonfixation frozen sections was almost similar to that in acetone fixation, although the sections were fragile. We have used acetone fixation technique in immunohistochemical study on localization of gangliosides (for example, GD1b and GQ1b)[10,23] in human peripheral nerves. Acetone fixation technique proved to be sufficient to investigate the localization of gangliosides.

A recent study showed that binding of human monoclonal antibodies cloned from peripheral blood lymphocytes of patients with motor neuropathy was enriched in clusters of small fibers in human dorsal root and around myelin-axon interface of myelinated fibers in human VR.[24] The antibodies had cholera toxin subunit β-like activity or anti-GM1, GA1, and GD1b activities. The distribution of the binding site of the monoclonal antibody is similar to that of purified anti-GalNAc-GD1a antibody in DR and VR in our study, although the purified antibody had no reactivity with GM1, GD1b, and GA1. As stated in the report,[24] factors other than antigen distribution, such as conformational properties of gangliosides and vulnerability of a particular place, may also influence distribution of peripheral nerve involvement in anti-ganglioside antibody-mediated neuropathy.

In another report, selective motor nerve fibers were immunostained by serum, containing IgG anti-GD1a antibody, of a patient with acute motor axonal neuropathy.[25] The serum used in the study appeared to have had antibody to GalNAc-GD1a as well. Our results provide partial confirmation of their results on motor roots. Immunostaining pattern in DR was, however, different between their and our studies, which is probably due to different specificity of each anti-GalNAc-GD1a antibody.

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Acute Axonal Polyneuropathy Associated with Alcoholism

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Introduction
We studied an alcoholic patient, who developed acute axonal polyneuropathy, mimicking Guillain-Barré syndrome (GBS). However, the clinical and laboratory findings support the idea of direct ethanol toxicity as a cause.

Case Report
A 42-year-old woman with a 9-year history of excessive ethanol consumption (daily average intake, 290 g ethanol) had limited her daily ethanol intake to 100 g during the 2 years prior to admission. However, she had resumed excessive drinking 1 month before admission. At the same time, she noticed bilateral myalgia in her calf muscles, and soon, developed progressive tetraparesis. She experienced severe nutritional deficiency due to loss of appetite during the 1-month intoxication period. On admission, her muscle strength, according to the MRC scale [1], was as follows: shoulder abduction, elbow flexion and extension, wrist extension, hip flexion, and knee flexion and extension: 4; wrist flexion: 3, and ankle dorsiflexion and plantar flexion: 1. Her grip strength was 0 kg bilaterally. There was severe paresthesia in both the proximal and distal portions of the upper and lower limbs. Her sense of vibration was severely reduced in the feet. There was no cranial nerve involvement or autonomic disturbances. Her glucose profile, thyroid function test results, and aminolevulinic acid level were normal. Monoclonal bands on immunoelectrophoresis, antinuclear antibody, and screening for antiganglioside antibodies (by ELISA), including anti-GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, GQ1b, GA1, Gal-C and GalNac-GD1a, were all negative. CSF examinations on the 3rd and 16th days of hospitalization revealed normal cell counts (1 and 0.67/mm^3), normal protein levels (25.6 and 24.9 mg/dl), and no oligoclonal bands or autochthonous IgG production. Vital lung capacity was normal. The electrophysiologic study (table 1) showed axonal motor and sensory neuropathy. None of the motor nerve conduction data were compatible with demyelination. Follow-up examinations showed a sharp increase in compound muscle action potential (CMAP) and sensory nerve action potential (SNAP) amplitudes (table 1). Needle electromyography (6th and 20th days) performed on the right biceps brachii and tibialis anterior showed denervation potentials (fibrillations and positive sharp waves, more potentials during the second examination than the first), and mild chronic neurogenic changes (high-amplitude and long-duration motor unit potentials and a high-amplitude incomplete interference pattern, no distinct difference between the first and second

Table 1. Nerve conduction study in a 42-year-old woman with alcohol-associated acute axonal neuropathy 6, 20, and 41 days after hospital admission

	Days after admission			Reference values
	6	20	41	
Motor nerve				
Median				
CMAP, mV	4.9	9.3	10.7	>4.5
Distal latency, ms	2.8	3.0	3.1	<3.7
Velocity, m/s	54.0	50.9	47.7	>50
F latency, ms	24.0	27.4	28.0	<30
Ulnar				
CMAP, mV	12.9	17.0	ND	>4.5
Distal latency, ms	2.9	2.9	ND	<3.4
Velocity, m/s	45.8	46.4	ND	>48
F latency, ms	23.6	ND	ND	<30
Tibial				
CMAP, mV	2.5	3.5	4.0	>7.0
Distal latency, ms	4.4	4.4	4.2	<5.2
Velocity, m/s	35.4	34.2	32.2	>38.2
F latency, ms	39.0	40.3	ND	<45
Sensory nerve				
Median				
SNAP, μV	2.7	2.9	4.2	>7.0
Velocity, m/s	52.4	44.4	44.2	>44
Ulnar				
SNAP, μV	1.6	2.0	ND	>5.0
Velocity, m/s	43.0	42.7	ND	>43
Sural				
SNAP, μV	0.5	1.2	2.0	>3.5
Velocity, m/s	39.9	39.9	40.1	>41

ND = Not determined. Abnormal data are underlined.

examination). Our patient received intravenous supplementation including vitamins B_1, B_6, and B_{12} beginning on the 1st day of hospitalization. Her muscle strength began to recover as early as day 2. Her grip strength recovered to 4 kg bilaterally by the 3rd day and had increased to 10 kg bilaterally by day 23. On the 7th day, shoulder abduction, elbow flexion and extension, wrist extension, and knee flexion and extension measured 5 on the MRC scale; wrist flexion, hip flexion and ankle plantar flexion measured 4; and ankle dorsiflexion measured 3. On the 15th day, ankle dorsiflexion was rated 4 and she regained the ability to walk. Her paresthesia began to disappear on day 9 and was limited in both the hands and feet by day 23.

Discussion

Whereas our patient mimicked acute axonal motor-sensory neuropathy (AMSAN), several signs were atypical for GBS, such as normal CSF findings, negative antiganglioside antibodies, the absence of conduction blocks, and no episodes of viral infection or diarrhea coinciding with those of previously reported alcoholic cases of acute axonal polyneuropathy [2–5]. Furthermore, early recovery of CMAP and SNAP contrasted with findings in the axonal GBS [6, 7]. The fact that the amplitudes in the nerve conduction studies rapidly increased in parallel with rapid functional recovery and the fact that the denervation signs in the needle electromyography increased suggest two possible pathomechanisms other than axonal degeneration. One is a partially neurapractic lesion. The other is a motor nerve terminal (very distally located) conduction block, although the distal motor latencies were within normal limits [7]. Interestingly, in contrast to alcoholics with painful small-fiber neuropathy and normal thiamine status, as reported by Koike et al. [8], our patient showed severe involvement of both large (vibration sense) and small (paresthesia) fibers. This may be due to her neuropathy being caused not only by alcohol abuse, but also by nutritional deficiency.

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□ EDITORIAL □

Anti-ganglioside Antibodies in Guillain-Barré Syndrome; Useful Diagnostic Markers as Well as Possible Pathogenetic Factors

Key words: Guillain-Barré syndrome, Miller Fisher syndrome, ganglioside

Guillain-Barré syndrome (GBS) is an acute motor-dominant monophasic polyneuropathy usually preceded by an infection. It has been considered that GBS is a demyelinative neuropathy, which is caused by the damage to myelin. Recently, in addition, the presence of an axonal form of GBS is recognized. There are also some variants of GBS, including Miller Fisher syndrome (MFS), characterized by the triads of ophthalmoplegia, ataxia and areflexia. Considering the effectiveness of plasmapheresis for the treatment of GBS, some humoral factors such as autoantibodies should have an important role in its pathogenesis.

Gangliosides are sialic-acid containing glycolipids, which are rich in the nervous system and are localized on the cell surface membrane. Among the gangliosides, there are diverse molecular species according to the carbohydrate sequence. Each ganglioside has a unique localization in the nervous system. Recent investigations have indicated that the increased titer of antibodies against glycolipids, including gangliosides, in sera is characteristic of autoimmune neuropathies, such as GBS, multifocal motor neuropathy, and IgM paraproteinemic neuropathy (1). The titer of the anti-ganglioside antibodies in GBS is highest in the acute-phase serum and decreases with time. This suggests that the production of anti-ganglioside antibodies is closely associated with the pathogenesis of GBS. Anti-ganglioside antibody therefore is a possible humoral factor that plays an important role in the pathogenesis.

Molecular mimicry between the microorganism of the antecedent infection and gangliosides is considered to be an important mechanism of the antibody production in GBS. The lipopolysaccharide of *Campylobacter jejuni* has been reported to have a ganglioside-like structure (2). In addition to gangliosides, galactocerebroside, a major myelin glycolipid, also is a target for serum antibody in some GBS patients. Most of GBS patients with anti-galactocerebroside antibody have an antecedent infection with *Mycoplasma pneumoniae*, which has been shown to have a glycolipid with a galactocerebroside-like carbohydrate structure (3).

Most of the anti-ganglioside antibodies are known to be associated with a certain clinical features. Anti-GQ1b IgG antibody is closely associated with ophthalmoplegia and ataxia (MFS) (4, 5). Anti-GM1 IgG antibody (6), anti-GalNAc-GD1a IgG antibody (7), and anti-GM1b IgG antibody (8) are associated with pure motor type of GBS. Anti-GD1a IgG antibody is associated with GBS of acute motor axonal neuropathy type (9). GBS patients with anti-GD1b IgG antibody have sensory as well as motor disturbance and are of demyelinating type (10). IgG antibody against LM1 is present in the sera from patients with demyelinating type of GBS. The association between anti-GT1a IgG antibody and the pharyngeal-cervical-brachial variant of GBS has been reported.

Some of these relationships can be explained by the distribution of target antigens in human peripheral nerves; that is, GQ1b is densely localized in the paranodal region of the three cranial nerves (oculomotor, trochlear, and abducens nerves) innervating extraocular muscles (4) and in some primary sensory neurons (5), GD1b is localized in the large primary sensory neurons and in the paranodal region of the peripheral nerves (11). LM1 is known to be the predominant ganglioside in human peripheral nervous system myelin. Anti-ganglioside antibodies may therefore determine the distribution of the damage by binding to the regions where respective ganglioside antigens are densely localized.

The pathogenetic role of anti-ganglioside antibodies has been confirmed by rabbit experimental sensory ataxic neuropathy induced by sensitization with GD1b ganglioside (12). GD1b is localized in large primary sensory neurons, which convey deep sensation. Disturbance in deep sensation cause sensory ataxia. Anti-GD1b antibody therefore may bind to those GD1b-positive sensory neurons to cause ataxia. In addition, motor neuropathy has been reported by sensitization with GM1 (13). The effect of anti-GQ1b antibody to the neurotransmitter release from the presynaptic terminal of neuromuscular junction of mouse diaphragm has also been reported (14). Thus, data suggesting that anti-ganglioside antibody is involved in the pathogenesis of autoimmune neuropathies have recently accumulated.

Anti-ganglioside antibodies also can be used as a diagnostic marker of autoimmune neuropathies, especially GBS and MFS, because early diagnosis is essential for appropriate therapy for the patients with GBS and MFS. For example, acute ophthalmoplegia or ataxia can be caused by many dis-

orders of various etiologies; cerebrovascular diseases, acute cerebellitis, brainstem encephalitis, multiple sclerosis, aneurysm, tumor, Tolosa-Hunt syndrome, diabetic ophthalmoplegia, myasthenia gravis, etc. In such cases, anti-GQ1b IgG antibody can be used as a diagnostic marker of MFS and GBS with ophthalmoplegia or ataxia.

The presence of anti-ganglioside antibodies is usually examined by enzyme-linked immunosorbent assay (ELISA) or thin-layer chromatogram (TLC) immunostaining procedure. Although those assays could be performed within one day, they need technical skill and are not included in the routine laboratory examination in most hospitals. Considering the usefulness of the antibody assay for the diagnosis of GBS, it is of importance to develop a method that can be easily performed by clinicians. Alaedini and Latov reported the latex agglutination assay for detection of anti-ganglioside antibodies (15). In this issue, Irie et al reported the results of anti-ganglioside antibody assay by the use of the above method with some modifications and compared the results with those of ELISA (16).

See also p 490.

This method is easy to perform and the results can be obtained within only a few minutes. The problem is its sensitivity. Anti-ganglioside antibodies with a high titer on ELISA were detected by this method, while those with medium or low titer were undetectable. Because this agglutination assay is a hopeful method for easy and rapid examination of anti-ganglioside antibody activities, further investigation is necessary to increase the sensitivity of this assay.

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

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Abstract—Compared with 87 unventilated patients with Guillain–Barré syndrome (GBS), 44 ventilated patients with GBS more frequently had multiple cranial nerve involvement (91 vs 50%; $p < 0.001$) and IgG anti-GQ1b antibody (27 vs 8%; $p = 0.006$). In GBS patients without ophthalmoparesis, the presence of IgG anti-GQ1b antibody was associated with respiratory failure (12 [3/25] vs 0% [0/67]; $p = 0.04$). The presence of the antibody may be a factor predictive of respiratory failure in GBS.

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Poor prognosis for disabilities and prolonged recovery are common for ventilated patients with Guillain–Barré syndrome (GBS).[1,2] Comparison of clinical features between ventilated and unventilated patients with GBS has identified factors predictive of progression to mechanical ventilation,[1,2] leading to an appropriate choice of therapy and improvement of prognoses in patients with severe GBS. Antiganglioside antibodies, which are frequently present in the acute-phase GBS sera, may be immunologic markers associated with certain neurologic features.[3] We investigated the clinical features of and antiganglioside antibodies in ventilated and unventilated patients with GBS to determine factors predictive of progression to artificial ventilation.

Methods. *Study population.* Between January 1998 and September 2000, acute-phase GBS sera, collected from 329 patients with GBS at various general and teaching hospitals throughout Japan, were sent to us. Those sera were obtained on admission and before treatment. Patients' clinical data, also sent to us at that time, were examined by neurologists. The GBS diagnosis was defined clinically by the criteria of Asbury and Cornblath.[4] Those with clinically defined GBS were divided into two groups: ventilated (GBS-AV[+] group) and unventilated patients. Control patients (GBS-AV[−] group) were selected systematically (one in every three consecutive cases in our files) from unventilated patients with GBS to reduce the effects of selection bias. Clinical and electrophysiologic features of the GBS-AV(+) and GBS-AV(−) patients were analyzed.

Analyses of clinical and electrophysiologic features. Patient disabilities were graded on the Hughes Functional Grading Scale.[5] Neurologic symptoms were analyzed during the course of the disease. Electrophysiologic data were evaluated as described previously[6] and categorized as "primary demyelinating," "primary axonal," "inexcitable," "equivocal," or "normal."

Analyses on antiganglioside antibodies. Serum antibodies to nine ganglioside antigens (GalNAc-GD1a, GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and GQ1b) were investigated by ELISA, as described elsewhere.[6] IgG anti-GT1a antibody was also investigated in some patients' sera. GalNAc-GD1a was prepared in our laboratory from bovine brain.[6] The other gangliosides were purchased from Sigma (St. Louis, MO).

Statistical analysis. Differences in proportions were tested by Fisher exact probability test or the χ^2 test. The Student t-test was used to compare ages and the Mann–Whitney test to compare onset with nadir (days) of the GBS-AV(+) and GBS-AV(−) groups. The time of nadir, when a patient was most severely affected, was determined by each attending physician. Frequency of antiganglioside antibody was analyzed with a multiple logistic regression model. The dependent variable was artificial ventilation, and the independent variables were IgG anti-ganglioside antibodies that were positive in the sera of >3 patients in 131 total subjects. Two-tailed p values of <0.05 were considered significant. These analyses were performed with StatView (SAS, Cary, NC) and Statistica (3.0; Statsoft, OK) software.

Results. *Study population.* Of 329 patients with clinically defined GBS, 44 (13%) required artificial ventilation (GBS-AV[+] group). Of the 285 unventilated GBS patients, 94 were selected systematically as described in Methods, 7 of whom were excluded because of incomplete clinical data. The GBS-AV(−) group therefore consisted of 57 unventilated male and 30 unventilated female patients with GBS. No significant differences were found between the two groups as to gender and age.

Clinical and electrophysiologic features. Clinical features of the patients in the two groups are given in table 1. Most of the GBS-AV(+) patients had cranial nerve deficits, in particular facial and bulbar palsies (see table 1). Thirty-one GBS-AV(+) patients had more than two of the extraocular, facial, and bulbar palsies. There was no difference between the two groups in the distribution of limb weak-

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Table 1 Clinical features of and electrophysiological findings for GBS patients with and without artificial ventilation

Clinical features	GBS-AV(+), n = 44	GBS-AV(−), n = 87	p Value*	Odds ratio
Age, y; mean (95% CI)	42 (36–48)	41 (38–45)†	0.8‡	
Male, no. (%)	27 (61)	57 (66)	>0.9	
Antecedent infections, no. (%)	§	‖		
RT	26 (60)	45 (56)	0.54	
GI	7 (16)	24 (30)	0.2	
Onset to nadir, d	¶	**		
Mean (95% CI)	5.9 (5.0–6.8)	6.8 (5.8–7.9)	0.74††	
Cranial nerve deficits, no. (%)	§	‡‡		
Positive	39 (91)	41 (50)	<0.0001	9.8
III, IV, VI	19 (44)	15 (18)	0.004	3.5
VII	33 (77)	25 (30)	<0.0001	7.5
IX, X	28 (65)	28 (34)	0.002	3.6
XI	6 (14)	1 (1)	0.01	13
XII	8 (19)	4 (5)	0.04	4.5
Sensory disturbances, no. (%)	§§	‖‖		
No	11 (28)	20 (25)	0.87	
Sensory loss	13 (33)	31 (39)	0.71	
Electrophysiology, no. (%)	‖‖	¶¶		
Primary demyelinating	16 (52)	22 (58)	>0.9	
Primary axonal	2 (7)	3 (8)	>0.9	
Inexcitable	3 (10)	0 (0)	0.17	
Normal	0 (0)	2 (5)	0.6	
Equivocal	10 (32)	11 (29)	>0.9	

* Two-tailed p value; † n = 85; ‡ Student t-test; § n = 43; ‖ n = 80; ¶ n = 36; ** n = 73; †† Mann–Whitney U test; ‡‡ n = 82; §§ n = 39; ‖‖ n = 31; ¶¶ n = 38.

GBS = Guillain–Barré syndrome; AV = artificial ventilation; RT = respiratory tract infection; GI = gastrointestinal tract infection.

ness and sensory disturbance. Detailed information on autonomic dysfunction was not obtained. Electrophysiologic findings were "primary demyelinating" type dominant in both groups (see table 1).

Treatment and prognosis. Treatment and prognosis in GBS-AV(+) and GBS-AV(−) groups are summarized in table 2. GBS-AV(+) group patients tended to be treated more extensively than GBS-AV(−) group patients. Because the long-term follow-up data often were incomplete, short-term prognoses were evaluated (see table 2).

Frequencies of antiganglioside antibodies. Antiganglioside antibody frequencies in the GBS-AV(+) and GBS-AV(−) groups are shown in table 3. IgG anti-GQ1b and anti-GT1a antibodies occurred significantly more often in the GBS-AV(+) group. The frequency of anti-GQ1b antibodies in GBS-AV(+) patients with ophthalmoparesis (9/19 = 47.4%) equaled that in GBS-AV(−) patients with ophthalmoparesis (7/15 = 46.7%), with no significance ($p >$ 0.9, χ^2 test). The frequency of anti-GQ1b antibodies in GBS-AV(+) patients without ophthalmoparesis (3/25 = 12%) was higher than that in GBS-AV(−) patients without ophthalmoparesis (0/67 = 0%; $p = 0.04$, Fisher exact probability). As for the frequency of IgG anti-GT1a antibody, however, there were no such significant differences: GBS-AV(+) with ophthalmoparesis (9/19 = 47.4%) vs GBS-

Table 2 Therapy and prognosis in GBS-AV(+) and GBS-AV(−) groups

Parameters	GBS-AV(+), n = 42	GBS-AV(−), n = 75	p Value
Treatment, no. (%)			
PE	15 (36)	12 (16)	0.03
IA	16 (38)	35 (47)	0.7
DFPP	12 (29)	15 (20)	0.6
IVIG	11 (26)	4 (5.3)	0.004
Combination therapy	11 (26)	7 (9.3)	0.03
Prognosis,* no. (n)			
1 mo	35† (37)‡	18 (28)	0.005
2 mo	23 (27)	10 (17)	0.1

* Scoring >2 on the Hughes Grading Scale (≧3).
† No. of patients scoring >2 on the Hughes Grading Scale 1 mo after disease onset.
‡ n = total no. of patients with available follow-up data.

GBS = Guillain–Barré syndrome; AV = artificial ventilation; PE = plasma exchange; IA = immunoadsorption; DFPP = double-filtered plasmapheresis; IVIG = IV immunoglobulin.

Table 3 *Antiganglioside antibody frequency*

	GBS-AV(+), n = 44	GBS-AV(−), n = 87	*p* value*	Odds ratio
Antibody positive, no. (%)	25 (57)	54 (62)	>0.9	
IgG class, no. (%)				
Anti-GM1	4 (9)	19 (22)	0.11	
Anti-GM2	0 (0)	0 (0)		
Anti-GM3	0 (0)	0 (0)		
Anti-GD1a	6 (14)	4 (5)	0.13	
Anti-GalNAc-GD1a	3 (7)	13 (15)	0.36	
Anti-GD1b	9 (20)	14 (16)	0.7	
Anti-GD3	2 (5)	1 (1)	0.44	
Anti-GT1b	3 (7)	2 (2)	0.4	
Anti-GQ1b	12 (27)	7 (8)	0.006	4.29
Anti-GM1b	2 (5)	3 (3)	>0.9	
Anti-GT1a	11 (31)*	11 (13)†	0.04	2.96
IgM class, no. (%)				
Anti-GM1	4 (9)	13 (15)	0.51	
Anti-GM2	1 (2)	7 (8)	0.36	
Anti-GM3	0 (0)	0 (0)		
Anti-GD1a	1 (2)	0 (0)	0.67	
Anti-GalNAc-GD1a	2 (5)	9 (10)	0.43	
Anti-GD1b	1 (2)	3 (3)	>0.9	
Anti-GD3	0 (0)	1 (1)	>0.9	
Anti-GT1b	1 (2)	0 (0)	0.67	
Anti-GQ1b	2 (5)	3 (3)	>0.9	
Anti-GM1b	1 (2)	2 (2)	>0.9	
Anti-GT1a	NS	NS		

* n = 35.
† n = 82.

GBS = Guillain–Barré syndrome; AV = artificial ventilation; NS = not studied.

AV(−) with ophthalmoparesis (7/15 = 46.7%; $p > 0.9$, χ^2 test), GBS-AV(+) without ophthalmoparesis (2/25 = 8.0%) vs GBS-AV(−) without ophthalmoparesis (4/67 = 6.3%; $p > 0.9$, Fisher exact probability). A multiple logistic regression model identified only IgG anti-GQ1b antibody as an independent variable correlated with artificial ventilation ($p = 0.04$, odds ratio 7.23).

Discussion. Our findings show that multiple cranial nerve involvement and the presence of IgG anti-GQ1b antibody are important factors predictive of respiratory muscle weakness in GBS. Multiple logistic testing may obscure statistical correlation when variables are not appropriately selected. We selected eight IgG antiganglioside antibodies as individual variables in view of the validity of a multiple logistic regression model. Although pure pharyngeal palsy without respiratory muscle weakness can be an important indication for artificial ventilation, all ventilated patients in our study had respiratory muscle weakness with or without pharyngeal palsy. Bulbar and facial palsies have been reported to be predictive features of respiratory muscle weakness in GBS.[1] An overlap of lower cranial nerve involvement, ophthalmoplegia, and facial diplegia is common in GBS.[7] Our results are consistent with these reports. Certain muscle groups (extraocular, levator, pharyngeal, neck, and respiratory muscles) are preferentially involved in such neuromuscular transmission disorders as myasthenia gravis and botulism. The inference based on clinical features is that neuromuscular junction (NMJ) involvement may contribute to oculomotor, facial, and respiratory muscle weakness in ventilated patients with GBS.

This inference is supported by our findings that the presence of anti-GQ1b antibody is associated with respiratory muscle weakness. In vitro electrophysiologic and morphologic studies show that human and mouse anti-GQ1b antibodies have an α-latrotoxin (LTx)–like blockade effect on neuromuscular transmission.[8] Moreover, passive transfer of sera from botulism or Miller–Fisher syndrome (MFS) patients produced respiratory muscle weakness in mice.[9] In GBS patients with respiratory paresis, the motor nerve terminals of the respiratory muscle may be a candidate for the target of anti-GQ1b antibodies. Because few MFS patients have respiratory paresis, respiratory muscle weakness cannot be explained by the presence of anti-GQ1b antibody alone. Infrequency of respiratory failure in MFS may be associated with differences in the vulnerability to and thresholds of the α-LTx-like effect of anti-GQ1b antibodies in the NMJ of various muscle groups, in addition to the fine specificity of anti-GQ1b antibody. On the other hand, the specific binding of anti-GQ1b IgG antibodies to the paranodal myelin of the oculomotor, trochlear, and abducens nerves may account for the exceptionally high occurrence of ophthalmoplegia in patients with anti-GQ1b IgG antibodies.[10]

Frequency of ventilated patients with GBS and their mortality rate in our series were lower than those in previous reports,[1,2] which might result from advances in supportive care and the recent widespread use of specific treatments such as plasmapheresis and IV immunoglobulin therapy.

Thus, the presence of IgG anti-GQ1b antibody may be a factor predictive of respiratory failure. Development of a more rapid and easier assay system of antiganglioside antibodies is desired for practical use.

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Ganglioside Complexes as New Target Antigens in Guillain–Barré Syndrome

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Antibodies specific for a complex of gangliosides GD1a and GD1b (GD1a/GD1b) were found in sera from eight of 100 patients with Guillain–Barré syndrome (GBS) by the use of enzyme-linked immunosorbent assay and thin-layer chromatogram immunostaining. Those sera also had antibody activities to such ganglioside complexes as GD1a/GM1, GD1b/GT1b, and GM1/GT1b but had little or no reactivity to the each isolated antigen. Clustered epitopes of the ganglioside complex in the plasma membrane may be targeted by such an antibody, and interaction between the antibody and ganglioside complex may induce the neuropathy.

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Antibodies to gangliosides are present in the sera of approximately 60% of patients with acute immune-mediated polyradiculoneuropathy, Guillain–Barré syndrome (GBS).[1] Antiganglioside antibody may function in the development of certain neurological signs and in the pathogenesis of GBS through its binding to ganglioside antigens in peripheral nerves.[2,3] Although various antiganglioside antibodies in GBS patients' sera have been investigated, no antibodies to the ganglioside complex with clustered glycoepitopes have been reported. We found antibodies to a mixture of two ganglioside antigens in the sera of some GBS patients.

Materials and Methods

Representative Serum and the Antiganglioside Antibody Assay

After several days of flu-like symptoms, a 31-year-old man (Patient A) developed acute flaccid tetraparesis. GBS was di-

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agnosed based on Asbury and Cornblath criteria.[4] His acute phase serum was checked for antiganglioside antibodies by an enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatogram (TLC) immunostaining.

TLC immunostaining was conducted on a high-performance TLC plate on gangliosides extracted from whole bovine brain gangliosides through an anion-exchange resin column, DEAE Sephadex A-25 (Amersham Biosciences, Uppsala, Sweden), as described elsewhere.[5] The gradients used were 0.05, 0.1, 0.2, and 0.4M ammonium acetate in methanol. TLC immunostaining also was done for GD1a, GD1b, and a mixture of GD1a and GD1b. The immunostained TLC plate was assayed in an Image analyzer (Luminescent Image Analyzer, LAS-1000plus; Fujifilm, Tokyo, Japan) to evaluate staining in the lane with the GD1a–GD1b mixture.

The ELISA was performed for antibodies to the gangliosides GalNAc-GD1a, GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and GQ1b, as described elsewhere.[6] Serum diluted 1:40 with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was added to wells coated with 0.2μg of antigen. Optical density (OD) values were corrected by subtracting the OD of a control well that had been similarly processed. When the corrected OD was more than 0.1, the serum was considered positive. Serum antibody to GD1a/GD1b, a mixture of 0.1μg each GD1a and GD1b, was investigated by the same methods. Responses of his serum to a 0.2μg mixture of GD1a and GD1b in various ratios (GD1a:GD1b = 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8) also were investigated with an ELISA. ELISAs were repeated twice in the same way, and the mean OD of the two experiments was calculated.

Sera from 100 Consecutive Patients with Guillain–Barré Syndrome and the Enzyme-Linked Immunosorbent Assays for Anti–GD1a/GD1b Antibodies

To investigate the frequency of anti–GD1a/GD1b antibody in GBS patients, between February and December 2002 acute phase sera were collected from 100 consecutive GBS patients who were diagnosed at various general and teaching hospitals throughout Japan, and their clinical data were sent to us at that time. All met the diagnostic criteria of Asbury and Cornblath.[4] Serum antiganglioside antibodies were investigated by ELISAs as described above. Anti-GD1a– or anti-GD1b–positive sera, in which the corrected anti–GD1a/GD1b antibody OD was 0.2 higher than the corrected anti-GD1a or anti–GD1b antibody OD, were considered anti-GD1a/GD1b antibody–positive. The cutoff value (0.2) for the anti-GD1a/GD1b antibody was decided arbitrarily. Anti-GD1a/GD1b antibody–positive sera were overlaid for TLC immunostaining, as described elsewhere.[5]

Anti–GD1a/GD1b antibody was surveyed in ELISAs of sera from16 normal subjects (normal control) and from 119 patients with neurological disorders other than GBS (disease control): cerebrovascular disease, 17; multiple sclerosis, 10; Parkinson's disease, 8; amyotrophic lateral sclerosis, 7; spinocerebellar degeneration, 7; myasthenia gravis, 6; chronic inflammatory demyelinating polyradiculoneuropathy, 5; other neuropathies, 3; and other neurological diseases, 56.

Antibody activities against a mixture of 0.1μg each of other gangliosides such as GM1/GD1a, GM1/GT1b, GD1a/GT1b, and GD1b/GT1b were investigated on the eight anti–GD1a/GD1b antibody–positive sera in the same way as used for GD1a/GD1b described above.

Results

Anti–GD1a/GD1b Antibody in Serum of Patient A

TLC immunostaining showed strong staining just below the position of GD1a in the lane with the ganglioside fraction extracted with 0.1M ammonium acetate (Fig 1A). Positive staining also was present in the lane for GD1a–GD1b mixture, but not in the lanes for GD1a and GD1b (see Fig 1A). In another developing solvent (C/M/0.2% $CaCl_2$ = 30/65/10) that separated the positions of GD1a and GD1b, the immunostaining in the lane of GD1a–GD1b mixture disappeared (see Fig 1B). The Image Analyzer assay showed specific immunostaining in the overlapping portion of the GD1a and GD1b antigens (see Fig 1C). ELISA results were negative for each of the test gangliosides but positive in the well coated with the mixture of GD1a and GD1b antigens (GD1a/GD1b). IgG anti–GD1a/GD1b antibody titer was 1:640 (see Fig 1D). ELISAs done with various combinations of the GD1a and GD1b (GD1a:GD1b) mixture showed that the corrected OD values were the highest at the ratios of 6 to 4 and 5 to 5 (see Fig 1E).

Anti–GD1a/GD1b Antibody Assay for the Consecutive GBS Patients and Controls

ELISAs showed that 8 of 100 consecutive patients (8%) with GBS had IgG anti–GD1a/GD1b antibodies, whereas none of the disease or normal control group patients did. Three of the eight GBS patients with IgG anti–GD1a/GD1b antibodies had neither the IgG anti–GD1a nor anti–GD1b antibody. Of 92 GBS patients without IgG anti–GD1a/GD1b antibody, 4 patients had IgG anti–GD1a antibody, and 11 had IgG anti–GD1b antibody.

TLC immunostaining of the anti–GD1a/GD1b antibody–positive sera from five patients with GBS gave the same results as for Patient A's serum (Fig 2). The quantities of sera obtained from the remaining two patients with anti–GD1a/GD1b antibody were insufficient to overlay for TLC immunostaining. The clinical features and antiganglioside antibodies of the eight GBS patients are given in the Table. Four anti–GD1a/GD1b antibody–positive patients required artificial ventilation. Electrophysiological studies showed "primary axonal" in Patients 2 and 8 and "equivocal" in Patient 3, which were classified as described elsewhere.[5] Five other patients had no available electrophysiological data.

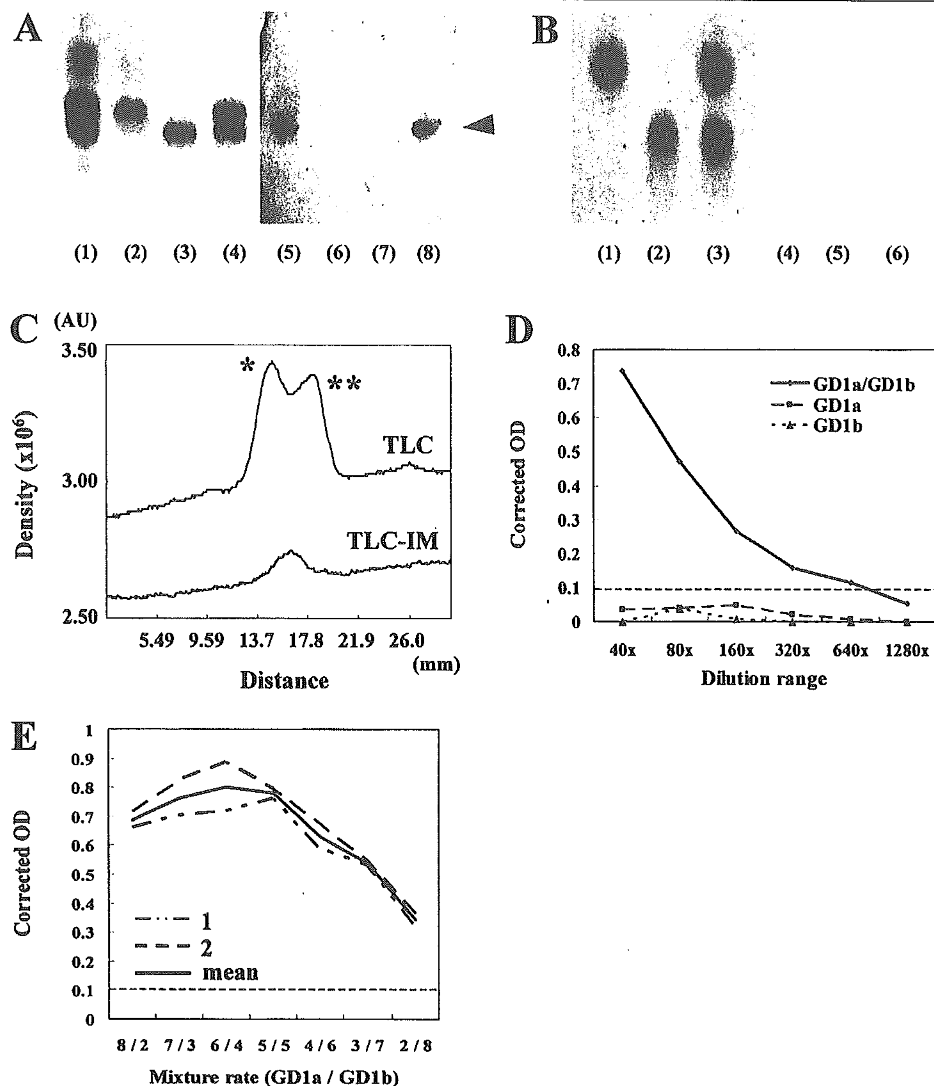

Fig 1. Enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatogram (TLC) studies of Patient A's serum. (A) Left panel (lanes 1–4): TLC results made visible by orcinol reagent, right panel (lanes 5–8): TLC immunostaining of his serum. The developing solvent consisted of chloroform, methanol, and 0.2%$CaCl_2$-$2H_2O$ (50:45:10, vol/vol). Serum was diluted 1:100. Peroxidase-conjugated goat anti–human immunoglobulin G Fc antibody (diluted 1:200; ICN Biomedicals Inc., Aurora, OH) was the second antibody. Immunoreactants were made visible with phosphate-buffered saline containing 0.01% H_2O_2 and 50mg/dl 3,3'-diaminobenzidine tetra-hydrochloride. Bovine brain gangliosides obtained by extraction with 0.1M ammonium acetate were applied to lanes 1 and 5, GD1a (3μg) to lanes 2 and 6, GD1b (3μg) to lanes 3 and 7, and both GD1a and GD1b (3μg each) to lanes 4 and 8. The arrowhead indicates the immunostaining on the overlapping portion of GD1a and GD1b. (B) These panels show TLC results using a solvent system, C/M/0.2%$CaCl_2$ = $2H_2O$ (30:65:10, vol/vol). Left panel (lanes 1–3): TLC results made visible by orcinol reagent, right panel (lanes 4–6): TLC immunostaining of his serum. GD1a (3μg) was applied to lanes 1 and 4, GD1b (3μg) to lanes 2 and 5, and both GD1a and GD1b (3μg each) to lanes 3 and 6. The immunostaining disappeared in the solvent system that allowed clear separation of GD1a and GD1b. (C) Analysis of TLC immunostaining with the Image Analyzer. The top line (TLC) indicates the densities of GD1a and GD1b on the HPTLC plate; the bottom line (TLC-IM) shows the density of the TLC immunostaining of Patient A's serum. The abscissa gives the distance from the base line. The single asterisk shows the GD1b density peak; the double asterisks the GD1a density peak. The peak in the lower line nearly corresponds to the bottom of the trough between the GD1a and GD1b peaks in the upper line. AU = arbitrary unit. (D) ELISA results. Serum was diluted serially from 1:40 to 1:1,280. IgG anti–GD1a and GD1b antibodies are negative. (E) ELISA results for various mixtures of GD1a and GD1b antigens. (line 1) first study results; (line 2) second study results; (solid line) *the mean of 1 and 2.*