Brief Definitive Report

A Bacterium Lipopolysaccharide That Elicits Guillain-Barré Syndrome Has a GM1 Ganglioside-like Structure

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Summary

There is a strong association between Guillain-Barré syndrome (GBS) and Penner's serotype 19 (PEN 19) of *Campylobacter jejuni*. Sera from patients with GBS after *C. jejuni* infection have autoantibodies to GM1 ganglioside in the acute phase of the illness. Our previous work has suggested that GBS results from an immune response to cross-reactive antigen between lipopolysaccharide (LPS) of the Gram-negative bacterium and membrane components of peripheral nerves. To clarify the pathogenesis of GBS, we have investigated whether GM1-oligosaccharide structure is present in the LPS of *C. jejuni* (PEN 19) that was isolated from a GBS patient. After extraction of the LPS, the LPS showing the binding activity of cholera toxin, that specifically recognizes the GM1-oligosaccharide was purified by a silica bead column chromatography. Gas-liquid chromatography–mass spectrometric analysis has shown that the purified LPS contained Gal, GalNAc, and NeuAc, which are sugar components of GM1 ganglioside. ^{1}H NMR methods [Carr-Purcell-Meiboom-Gill (CPMG), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY)] have revealed that the oligosaccharide structure [Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ] protrude from the LPS core. This terminal structure [Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ] is identical to the terminal tetrasaccharide of the GM1 ganglioside. This is the first study to demonstrate the existence of molecular mimicry between nerve tissue and the infectious agent that elicits GBS.

Guillain-Barré syndrome (GBS) is the most common cause of acute neuromuscular paralysis in developed countries and it affects 1–2/100,000 people annually (1). Anti-neural antibodies may function in the development of GBS because plasma exchange elicits a beneficial response (1). Gangliosides, cell surface components of nerve tissue, are considered the target antigens of anti-neural antibodies because some patients develop GBS after being administered them (2–6). The dependence of GBS on "molecular mimicry" between infectious agents and surface components of peripheral nerves has been postulated (1). 15–40% of the GBS patients develop the syndrome after being infected by the Gram-negative bacterium *Campylobacter jejuni*, a leading cause of acute gastroenteritis in humans (7, 8). Sera from GBS patients who have had *C. jejuni* enteritis contain autoantibodies to GM1 ganglioside (5, 6, 9–11). The bacterium can be serotyped on the difference in the carbohydrate structure in the lipopolysaccharides (LPS) which are major constituents of the outer membrane of Gram-negative bacteria (12, 13, 14, 15). The specific serotype of Penner's 19 (PEN 19) of *C. jejuni* is very frequently isolated from GBS patients (8, 16); whereas, it is rarely isolated in the case of *C. jejuni* enteritis. We recently found antigenic similarity between the GM1 ganglioside and the LPS of *C. jejuni* (PEN 19) (17). Present study of immunochemical and NMR analyses on the LPS of *C. jejuni* has now confirmed molecular mimicry between the carbohydrate structure of this LPS and that of GM1 ganglioside.

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Materials and Methods

Case Report. A 24-yr-old man developed watery diarrhea with fever, which improved over 4 d. 6 d after the resolution of this illness, he developed distal muscle weakness, and on day 4, proximal muscle weakness. He was admitted to the hospital 7 d after the onset of neurologic symptoms. Neurologic examination was significant for areflexia and distal-dominant muscle weakness without superficial or deep sensory disturbances. Muscles of respiration and cranial nerves were unaffected. The cerebrospinal fluid protein level was increased with normal cellularity. Serial electrophysiological studies indicated that the predominant mechanism was axonal degeneration of the motor nerve. *C. jejuni* was isolated from his stool. The agent of antecedent infection was serologically determined using a complement fixation test; a high serum antibody titer against *C. jejuni* and a more than fourfold change in titer indicated a preceding *C. jejuni* infection. In the acute phase of this neurologic disease, the patient's serum antibody reacted strongly with GM1, and very faintly with GD1b as previously reported (17), but did not react with GD1a, GT1b, GM2, and GQ1b.

Bacterium. The bacterium used in this study was *C. jejuni* (CF 90-26) from this GBS patient. Serotyping of *C. jejuni* was performed by the method of Penner and Hennessy (12) and the strain belonged to PEN 19. Stock culture was maintained at −80°C in glycerol and brain heart infusion broth (15:85, by volume, Difco Laboratories Inc., Detroit, MI). Culture was grown on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, UK) at 37°C for 48 h in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. The bacterium was harvested in physiological saline, centrifuged at 10,000 *g* (4°C, 30 min), and washed three times with physiological saline.

Extraction of Crude LPS. LPS was extracted from 20 g (wet weight) of *C. jejuni* (PEN 19) by the hot phenol-water technique (18). After keeping the fraction at 4°C overnight, the aqueous layer was dialyzed against distilled water. 2 vol of methanol and 1 vol of chloroform were added to the aqueous phase, after which 1 vol of chloroform, followed by 1 vol of water was added. The chloroform layer was evaporated, and the dry residue was dissolved in 25% NH_4OH, and then incubated at 56°C for 48 h. After dialysis against distilled water, the crude LPS was lyophilized.

Purification of LPS with GM1-oligosaccharide Structure. 100 mg crude LPS was dissolved in a solvent mixture of *n*-propanol-water-25% NH_4OH (75:15:10, by volume). This LPS solution was applied to a column (2 × 100 cm) packed with Iatrobeads (6RS-8060; Iatron, Co., Tokyo, Japan) and eluted with a gradient of *n*-propanol-water-25% NH_4OH [75:15:10 (900 ml) to 6:4:1 (900 ml), by volume]. Each fraction eluted from the column was subjected to thin-layer chromatography (TLC) and made visible by spraying precoated Silica Gel 60 plates (E. Merck, Darmstadt, Germany) with ninhydrin, orcinol, resorcinol, and Dittmer reagents, and by binding of the peroxidase-conjugated cholera toxin subunit B (Vector Laboratories, Burlingame, CA) which specifically recognizes the GM1-oligosaccharide. A solvent system of *n*-propanol-water-25% NH_4OH (6:3:1, by volume) was the developer used. Fractions that showed homogeneous bands with the binding activity of the cholera toxin were pooled, evaporated, and the dry residue dissolved in 20 ml of physiological saline. The solution containing the LPS was applied to a C_{18} Sep-Pak cartridge (Millipore Corp., Bedford, MA), which was washed with 20 ml of water, and the pure LPS fraction was eluted with 20 ml methanol.

Gas-Liquid Chromatography–Mass Spectrometry (GC/MS). 50 μg purified LPS was subjected to methanolysis with 200 μl 5% methanolic HCl and 50 μl methylacetate at 70°C for 16 h. Fatty acid methylesters were extracted with *n*-hexane, and the aqueous layer was used for sugar analysis. Fatty acid methylesters were analyzed by GC/MS using a Varian 3400 gas chromatograph (Varian Associates, Inc., Palo Alto, CA) interfaced to a TSQ 70 triple-stage quadrupole mass spectrometer (Finnegan MAT, San Jose, CA). The GC column was a DB-5 (0.25 mm i.d. × 30 m; J&W Scientific Folsom, CA) chemically bonded, fused silica, capillary column. The temperature was programmed from 180 to 270°C at the rate of 4°C/min. Electron impact mass spectra were acquired from *m/z* 40–650 at 1 s/scan. Electron energy was 70 eV. Ionization current was 200 μA. Ion source temperature was maintained at 150°C.

The aqueous layer–removed fatty acid methylesters was evaporated to dryness by the addition of 50 μl of *tert*-butanol. Acetylation was carried out for 20 min by the addition of 100 μl of methanol, 10 μl of pyridine, and 10 μl of acetic anhydride at room temperature, and then the solvent was evaporated under nitrogen stream (19). 50 μl of TMS reagent (consisting of 100 μl of pyridine, 20 μl of hexamethyldisilazane, and 20 μl of trimethylchlorosilane) was added, and the mixture was left for 20 min at room temperature. TMS sugars were analyzed by GC/MS. GC/MS conditions were similar to analysis of fatty acid methylesters.

Proton NMR Spectroscopy. NMR experiments were performed on a JEOL JNM α600 600 MHz ^1H NMR spectrometer (Jeol Ltd., Tokyo, Japan). A 1.5-mg sample of the purified LPS was dissolved in 500 μl of ^2H_2O and incubated at 60°C for 10 min to replace exchangeable protons with deuterons. Then, the sample solution was lyophilized. The resulting residue was dissolved in 500 μl of ^2H_2O, and the pH was ~7. All the measurements were made using 5-mm diameter sample tubes. 2,2-Dimethyl 2-silapentane-5-sulfonate was used as the internal standard of chemical shift. Delays alternating with nutation for tailored excitation (DANTE) decoupling was performed to suppress residual HDO resonance. One-dimensional NMR experiments were performed at 60°C. Carr-Purcell-Meiboom-Gill (CPMG) spectrum (20) was measured with a 100-ms delay time.

Two-dimensional NMR spectra were recorded in the phase sensitive mode with 512(t_1) × 1024(t_2) data points (21, 22). Total correlation spectroscopy (TOCSY) spectrum was measured with a mixing time of 120 ms at 60°C. Nuclear Overhauser effect spectroscopy (NOESY) spectrum was measured with a mixing time of 200 ms at 40°C. In total, 64 scans were made for each t_1 with a spectral width of 6,000 Hz. The digital resolution was 5.9 Hz in both dimensions with zero-filling in the t_1 dimension. The sine bell function was applied to both the t_1 and t_2 dimensions.

Results and Discussion

Fig. 1 shows chromatographic elution profile of the LPS from the Iatrobeads column. Fractions from 94 to 104 showed homogeneous bands when they were stained by the binding activity of cholera toxin and by ninhydrin reagents for amino groups, orcinol reagents for hexose, and resorcinol reagents for deoxy-keto sugar (Fig. 1), as well as Dittmer reagents for phosphorous groups (data not shown). GC/MS analysis showed that the purified LPS from *C. jejuni* (PEN 19) contained the following sugar components galactose (Gal), glucose, *N*-acetylgalactosamine (GalNAc), *N*-acetylneuraminic acid (NeuAc), and heptose (Hep), as well as 3-deoxy-2-octulosonic acid and the fatty acid components, 3-hydroxymyristic acid and palmitic acid. These fatty acids are known to be the components of the lipid A from *C. jejuni* (23).

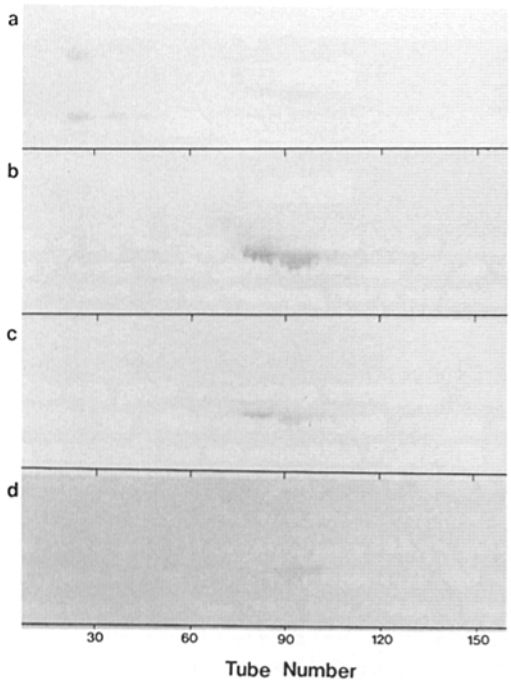

Figure 1. Chromatographic profile of the LPS. Fractions eluted from the Iatrobeads column were subjected to TLC and made visible by spraying the plate with (*a*) ninhydrin, (*b*) orcinol, and (*c*) resorcinol reagents, and by the binding of (*d*) cholera toxin.

Ethanolamine also was detected by the amino acid analysis (data not shown).

Fig. 2 *a* shows the normal NMR spectrum of the purified LPS. The 3e and 3a proton resonances of NeuAc are present. Anomeric proton resonances a, b, c, and d suggests the presence of sugars Galβ, Galβ, GalNAcβ, and Hepα, respectively. The spectrum in Fig. 2 *a* has resonances with narrow and broad line widths, indicative of two components with different mobilities. This is supported by measurement of the CPMG spectrum (Fig. 2 *b*), in which resonances with broad line widths were suppressed due to fast spin–spin relaxation times, whereas those with sharp line widths were enhanced. The resonances in Fig. 2 *b* correspond to protons located in the mobile region of the LPS. From the intensity of the anomeric protons in the CPMG spectrum, the mobility of the sugar residues was estimated to the Galβ(a)>GalNAcβ(c)> Galβ(b)-Hepα (d), which supports the contention that these oligosaccharides extend from the LPS core. Fig. 3 *a* shows TOCSY spectrum of the LPS sample. Cross peaks produced by magnetization transfer from the anomeric protons are present. On the basis of these cross peak patterns, the anomeric protons a, b, c, and d were assigned to the sugar types Galβ, Galβ, GalNAcβ, and Hepα shown in Fig. 3 *a*, respectively (21, 22). It should be noted that the H3 of Galβ(b) shifted as much as 0.5 ppm to the lower field, which is characteristic of the chemical shift change caused by the linkage formation of NeuAcα2-3Gal. We therefore concluded that the LPS sample had the NeuAcα2-3Galβ(b) moiety. The other sugar linkages then were studied by NOESY. The cross peaks

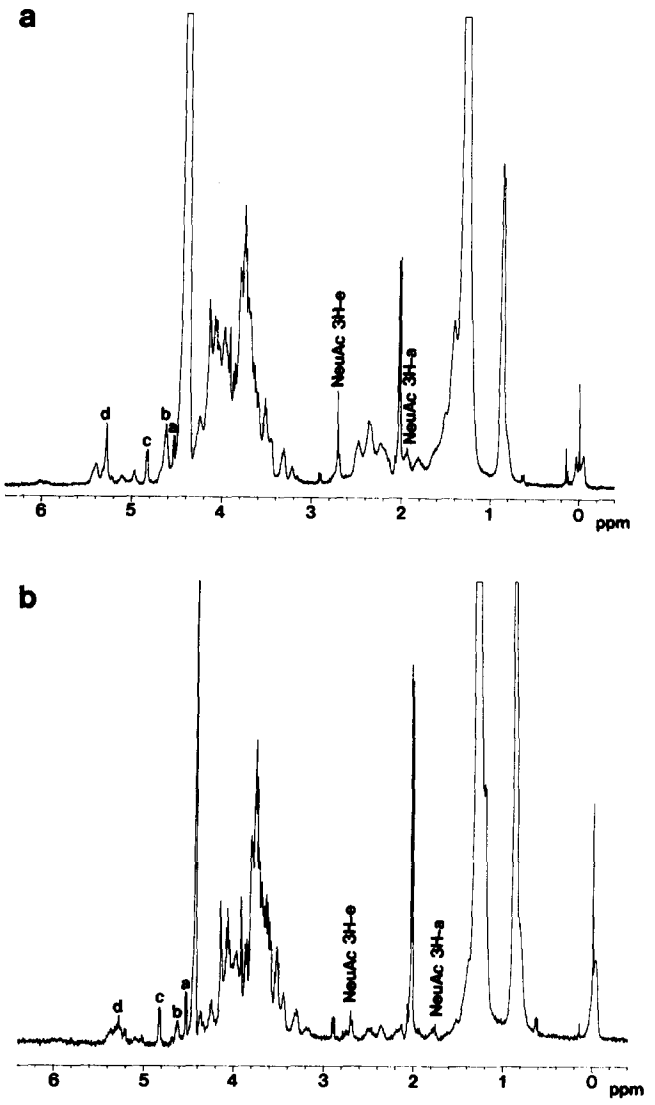

Figure 2. One-dimensional ^{1}H NMR spectra of the LPS sample. (*a*) normal spectrum, (*b*) CPMG spectrum.

developed from the anomeric protons are connected by dotted lines in Fig. 3. Taking into account the chemical shifts of the individual sugar proton resonances obtained in the TOCSY experiment, we identified the interresidual (boxed with solid lines) as well as the intraresidual NOESY cross peaks. Galβ(b) linked to the 2-position of the manno-type sugar. No mannose was detected by GC/MS, Hep being the only sugar with the manno-configuration in this sample. The linkage of Galβ(a)1-3GalNAcβ(c)1-4(NeuAcα2-3)Galβ(b)1-2Hepα(d) was confirmed from the interresidual cross peaks. On the basis of the mobility of the sugar residues (shown in Figs. 2 and 3), this oligosaccharide chain must protrude from the LPS core, because of which the GM1-oligosaccharide moiety [Galβ(a)1-3GalNAcβ(c)1-4(NeuAcα2-3)Galβ(b)] may be exposed (Fig. 4). This terminal structure [Galβ1-3GalNAcβ1-4 (NeuAcα2-3)Galβ] is identical to the terminal tetrasaccharide of GM1 ganglioside (Fig. 4), thereby enabling this LPS to interact with the cholera toxin.

GM1 is enriched in the membrane of the motor nerve ter-

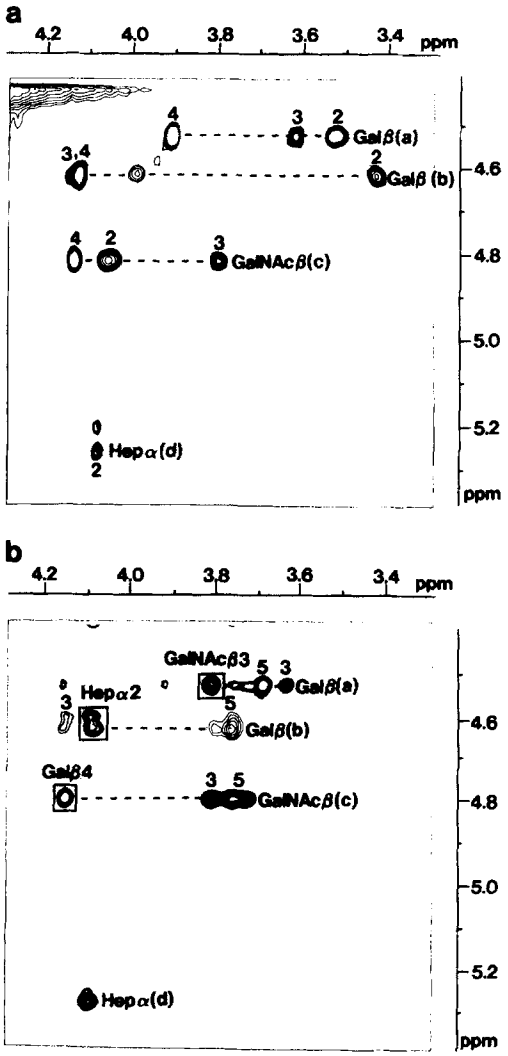

Figure 3. Two-dimensional ^{1}H NMR spectra of the LPS sample. (*a*) TOCSY spectrum. TOCSY cross peaks developed from the anomeric protons are connected by dotted lines. The assignments of the cross peaks also are shown. (*b*) NOESY spectrum. Interresidual NOESY cross peaks are boxed with solid lines. The sugar linkages are identified as Galβ(a)1-3GalNAcβ(*c*)1-4(NeuAcα2-3)Galβ(b)1-2Hepα(d) from the interresidual nuclear Overhauser effects.

minal which lacks the blood-nerve barrier, and to which anti-neutral antibodies easily gain access (24). In a coculture system of rat motoneurons and human muscle cells in monolayer (25), monoclonal anti-GM1 antibodies and serum anti-GM1 antibodies from GBS patients, who previously had been infected by *C. jejuni*, suppressed spontaneous firing and end-plate potentials; whereas, monoclonal anti-GD1a, anti-GD1b, anti-GT1b, and anti-GQ1b antibodies did not (Kobayashi et al., unpublished data). This finding indicates that the anti-GM1 antibody inhibits motoneuron excitability.

GM1 ganglioside Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer
3
NeuAcα2

LPS (PEN 19) Galβ1-3GalNAcβ1-4Galβ1-2Hepα-LPS core
3
NeuAcα2

Figure 4. Molecular mimicry between GM1 ganglioside and the LPS of *C. jejuni* (PEN 19). The same terminal tetrasaccharide (*underline*) occupies the nonreducing end of GM1 ganglioside and the LPS (PEN 19).

Administration of GM1 ganglioside sometimes causes the development of GBS associated with anti-GM1 antibody (2, 5, 6). Inoculation of rabbits with GM1 and certain adjuvants can cause peripheral neuropathy (26, 27). LPS is a potent polyclonal adjuvant of immune responses, its lipid A moiety being a known B cell mitogen (28). The LPS of *C. jejuni* (PEN 19), which bears the GM1-oligosaccharide structure, appears to have high immunogenic activity to produce anti-GM1 antibody. Sera from the GBS patient from whom *C. jejuni* (PEN 19) was isolated had antibodies to GM1, but did not have antibodies to GD1a, GT1b, GM2, or GQ1b. In contrast, rabbit anti-GM1 antibody did not react with LPSs from other serotypes of *C. jejuni*, PEN 3, 5, or 11 (Yuki et al., unpublished data). Very recently, Aspinall et al. determined chemical structures of side chains of LPSs from *C. jejuni* PEN 1, 2, 4, 23, and 36. The side chains described by them have terminal sugar structures of GM2, GM3, GD1a, or GM1b; they do not have that of GM1. Therefore, we speculate that infection by *C. jejuni* (PEN 19) induces high production of anti-GM1 antibody in patients with an immunogenetic background, thereby leading to the abolishment of tolerance, and that the anti-GM1 antibody binds to motor nerve terminals causing motoneuron inexcitability and the eventual development of muscular weakness. Ours is the first study to demonstrate the existence of molecular mimicry between nerve tissue and the infectious agent that elicits GBS (Fig. 4). The results reported here should be of use in establishing the mechanism of the pathogenesis of GBS after infection as well as the mechanisms of other autoimmune diseases.

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