Published in final edited form as:

J Neurosci Res. 2006 February 1; 83(2): 274–284. doi:10.1002/jnr.20717.

Molecular Mimicry: Sensitization of Lewis Rats With *Campylobacter jejuni* Lipopolysaccharides Induces Formation of Antibody Toward GD3 Ganglioside

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Abstract

Recently we have reported cases of demyelinating inflammatory neuropathy showing elevated titers of anti-GD3 antibodies, which occurs rarely in Guillain-Barré syndrome. To examine the correlation between the anti-GD3 antibody titer and *Campylobacter jejuni* infection, we sensitized female Lewis rats with lipopolysaccharides (LPSs) from serotype HS19 of *C. jejuni* and examined changes in nerve conduction velocity and nerve conduction block (P/D ratio). After 16 weeks of sensitization, animals revealed decreases of nerve conduction velocity and conduction block (P/D ratio) and high titer of anti-GD3 antibodies. These anti-GD3 antibodies also blocked transmission in neuromuscular junctions of spinal cord-muscle cells cocultures. The GD3 epitope was verified to be located on the Schwann cell surface and nodes of Ranvier in rat sciatic nerve. To determine the target epitope for GD3 antibodies in causing nerve dysfunction, the LPS fraction containing the GD3 epitope was purified from the total LPS by using an anti-GD3 monoclonal antibody-immobilized affinity column. Subsequently, chemical analysis of the oligosaccharide portion was performed and confirmed the presence of a GD3-like epitope as having the following tetrasaccharide structure: NeuAca2-8NeuAc2-3Galβ1-4Hep. Our data thus support the possibility of a contribution of GD3 mimicry as a potential pathogenic mechanism of peripheral nerve dysfunction.

Keywords

ganglioside; Guillain-Barré; syndrome; anti-ganglioside antibody; GD3; *Campylobacter jejuni*; LPS

Guillain-Barré syndrome (GBS) is an acute immune-mediated peripheral neuropathy consisting of acute inflammatory demyelinating polyneuropathy (AIDP), acute motor neuropathy (AMAN), and related disorders. Anti-GM1 antibodies are often detected in patients with AIDP and AMAN (Kornberg et al., 1994; Arasaki et al., 1998; Hiraga et al., 2005). Many investigators have reported the association between an antecedent *Campylobacter jejuni* (*C. jejuni*) infection and subsequent development of GBS. The lipopolysaccharides (LPSs) of a certain serotype of *C. jejuni* have been shown to possess carbohydrate epitopes similar to

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gangliosides, which may serve as the putative pathogenic triggers for subsequent GBS onset. Thus, these disorders may develop as a result of *C. jejuni* infection. In addition to anti-GQ1b Abs, anti-GD3 Abs have been detected in the Miller Fisher variant of GBS (Chiba et al., 1992; Carpo et al., 1998; Susuki et al., 2001; Willison and Yuki, 2002; Willison et al., 2004). Recently we have reported cases of AIDP and chronic inflammatory demyelinating polyneuropathy (CIDP) showing high elevations of anti-GD3 Abs, which rarely occurs in GBS (Usuki et al., 2005). The question then arises of the origin of the antiglycolipid (anti-GSL) Abs in GBS, in particular, the mechanism of elevation of anti-GD3 Abs in these patients. A plausible source, we reasoned, could arise from infectious agents such as bacteria, viruses, or foodstuffs, insofar as most GBS cases occur with an antecedent infectious event that precipitates an allergic and immunological response. In light of the wide occurrence of anti-GSL Abs in these patients, recent research on GBS has focused on GSL mimicry between LPSs in *C. jejuni* and endogenous GSLs in the peripheral nervous system that may be involved in the pathological mechanisms of GBS (Ang et al., 2004). Strain HS19 of *C. jejuni* was isolated from GBS patients and paralytic chickens (Li et al., 1996) and found to be associated with 29% of *C. jejuni*-associated GBS patients in the United States and 83% of cases in Japan (Nishimura et al., 1997; Allos et al., 1998). Although GSL mimicry as a causative mechanism remains uncertain for GBS and related diseases, the terminal tetrasaccharides Gal β 1-3GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4 of a GM1-like epitope and NeuAc α 2-8NeuAc α 2-3Gal β 1-4Hep of a GD3-like epitope have been chemically determined in the LPSs from HS19 (Aspinall et al., 1992, 1994; Yuki et al., 1993). To provide direct proof, we initiated the current study by sensitizing Lewis rats with a highly purified and structurally defined LPS fraction of the HS19 strain of *C. jejuni*.

Materials and Methods

Preparation of LPS

C. jejuni ATCC-43446 (serotype HS19) was grown in *Brucella* broth with gentle shaking (100–150 rpm) at 37°C for 48 hr under microaerophilic conditions. The cells were recovered by centrifugation at 4,000 rpm for 30 min and washed twice with saline. The cell pellets were kept frozen at –20°C until use.

The LPS fraction was extracted from pellets by the hot phenol-water procedure (Westphal et al., 1952). An aqueous phase and a phenolic phase were obtained. The aqueous phase was dialyzed against water. The dialysate was treated with 2 vol of methanol and 1 vol of chloroform. After the chloroform layer was recovered by separatory funnel partitioning, the aqueous layer was again partitioned with 1 vol of chloroform and 1 vol of water. The chloroform layer was recovered and combined with the previous chloroform layer. Most of the LPSs were recovered in the combined chloroform fraction. An additional minor amount of LPS was precipitated from the remaining phenolic phase by addition of 9 vol of acetone. Both LPS fractions were combined, dried, and then subjected to alkaline hydrolysis with 25% ammonia at 56°C for 48 hr. The solution was then dialyzed against water and the retentate lyophilized.

Sensitization of Rats

Immunization was performed according to our previous procedure (Yamawaki et al., 1996). Eight-week-old female Lewis rats, weighing 200–250 g each, were used. One hundred micrograms of LPS were dissolved in 50 mM of phosphate-buffered saline (PBS) with 0.05 ml of keyhole limpet hemocyanin (KLH; 2 mg/ml) and emulsified with an equal volume of complete Freund's adjuvant. Rats were given a single subcutaneous injection of 0.1 ml of inoculum (or vehicle) into the footpads of hind limbs and shoulders. Booster injections were administered similarly to LPS (or vehicle)-treated animals three times at 2-week intervals with 100 μ g of LPS (or no additive) and 2 mg/ml of KLH in PBS emulsified with an equal volume of incomplete Freund's adjuvant. The experimental animals were divided into three groups: 1)

eight rats (LPS-treated rats) injected with the LPS and KLH; 2) eight rats (vehicle-treated rats) injected with KLH; and 3) four rats (untreated rats) without injection.

The animals were weighed weekly and assessed for clinical signs of peripheral nerve abnormality. Blood samples were collected retroorbitally by bleeding with a capillary tube periodically. The animals were sacrificed at 24 weeks following the initial sensitization.

Nerve Conduction Velocity Measurement

Animals underwent electrophysiological measurement by recording nerve conduction velocity (NCV; m/sec) in the tail nerve, without surgical operation, as described previously (Andersen et al., 1994). Digital ring electrodes with twisted wires (Medtronic, Shoreview, MN) were placed on the proximal (5 cm) and distal (2 cm) segments from the recording position as shown in Figure 1A. NCV was recorded by using a diagnostic electromyography (NeuroMax 1002R System; XLTEK, Oakville, Ontario, Canada) and determined by subtracting proximal from distal latency of nerve conduction wave. Proximal or distal amplitudes were also measured from the nerve conduction waves, and then the nerve conduction block (P/D ratio) was calculated as proximal to distal amplitude ratio as shown in Figure 1B. During each measurement, the surface temperature of rat tail was kept constant within 34–35°C.

Antibody Titration

The titers of antiglycolipid or anti-LPS Abs in rat sera were determined by conventional enzyme-linked immunosorbent assay (ELISA) as modified in our laboratory (Usuki et al., 2005). Briefly, each well of a 96-well flat-bottom polystyrene microtiter plate (Immunlon 1B; Lab System, Franklin, MA) was individually coated with 0.1 µg of each of the following glycolipids: GM1, GM2, GD1a, GD1b, GD3, GT1b, GQ1b, GA1, or LPS [the nomenclature for gangliosides is based on that of Svennerholm (1964)]. Nonspecific binding sites in the wells were blocked by 1% bovine serum albumin (BSA) in PBS. Antisera were applied to the plate at various dilutions (1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, 1:12,800 in 1% BSA/PBS), and the plate was subjected to incubation for 1 hr at room temperature. To each of the wells was added a secondary Ab (horseradish peroxidase-conjugated anti-rat IgG or M, respectively, 1:5,000 dilution in 1% BSA/PBS) for 1 hr. Finally, the bound secondary Ab was visualized by addition of a coloring reagent (OPD Peroxidase Substrate in PBS; Sigma, St. Louis, MO). The absorbance was measured at 492 nm with a microplate spectrophotometer (Bio-Rad, Hemel Hempstead, United Kingdom). Ab titers were assigned as the highest dilution in which the absorbance was 0.1 or less.

Thin-Layer Chromatography Overlay

Rat antisera were analyzed by our procedure of thin-layer chromatography (TLC) overlay (Usuki et al., 2005). Briefly, purified glycolipid samples (0.1 µg each) were applied to a TLC plate (HPTLC aluminum sheet silica gel 60; E. Merck, Darmstadt, Germany). The plate was developed with the solvent system of chloroform:methanol:0.2% CaCl₂ in water (55:45:10, v/v/v). After drying, the plate was coated with 0.1% polyisobutylmethacrylate in n-hexane for 1 min. The plate was overlaid with rat antisera (1:100 dilution) for 1 hr. After washing with 1% BSA in PBS solution containing 0.05% Tween 20, the plate was reacted with a secondary Ab solution (horseradish peroxidase-conjugated anti-rat IgG or M Ab, respectively, 1:5,000 dilution in 1% BSA/PBS) for 1 hr, followed by detection with OPD Peroxidase Substrate. After washing of the plate with water, bands were recorded by an imaging scanner.

Immunoabsorption

To remove anti-GM1 or -GD3 Ab activities from the heterogeneous Ab mixtures in rat sera, immunoabsorption with GM1 or GD3 was performed as previously described (Usuki et al.,

2005). After immunoabsorption with GM1, the treated sera were further subjected to immunoabsorption with GD3 on the ELISA plate.

Pathological Analysis

Animals were anesthetized by injection with ketamine and xylazine intraperitoneally and perfused through the left cardiac ventricle with 4% paraformaldehyde in PBS. The sciatic nerves were removed from both sides, and immersed in 10% neutral buffered formalin v/v (Richard-Allan Scientific, Kalamazoo, MI). The fixed tissues were embedded in paraffin to make 4- μ m-thick cross-sections. Each section was stained with luxol fast blue and examined with a light microscope.

Preparation for Frozen Cryosections and Teased Fibers

Transverse cryosections—Sciatic nerve specimens were excised after perfusion of anesthetized rats, then washed three times in 10 mM PBS. The nerve samples were embedded in O.C.T. compound (Sakura Finetek, Torrance, CA) and then frozen in liquid nitrogen. Transverse cryosections (10 μ m) were rinsed four times in PBS after fixation in cold acetone for 3 min.

Teased fibers—Sciatic nerve specimens were removed as described above, washed in PBS, and incubated in collagenase (1 mg/ml; Wako Chemicals, Tokyo, Japan) in PBS for 20 min at 37°C. After washing three times with ice-cold PBS, the nerve samples were fixed in 4% fresh paraformaldehyde in PBS. After three washes, the samples were desheathed and teased into small bundles of fibers under a stereomicroscope.

Double Immunofluorescence Staining

The teased fibers and frozen cryosections were first incubated with 10% normal goat serum in Block Ace solution (Dainippon Pharmaceutical Co., Osaka, Japan) to block nonspecific binding for 30 min at room temperature. The teased fibers were incubated with tetramethylrhodamine isothiocyanate (TRITC)-peanut agglutinin (1:100 dilution; Sigma) overnight at 4°C and treated with anti-GD3 monoclonal antibody (mAb) from hybridoma cell line R24 (anti-GD3 mAb R24) from the American Type Culture Collection (ATCC No. HB8445; 1:5 dilution) overnight at 4°C, followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG Ab (1:100 dilution; Sigma) overnight at 4°C.

The cryosections were incubated overnight at 4°C with anti-GD3 mAb R24 (1:5 dilution) and subjected to labeling with anti-human neurofilament Ab (1:100 dilution; Dako, Glostrup, Denmark) overnight at 4°C, followed by both FITC-conjugated goat anti-mouse IgG Ab (1:100 dilution) and TRITC-conjugated goat anti-human IgG Ab (1:100 dilution; Sigma). Both teased fibers and cryosections were mounted with Aqua Poly/Mount (Polysciences Inc., Warrington, PA) on slides, covered with microslips, and observed with an Olympus laser scanning confocal microscope (Fluoview BW50; Olympus, Tokyo, Japan) at a wavelength of 488 or 568 nm.

Neuromuscular Junction (NMJ) Electrophysiology

Spinal cord-muscle coculture was performed according to the method of Taguchi et al. (2004). Briefly, spinal cord explants and muscle cells were prepared from spinal cord containing dorsal root ganglia and muscle, respectively, of 17-day-old fetal rats. Muscle cells and spinal cord explants were cocultured and maintained for up to 1 week in the medium (67% Dulbecco's modified Eagle's medium and 23% medium 199) containing 10% fetal calf serum supplemented with 25 ng/ml fibroblast growth factor and 20 μ g/ml insulin. Functional NMJ formation was observed by an inverted microscope (IX-70; Olympus) with an experimental

chamber on the stage perfused continuously with the above-mentioned medium at a rate of 1–2 ml/min. Spontaneous muscle action potential frequency was recorded by glass microelectrode (Ag/AgCl, 30–40 M Ω) and recording electrode (3 M KCl). The recording system consisted of a Microelectrode Amplifier MEZ-8301, Memory Oscilloscope VC-11 (Nihon Kohden, Tokyo, Japan), and A-D Converter Digidata-1200 Interface (Axon Instrument, Union City, CA). The spontaneous muscle action potential was low-pass filtered at 1 kHz. An antiserum solution, 10 μ l, was delivered directly near the innervated muscle cells with a micropipette.

Purification of GD3-Like Oligosaccharide From LPS Fraction

The LPS fraction described above was further fractionated by stepwise elution from a silica gel column (13 $\times$ 1.5 cm i.d.; Iatrobeads 6RS-8060; Iatron Laboratories, Inc., Tokyo, Japan) using the following solvents: 1) 60 ml of n-propanol, 2) 40 ml of n-propanol:H₂O (75:20, v/v), 3) 100 ml of n-propanol: H₂O:triethylamine (75:20:5, v/v/v), and 4) 100 ml of n-propanol:H₂O:triethylamine (60:20:20, v/v/v). The fraction eluted with solvent 3 was collected. An aliquot of this fraction was tested by the TLC-overlay method, and the bulk of the sample was evaporated to dryness. For TLC overlay, the sample was first developed using the solvent system of n-propanol:H₂O:25% NH₃ (6:3:1, v/v/v). After the plate was dried, it was overlaid with anti-GD3 mAb R24 (1:5 dilution) as described previously (Usuki et al., 2005).

For affinity purification, IgG protein recognizing GD3 was first purified by HiTrap Protein G HP affinity column chromatography (Amersham Biosciences, Uppsala, Sweden) from the conditioned medium of hybridoma R24 (Tai et al., 1988). The IgG protein thus obtained was dialyzed in a coupling buffer (0.1 M NaHCO₃, pH 8.0) and coupled with an affinity gel, CNBr-Sepharose 4B (Sigma), by using a procedure previously described (Cameron-Smith and Harbour, 2001). The column was preeluted with 3 M potassium thiocyanate in 0.5 M ammonium hydroxide and washed well with 1 mM PBS. The LPS fraction eluted with solvent 3 (100 mg) was applied onto an affinity column (column volume, 1 ml) and subjected to stepwise elution with the following solvents: 1) 10 ml of 1, 10, and 50 mM PBS; 2) 5 ml of 50 mM diethylamine/HCl buffer, pH 8.6, containing 0.1 M NaCl; and 3) 5 ml of 50 mM diethylamine/HCl buffer, pH 10.5, containing 0.1 M NaCl. The bound LPS fraction was eluted with solvents 2 and 3 and recovered in 10 1-ml fractions. Anti-GD3 mAb-binding LPS was present in the sixth and seventh tubes. They were collected, dialyzed against water, and lyophilized. The LPS lyophilizate was stored at –20°C before use.

The LPS fraction, 10 mg, was subjected to mild acid hydrolysis with 10 ml of 1% acetic acid in water for 1 hr at 90°C. After neutralizing with 1 M NaOH, the reaction mixture was ultracentrifuged at 4°C, 100,000g for 1 hr. The supernatant was recovered and then washed with an equivalent volume of chloroform. After partitioning, an aqueous solution was recovered and freeze dried. The residue was dissolved in a small amount of water, applied onto a BioGel P-2 column (45 $\times$ 1 cm i.d.; Bio-Rad, Hercules, CA), and eluted with 0.1 M pyridine-acetic acid buffer (pH 5.3). One-milliliter fractions were collected with a fraction collector. The sugar content in each fraction was determined by the resorcinol-sulfuric acid method using mannose as a standard (Monsigny et al., 1998). The resorcinol-sulfuric acid reaction-positive fractions containing oligosaccharides were pooled and dried in vacuo.

Carbohydrate Composition Analysis

Galactose (Gal) and heptose (Hep) composition was analyzed by the method of Fujii and colleagues (Fujii et al., 1999; Yasuno et al., 1999). Briefly, the purified oligosaccharide fraction was hydrolyzed in 2 N HCl and 2 M trifluoroacetic acid at 100°C for 4 hr, then converted to p-aminobenzoic acid ethyl ester (ABEE)-labeled monosaccharides. The labeled

monosaccharides were then analyzed by high-performance liquid chromatography (HPLC; model LC-VP, equipped with a model RF 10AXL fluorescence detector; Shimadzu, Kyoto, Japan) with isocratic elution of 6% CH₃CN in 0.2 M potassium borate buffer (pH 9.0). The eluent was monitored by fluorescence detection using an excitation wavelength of 305 nm and an emission wavelength of 360 nm.

For α -keto acid monosaccharide analysis, the above-mentioned oligosaccharide fraction was subjected to hydrolysis with 25 mM sulfuric acid at 80°C for 2 hr. The released α -keto acid monosaccharide was derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) by using a procedure previously described (Ito et al., 2002). DMB-labeled α -keto acid monosaccharides, such as N-acetyl neuraminic acid (NeuAc), N-glycolyl neuraminic acid (NeuGc), and 2-keto-3-deoxyoctanoate (Kdo), were analyzed by HPLC using an ODS-column (250 $\times$ 4.6 mm i.d., particle size 5 μ m; Axxiom Chromatography, Moorpark, CA) with a fluorescence detector at an excitation wavelength of 373 nm and an emission wavelength of 448 nm. The molar ratio of DMB-NeuAc and ABEE-Gal was calibrated from estimating correction factor between fluorescence intensities using labeled monosaccharides from sialyllactose.

Oligosaccharide Analysis by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

The oligosaccharide fraction was derivatized by using a p-aminobenzoic acid octyl ester (ABOE) labeling kit, and the derivatives were separated by a BioGel P-2 column (45 $\times$ 1 cm i.d.; Bio-Rad) by elution with 0.1 M pyridine-acetic acid buffer (pH 5.3). Fractions of 1 ml each were collected. The fluorescence intensity in each fraction was measured by using a fluorescence spectrophotometer (model F-2500; Hitachi, Tokyo, Japan). Fractions containing ABOE-labeled oligosaccharides were collected, pooled, and evaporated to dryness in vacuo. The dried residue was dissolved in water and analyzed by HPLC on a Honen Pak C18 column (Seikagaku Corp. Tokyo, Japan), programmed with a linear gradient elution from 10% of 0.1 M aqueous ammonium acetate (pH 4.0):CH₃CN [55:45 (v/v) in 75:25 (v/v)] to 80% of 0.1 M aqueous ammonium acetate (pH 4.0):CH₃CN [55:45 (v/v) in 75:25 (v/v)] for 40 min. Elution was monitored by a fluorescence detector at an excitation wavelength of 314 nm and an emission wavelength of 368 nm (Murata et al., 1997). The fluorescent peak at retention time 28.5 min, corresponding to the ABOE-labeled oligosaccharide, was recovered from HPLC and desalted by a Sep-Pak C18 cartridge. The ABOE-labeled oligosaccharide was analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) equipped with a nitrogen UV laser (λ = 337 nm) with a matrix of 2,5-dihydroxybenzoic acid at a concentration of 10 mg/ml in 10% aqueous ethanol.

Results

Antibody Titers

Titers of antiglycolipid or -LPS in rat sera were measured by ELISA. Anti-LPS IgM and IgG showed maximal titers at 4 and 8 weeks, respectively, after immunization with LPS (Fig. 2). Anti-GD3 and -GM1 (IgM or IgG) Ab titer elevation appeared later than elevation of anti-LPS Abs and showed maximal titers at 8 or 16 weeks, respectively. Vehicle-treated animals did not reveal any significant titer elevation (less than 100) against LPS or gangliosides (data not shown). Table I shows the IgG Ab titers against glycoconjugates of LPS-treated rats at 16 weeks. Three animals (LPS-1–3) were found to possess high titers of anti-GM1 and -GD3 Abs accompanied by severe decreases of NCV and P/D ratio.

Time Course of Changes in NCV and P/D Ratio

During the entire course of immunization, rats did not develop any noticeable weight loss or clinical symptoms such as flaccid limb weakness or paralysis. At 24 weeks, slightly age-dependent increases were observed in NCV of all groups (Fig. 3A, left), as previously reported (Birren and Wall, 1956; Biessels et al., 1999). As shown in Figure 3A, LPS-treated rats at 16 weeks postinoculation showed statistically significant decreases in NCV and P/D ratio as compared with the non- or vehicle-treated groups. The NCV of the LPS-treated group was 44.3 ± 9.4 m/sec ($N = 8$). The NCVs of the non- or vehicle-treated groups were 60.0 ± 10.8 ($*P < 0.05$, $N = 4$) and 58.5 ± 9.3 ($**P < 0.01$, $N = 8$), respectively. The P/D ratio of the LPS-treated group was 0.73 ± 0.11 ($N = 8$). The P/D ratios of non- or vehicle-treated groups were 0.89 ± 0.07 ($*P < 0.05$, $N = 4$) and 0.92 ± 0.06 ($**P < 0.01$, $N = 8$), respectively. However, the decreases of NCV and P/D ratio were transient, and LPS-treated animals recovered to normal ranges at 24 weeks postinoculation.

The Ab titers during the course of immunization are important indices when considering the relationship between anti-GSL Abs and peripheral nerve dysfunction. The titers of anti-GM1 or -GD3 Abs showed remarkable elevation at 16 weeks, as shown in Figure 2. Correlation diagrams were drawn for each Ab titer and NCV (or P/D ratio). Although the P/D ratio had a low correlation with either anti-GM1 or -LPS Ab titer (Fig. 4B2,3), NCV was not correlated with anti-GM1 or -LPS Ab titer (Fig. 4A2,3). On the contrary, anti-GD3 Ab titers had highly significant correlation coefficients compared with peripheral nerve dysfunction (NCV: $r = -0.791$; P/D ratio: $r = -0.870$) as shown in Figure 4A1,B1. No other Ab titers showed any statistically significant correlation with NCV or P/D ratio during the entire 24 weeks of inoculation. Thus, our data suggest that the anti-GD3 Ab response elicited by LPS immunization may be responsible for the reduction of NCV or P/D ratio in the GBS rat model.

To examine any pathological changes in the sciatic nerve, all rats were sacrificed 24 weeks postimmunization, and the nerve transverse sections were subjected to histological determination. For each animal shown in Table I, five sections were prepared and examined with a light microscope. There were indications of demyelination of nerve fibers in each of the sections of three experimental animals (Nos. 1–3), but no clear demyelination was observed in the other five experimental animals (Nos. 4–8) or in the vehicle- or nontreated animals. Figure 3Ba shows the case of animal No. 1. On the other hand, we did not notice any clinical signs in the LPS- or vehicle-treated animals.

Correlation Between NCV and P/D Ratio and Elevation of Anti-GD3 Ganglioside Ab

Although the NCV and P/D ratio revealed statistically significant differences among LPS- and vehicle-treated groups, there is a large individual variation, particularly among the 16-week rats of the LPS-treated group (NCV, 44.3 ± 9.4 m/sec; P/D ratio, 0.73 ± 0.11). In particular, we have focused on three animals (LPS-1–3) that showed both high titers of anti-GM1 and -GD3 Abs and demyelination of nerve fibers (Table I, Figs. 3B, 4). In these animals, the NCV and P/D ratio values decreased dramatically to 39.0 ± 10.1 m/sec and 0.64 ± 0.11 m/sec, respectively, compared with control.

Specificity and Cross-Reactivity of GM1 and GD3 Abs

Figure 5A shows the specificity of the three (LPS-1–3) sera IgG Abs against authentic GSLs as examined by the TLC-overlay method (Fig. 5A). TLC overlay of LPS-1 and -2 revealed dense dark staining against GM1, GM2, GD1a, GQ1b, and GD3. LPS-3 showed light staining against GM1 and dark staining against GQ1b and GD3. The binding profile of TLC overlay is similar to that from the ELISA titration (Table I).

To investigate the cross-reactivity of the IgG Ab with GM1 or GD3, sera were at first immunoabsorbed by GM1, followed by GD3. All activities of ganglioside Abs in the serum of LPS-2 disappeared by absorption treatment with GM1. However, activities of anti-GD3 and -GQ1b Abs remained in the sera of LPS-1' and -3' (LPS-1 and -3 immunoabsorbed with GM1). Subsequent to immunoabsorption with GD3, the activities of anti-GD3 and -GQ1b Abs disappeared in LPS-1'' and -3'' (LPS-1 and -3 immunoabsorbed with GM1/GD3). Thus, LPS-1 and -3 appear to contain two major polyclonal Abs: one with high avidity to GM1, GM2, and GD1a and the other to GD3 and GQ1b. The titer of anti-GD3 Abs in serum of LPS-2 was lower than the others. Therefore, the possibility is suggested that GD3 could not be detected by the TLC-overlay method because of the low titer of anti-GD3 Abs.

NMJ Electrophysiology

To determine the nature of the anti-GSL Ab in LPS-1–3 that blocks NMJ activity, spontaneous muscle action potential frequencies were examined by addition of individual serum to cocultures of spinal cord–muscle cells (Fig. 5Ba). LPS-1–3 all revealed significant blockade immediately after the addition of serum. However, the inhibition could be removed from LPS-2' by immunoabsorption with GM1, but not from LPS-1' and -3' (Fig. 5Bb). On the other hand, immunoabsorption of sera with GD3 removed NMJ-blocking activities in all LPS-treated sera (Fig. 5Bc). Before immunoabsorption with GD3, NMJ-blocking activity has already disappeared in LPS-2'. Anti-GD3 Abs titer of LPS-2 may not be sufficient to induce NMJ-blocking activity for the same reason that GD3 could not be detected by TLC overlay.

Subcellular Localization of GD3 Ganglioside

Figure 6A shows the subcellular localization of the GD3 epitope in the nodal and paranodal gap of the teased fiber by immunocytochemical analysis using anti-GD3 mAb R24. In frozen cross-sections (Fig. 6D), the GD3 epitope was found in the nodal gaps and the outer surface of Schwann cells, but not in compact myelin and axoplasm. Peanut agglutinin staining revealed that the Gal β 1-3GalNAc epitope was concentrated at the nodal region, and to a lesser degree in the paranodal gap (Fig. 6B; Sheikh et al., 1999). Anti-human neurofilament Ab staining revealed that the epitope was localized in the axoplasm and faintly on the abaxonal Schwann cell surface (Fig. 6E). Double-fluorescence staining demonstrated that a part of the GD3 epitope was also colocalized with those of Gal β 1-3GalNAc epitope in the nodal regions but not seen in the axoplasm at all (Fig. 6C,F).

Characterization of the Carbohydrate Epitope in LPS That Is Recognized by Anti-GD3 mAb R24

The LPS fraction that was eluted with solvent 3 of the silica gel chromatographic column was examined by TLC overlay using anti-GD3 mAb R24 (Fig. 7A). The LPS fraction reacted strongly with anti-GD3 mAb R24 compared with authentic GD3. Authentic GM1 had no staining. To isolate anti-GD3 mAb R24-binding LPS, the LPS fraction was applied to an affinity gel column immobilized with anti-GD3 mAb R24, and the bound LPS was eluted as described in Materials and Methods. The recovery of anti-GD3 mAb binding LPS was about 2.5%. After oligosaccharide release by mild acetic acid hydrolysis, the oligosaccharide was subjected to carbohydrate compositional analysis by fluorescence labeling and structural determination using HPLC and MALDI-TOF mass spectrometry. As shown in Table II, ABEE-labeled carbohydrates resulted in equivalent moles of Gal and Hep. DMB-labeled carbohydrates consisted of only NeuAc, but not NeuGc or Kdo. Table II shows the monosaccharide composition of the oligosaccharide of the LPS fraction as Neu-Ac:Gal:Hep in the molar ratio of 2:1:1.

Figure 7B shows the MALDI-TOF mass spectrum of ABOE-labeled oligosaccharide released from the anti-GD3 mAb R24-binding LPS. The strong monosodium-adduct ion $[M + Na]^+$ was

detected at m/z 1,208.1. The peak at m/z 1,229.0 was assigned to the disodium-adduct ion $[M + 2Na - 2H]^+$. The peak at m/z 1,186.1 was assigned to the protonated molecular ion $[M + H]^+$. The peak at m/z 916.3 was assigned to the fragment ion $[M + Na - NeuAc]^+$ that corresponds to the loss of a NeuAc residue from the monosodium-adduct ion. The peak at m/z 625.6 was assigned to the fragment ion $[M + Na - 2NeuAc]^+$ that corresponds to the loss of two NeuAc residues from the monosodium-adduct ion. We propose that the carbohydrate structure of anti-GD3 R24-binding LPS is a tetrasaccharide (NeuAc α 2-8NeuAc α 2-3Gal β 1-4Hep; Fig. 7C).

Discussion

It has been well established that anti-GSL Abs occur in about 60% of patients with GBS (Ariga and Yu, 2005). For this reason, it has been proposed that the pathogenic mechanisms of GBS may be related to abnormal elevation of anti-GSL Abs. To validate this mechanism, many attempts have been made to establish animal models of GBS by sensitizing laboratory animals (e.g., rabbit, rat, chicken, or mouse) with a variety of GSLs, such as sulfated glucuronosyl paragloboside (SGPG), GM1, bovine brain ganglioside mixture (BBG), or LPS (Yuki et al., 2004; Ariga and Yu, 2005). We have previously established rat and rabbit models of demyelinating paraproteinemia by sensitization of these animals with SGPG that revealed minor but clear clinical signs of neuropathy, including muscle weakness and conduction blocks in sciatic nerve (Kohriyama et al., 1988; Yamawaki et al., 1996).

Animal models of axonal injury with Wallerian-like degeneration in the peripheral nervous system have been reported to be induced in rabbits (sensitization with GM1, BBG, or LPS) and rats (sensitization with SGPG; Saida et al., 1979; Maeda et al., 1991; Yamawaki et al., 1996; Yuki et al., 2001, 2004). Kusunoki et al. (1996) also reported a model for experimental sensory neuropathy induced by sensitization of rabbits using purified GD1b. However, in our opinion, most of these claims should be carefully reexamined, owing largely to the use of GSL of questionable purity, particularly from contamination by proteins that are highly encephalitogenic (Ariga and Yu, 2005). In fact, our recent investigation on sensitization of New Zealand white rabbits using highly purified, protein-free GM1 or GD1a did not result in any apparent clinical abnormalities, despite the presence of high-titer Abs in the experimental animals (Dasgupta et al., 2004).

In the present study, we found evidence for peripheral nerve dysfunction by electromyography as well as demyelination of small nerve fibers in the sciatic nerve (Fig. 3B). The electrophysiological abnormalities occur at 16 weeks postinoculation, which coincides with the elevation of Abs against gangliosides GM1, GM2, GD1a, GQ1b, and GD3 (Table I). Anti-LPS Ab titers, however, increased 2 weeks before anti-GM1 and -GD3 Abs, but the peak titer was not consistent with the time when peripheral nerve dysfunction occurred. Therefore, anti-GD3 or -GM1 Abs, rather than the LPS Abs, are presumed to be associated with nerve dysfunction; in particular, the remarkably high titers of anti-GM1 and -GD3 Abs in 16-week sera, which coincides with the occurrence of the electrophysiological evidence of decreased NCV and P/D ratio in the peripheral nerves of the experimental animals.

Immunoabsorption and NMJ electrophysiology showed that each of the three experimental animals (LPS-1–3) possesses NMJ-blocking activity and high titers of Abs against GM1 or GD3 (Fig. 5A,Ba). This observation implies the possibility that the two terminal epitope structures, namely, the disaccharide (Gal β 1-3GalNAc) and the tetrasaccharide (NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc) might be important in the antigenic determinant and the functional neuromuscular synapse. Immunoabsorption experiments using GM1 eliminated anti-GM1, -GM2, and -GD1a Ab activities and retained NMJ-blocking activity. Further treatment with GD3 resulted in the loss of anti-GD3 and -GQ1b Ab activities and in the

complete elimination of NMJ-blocking activity, suggesting that the tetrasaccharide epitope (NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc) may be involved in NMJ function.

GD3 is known to be located in human peripheral nerve and rat Schwann cell surface (Levison and McCarthy, 1989; Svennerholm et al., 1994). Goodyear et al. (1999) showed that anti-GD3 mAbs were elicited from mice sensitized with LPS of *C. jejuni* from patients with Miller Fisher syndrome and that they possess a binding activity to the NMJ region of mice.

In our immunohistochemical study using anti-GD3 mAb R24, GD3 was verified to be located on the Schwann cell surface and nodes of Ranvier in rat peripheral nerve. Double staining with peanut agglutinin revealed that the GD3 epitope was colocalized with the Gal β 1-3GalNAc epitope in paranodal gap regions, consistent with the result of Sheikh et al. (1999).

We reported nontypical forms of AIDP and CIDP patients with elevation of anti-GD3 Abs with continuing neurological deterioration in spite of several rounds of plasma exchanges (Usuki et al., 2005). Both sera showed high cross-reactivity with other gangliosides, especially GM3 or GT3, suggesting that the tetrasaccharide Neu-Ac α 2-8NeuAc α 2-3Gal β 1-4Glc was a common epitope expressing cross-reactivity. In our cases of AIDP and CIDP, specific infection of *C. jejuni* or any other bacteria has not been identified. Therefore, it may be suggested that our rat anti-GD3 Abs recognize the tetrasaccharide structure (NeuAc α 2-8NeuAc α 2-3Gal β 1-4Hep) of LPS that has an epitope similar to GD3 (NeuAc α 2-8Neu-Ac α 2-3Gal β 1-4Glc). Alteration of the reducing end-terminal sugar, Glc to Hep, may result in additional fine specificity of rat antibodies to recognize an unknown NMJ-function-related molecule.

In conclusion, we have provided a direct proof that molecular mimicry may play a crucial role in eliciting GSL-induced and Ab-mediated neuropathies. The finding that sensitization of rats with a purified and chemically defined LPS of HS19 causes peripheral nervous dysfunction together with elevated titers of anti-GD3 Abs lends strong support to this concept. Additional studies are needed to examine other related LPS antigens that may arise from a similar causative mechanism.

Acknowledgments

This work was supported by an NIH grant NS-26994 to R.K.Y. We thank Dr. Nancy Rodriguez (Laboratory Animal Services), Ms. Anke Beckedorf (Mass Spectrometry/Proteomics Core Facility), and Ms. Kimberly K. Smith (Histopathology Laboratory) at the Medical College of Georgia for providing technical assistance.

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Fig. 1.

Animals were subjected to diagnostic electromyography measurement in the tail nerve without surgical operation. **A:** Nerve conduction waves were recorded from stimulations of the distal and proximal electrode positionings on the tail. **B:** Latency_{proximal-distal} was determined by subtracting proximal from distal latency of nerve conduction waves. NCV and nerve conduction block (P/D ratio) were calculated by the following formulas, respectively: NCV (m/sec) = 0.03/latency_{proximal-distal}; P/D ratio = proximal to distal amplitude ratio among nerve conduction waves. If P/D ratio is less than 1.0, nerve conduction blocks may occur in the segment between electrode positionings.

Fig. 2.
Time course of Ab titers after sensitization. Average IgG (solid columns) and IgM (open columns) Ab titers against GM1, GD3, LPS, or KLH in the sera of LPS-treated animals (N = 8).

Fig. 3. Nerve dysfunction and demyelination. **A:** Time course of changes in NCV and P/D ratio. Solid columns, LPS-treated animals ($N = 8$); open columns, vehicle-treated animals ($N = 8$); stippled columns, nontreated animals ($N = 4$). Bars are standard deviations, and statistical differences are determined by Student's t -test ($*P < 0.05$, $**P < 0.01$). **B:** Luxol fast blue staining in the transverse sections of rat sciatic nerve. a: Arrows show areas of demyelination (LPS-1). b: Control animals. Scale bar = 10 μ m.

Fig. 4.

NCV (**A**) and P/D ratio (**B**) are plotted against anti-GD3 (1), -GM1 (2), or -LPS (3) Ab titers in LPS-treated rats at 16 weeks after immunization. Circles correspond to each of the animals; in particular, the three arrowed animals (LPS-1–3) are characterized by high titers of anti-GM1 and -GD3 Abs with severity of nerve conduction dysfunctions as described in Results. r Value is the correlation coefficient between Ab titer and nerve conduction (NCV or P/D ratio).

Fig. 5.

Effect of immunoabsorption on Ab-binding and NMJ-blocking activities. **A:** TLC overlay with IgG GSL Abs of rat serum (LPS-1–3 in Fig. 4B), serum immunoabsorbed with GM1 (LPS-1'–3'), and serum immunoabsorbed with GM1 and GD3 (LPS-1''–3''). Lanes 1, GM1; 2, GM2; 3, GD1a; 4, GD1b; 5, GT1b; 6, GQ1b; 7, GD3; 8, GA1. **B:** Time course of inhibitory effect on muscle action potential frequencies in spinal cord–muscle cocultured cells. Arrow shows addition of rat serum, 10 μ l of LPS-1–3 (a); serum immunoabsorbed with GM1 only, 10 μ l of LPS-1'–3' (b); and serum immunoabsorbed with GM1 and GD3, 10 μ l of LPS-1''–3'' (c).

Fig. 6. Localization of GD3 epitope in rat sciatic nerve. Teased fibers of rat sciatic nerve immunofluorescence stained by anti-GD3 mAb R24 (A), TRITC-peanut agglutinin (B), or anti-GD3 mAb R24 and TRITC-peanut agglutinin (C). Cryosections of rat sciatic nerve immunofluorescence stained by anti-GD3 mAb R24 (D), anti-NF (E), or anti-GD3 mAb R24 and anti-human neurofilament Ab (F). Scale bar = 10 μ m.

Fig. 7. Chemical analysis of the epitopic structure of HS19-derived LPS recognized by anti-GD3 mAb R24. **A:** The fraction (100 ng) eluted with n-propanol:H₂O:triethylamine (75:20:5, v/v/v; solvent 3) by silica gel column chromatography was developed on an HPTLC plate and examined by TLC overlay using anti-GD3 mAb R24. **B:** After purification by anti-GD3 mAb R24-immobilized affinity chromatography, the oligosaccharides of LPS were converted to ABOE derivatives and analyzed by MALDI-TOF mass spectrometry. The arrowed peak at m/z 1,186.1 is assigned to protonated molecular ion [M + H]⁺. The peaks at m/z 1,229.0 and 1,208.1 are sodium adduct ions [M + 2Na-2H]⁺ or [M + Na]⁺, respectively. The peaks at m/z 916.3 and 625.6 correspond to loss of one or two NeuAc residues from the sodium adduct ions [M + Na-NeuAc]⁺ and [M + Na-2NeuAc]⁺, respectively. **C:** The proposed carbohydrate structure of the epitope recognized by anti-GD3 mAb R24.

TABLE I
IgG Ab titers against glycoconjugates of LPS-treated rats at 16 weeks after immunization

Rat No.	LPS	GM1	GM2	GD1a	GD1b	GT1b	GQ1b	GD3	GAI
LPS-1	6400	12800	1600	1600	-	-	1600	12800	-
LPS-2	3200	12800	1600	1600	-	-	1600	6400	-
LPS-3	400	1600	-	-	-	-	400	12800	-
LPS-4	1600	6400	-	-	-	-	-	1600	-
LPS-5	800	3200	-	-	-	-	-	6400	-
LPS-6	800	1600	-	-	-	-	-	400	-
LPS-7	1600	6400	-	-	-	-	-	1600	-
LPS-8	1600	400	-	-	-	-	800	400	-

TABLE II
Carbohydrate composition (molar ratio) of GD3-like epitope

Method	Gal	Hep	NeuAc
ABEE	1.0	0.94	—
DMB	—	—	1.97

Carbohydrate composition analysis of the oligosaccharide of anti-GD3 mAb R24 binding-LPS fraction. Neutral or α -keto acid monosaccharides were converted into ABEE-or DMB-derivatives, respectively, and analyzed by HPLC with a fluorescence detector. Molar ratio was estimated by fluorescence intensity ratio and was corrected with NeuAc/Gal fluorescence intensity ratio of authentic sialyllactose.