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Miller-Fisher Syndrome Associated with *Campylobacter jejuni* Bearing Lipopolysaccharide Molecules That Mimic Human Ganglioside GD_3

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A *Campylobacter jejuni* strain of serotype O:10 was isolated from a patient who had Miller-Fisher syndrome. In its biochemical reactions and cellular morphology, the isolate was characteristic of typical *C. jejuni*. Antibodies against extracted lipopolysaccharide (LPS) were detected by passive hemagglutination in the acute- and convalescent-phase patient sera. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with the O:10 antiserum, it was demonstrated that the strain possessed both low- and high-molecular-weight molecules. Chemical analysis of the LPS revealed that the core oligosaccharide has a terminal trisaccharide epitope consisting of two molecules of sialic acid linked to galactose, a structure reflecting the terminal region of human ganglioside GD_3. As this trisaccharide is also present in LPS cores of serotype O:19 strains from patients with Guillain-Barré syndrome but not in cores of nonneuropathic *C. jejuni*, a possible role for the trisaccharide in the etiology of neuropathies is indicated, and a difference for distinguishing neuropathic strains from nonneuropathic strains may be the presence of a sialyltransferase required for the synthesis of this trisaccharide.

Miller-Fisher syndrome (MFS) is a neuropathy associated with ataxia, areflexia, and ophthalmoplegia and is considered to be an infrequent variation of the more common form known as Guillain-Barré syndrome (GBS) (19). Renewed interest in the etiology of these neuropathies has stemmed from the findings that the onset of both MFS and GBS in some patients is preceded by intestinal infections with *Campylobacter jejuni* (10, 12, 18, 20, 24, 25). Kuroki et al. (11) reported in a study of 11 GBS patients that 7 of the 8 who were cultured were positive for *C. jejuni* and that 5 of the 7 experienced abdominal pain or diarrhea or both 5 to 15 days before onset of GBS symptoms. Although only four isolates were available for serotyping, it is interesting that they all belonged to serotype O:19. In an extended study, Kuroki et al. (12) reported that *C. jejuni* strains were isolated from 14 (30%) of 46 patients with GBS. Twelve of the 14 were serotyped, and of the 12, 10 were found to belong to serotype O:10 and 2 were found to belong to serotype O:2. Also, the four isolates described by Fujimoto et al. (6) were of serotype O:19. However, strains of other serotypes, including O:1 and O:64, have been identified by Rees et al. (17) among isolates associated with GBS. In studies on MFS, Yuki et al. (25) reported that diarrhea due to *C. jejuni* of serotype O:2 preceded the onset of symptoms.

It is known that lipopolysaccharides (LPSs) from some strains of *C. jejuni* mimic the structure of human gangliosides (4), and attention has focused on the possibility that molecular mimicry is a factor in the pathogenesis of human neurological disease (1, 6, 26). The ganglioside-mimicking structures have been found to be located at the terminal regions of the core oligosaccharides (OSs) of the LPS molecule (1, 4). OS structures mimicking human gangliosides G_{M1} and G_{D1a} have been found in serostrains (serotype reference strains) for serotypes O:4 and O:19 (2, 4), and G_{M2}-like OS structures occur in LPSs from serostrains for serotypes O:1, O:23, and O:36 (4). The complete molecular structure of the core OS of the serostrain for serotype O:2 has been determined (3). This core OS is terminated with a single sialic acid residue and does not resemble any of the gangliosides mentioned above. No patient isolate of serotype O:2 has been subjected to chemical and structural analysis, but the isolates of this serotype from the MFS patients described by Yuki et al. (26) were examined with monoclonal antibodies and reported to have LPS that mimics the ganglioside GQ_{1b}, which has two trisaccharide epitopes, one terminal and one lateral.

In this study, we describe an isolate of *C. jejuni* belonging to serotype O:10 that was recovered from a patient who developed MFS and compare its characteristics with those of *C. jejuni* of serotype O:19 isolated from patients with GBS described in a previous study (1).

MATERIALS AND METHODS

Patient. A 20-year-old male marine developed diarrhea while on maneuvers in Puerto Rico. Ten days later he developed diplopia, and 48 h later he was observed to have ophthalmoplegia, fixed and dilated pupils, areflexia, and ataxia. The cerebrospinal fluid was acellular, and the protein level was 40 mg/dl, with a normal range of 15 to 35 mg/dl. Stool cultures grew *C. jejuni*. The patient was diagnosed as having MFS and received plasmapheresis and clarithromycin. The ataxia resolved after 2 weeks, and eye movements returned to normal after 10 months.

Bacteria. *C. jejuni* PG836 was isolated from the above-described patient at the Rhode Island Hospital, Providence. Isolates *C. jejuni* OH4382 and *C. jejuni* OH4384 of serotype O:19 were obtained from patients in Japan who later acquired GBS as reported previously (1, 2). The *C. jejuni* serostrains for serotypes O:10 and O:19 are from the serotyping scheme for *C. jejuni* (15). Bacteriological studies were performed in accordance with procedures described previously (14), and cell morphology was examined by electron microscopy.

Serotyping and serological experiments. *C. jejuni* PG836 was serotyped on the

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basis of its thermostable (O) LPS antigens, using a passive hemagglutination system as described previously (15). Thermostable antigens extracted by heating saline suspensions of the bacteria or by the hot phenol-water method of Westphal and Jann (23) were used to sensitize erythrocytes for passive hemagglutination tests. In passive hemagglutination tests with rabbit serotyping antisera, sheep erythrocytes were used as the antigen carrier, but type O Rh-positive erythrocytes were used when tests were performed with human sera.

Proteinase K digestion. A procedure modified from that of Hitchcock and Brown (7) was used for the enzymatic digestion of whole-cell lysates. Bacteria were cultured on blood agar plates (Columbia agar base; Oxoid Ltd., London, England) with 7% defibrinated horse blood (PML Microbiologicals, Tualatin, Oreg.) in an atmosphere containing 7% CO_2 at 37°C for 24 to 48 h. Growth from one plate was harvested in 5 ml of cold isotonic phosphate-buffered saline (10 mM Na_2HPO_4 [pH 7.0] in 0.85% [wt/vol] saline), washed once and then diluted to an optical density of 0.375 at a wavelength of 625 nm; 1.5 ml was transferred to an Eppendorf tube and centrifuged. The pellet was solubilized in 150 μl of lysing buffer (20% glycerol, 5% 2-mercaptoethanol, 4.6% sodium dodecyl sulfate [SDS], 0.125 M Tris-HCl [pH 6.8], 0.004% bromophenol blue). After the lysate was boiled for 10 min and cooled to room temperature, 150 μl of proteinase K (Boehringer Mannheim, Canada, Dorval, Quebec, Canada), which was dissolved in the lysis buffer at a concentration of 1.0 mg/ml, was added to the lysate. The enzyme-treated cell lysates were incubated at 37°C overnight and then at 65°C for 1 h prior to electrophoresis.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The discontinuous buffer system of Laemmli (13) was used to fractionate the LPS prepared by proteinase K digestion of whole-cell lysates. A stacking gel of 5% acrylamide, a separation gel of 12.5% acrylamide, and a constant current of 35 mA were used for electrophoresis. Electrophoresis was continued until a tracking dye was within 1 cm of the bottom of the gel. The gels were fixed and stained for LPS after electrophoresis by the method of Tsai and Frasch (22) or were transferred to nitrocellulose for immunoblotting.

After transfer to nitrocellulose with the buffer described by Towbin et al. (21), the nitrocellulose sheets were immersed in Tris-hydrochloride-buffered saline (TBS) (20 mM Tris, 500 mM NaCl [pH 7.50]) containing 1% gelatin and rabbit serum diluted 1:50 and incubated overnight. After the sheets were rinsed in deionized water, they were rinsed twice for 10 min in TBS containing Tween 20 (0.05%) and then incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Bio-Rad, Mississauga, Ontario, Canada) diluted 1:1,000 in TBS containing gelatin (1%) for 3 h. The sheets were then rinsed as described above and immersed in the horseradish peroxidase color development solution (60 mg of 4-chloro-1-naphthol [Bio-Rad] dissolved in 20 ml of methanol added to 100 ml of TBS containing 60 μl of cold hydrogen peroxide [30%]). The nitrocellulose sheets were rinsed in deionized water to halt color development.

Determination of the chemical composition and molecular structure of *C. jejuni* LPS. *C. jejuni* PG836 LPS was isolated by the phenol-water extraction procedure of Westphal and Jann (23). The aqueous layer was removed, dialyzed for 48 h against distilled water, and ultracentrifuged (100,000 × *g* for 4 h) to produce LPS with a low M_r as a water-insoluble gel and a fraction with a high M_r from the aqueous supernatant solution. Structural studies on the low-M_r LPS and core OS derivatives used chemical analyses, nuclear magnetic resonance spectroscopy, mass spectrometry (fast atom bombardment), and electron impact-mass spectrometry as reported previously for LPS from *C. jejuni* O:19 (2).

RESULTS

Bacterial identification and classification. Biochemical reactions of isolate *C. jejuni* PG836 were typical of the species (14), and transmission electron microscopy showed cells curved to spiral in shape with single unsheathed flagella either at one end or at both ends of the cell (Fig. 1). Thermostable antigenic preparations of the isolate reacted in O:10 antiserum (titer of 1:640) and in none of the other *C. jejuni* typing antisera.

Detection of antibodies against *C. jejuni* PG836 in patient sera. An acute-phase serum sample and a convalescent-phase sample that was taken after plasmapheresis were titrated by passive hemagglutination against thermostable antigenic preparations from the patient isolate, *C. jejuni* PG836. The results indicate that the acute serum had a titer one twofold dilution higher than the sample taken during convalescence. Titers for heat-extracted LPS were 1:160 and 1:80 and those for phenol water-extracted LPS were 1:320 and 1:160 in samples taken on 5 May 1993 and 23 February 1994, respectively.

Characteristics of LPS from *C. jejuni* PG836. Silver-stained LPS from *C. jejuni* PG836 after electrophoresis revealed a pattern of several bands migrating near the bottom of the gel that correspond to core OS, but bands characteristic of high-M_r LPS with O chains were not present (Fig. 2). This finding is consistent with earlier observations (16) that *C. jejuni* high-M_r LPS is not visualized with the staining procedure of Tsai and Frasch (22). Moreover, the banding patterns of the low-M_r LPS differed from patterns for LPSs from the O:10 serostrain, the two O:19 patient isolates, and the O:19 serostrain, providing further evidence that the core OSs not only are not conserved within the species but also are not conserved within the same serotype.

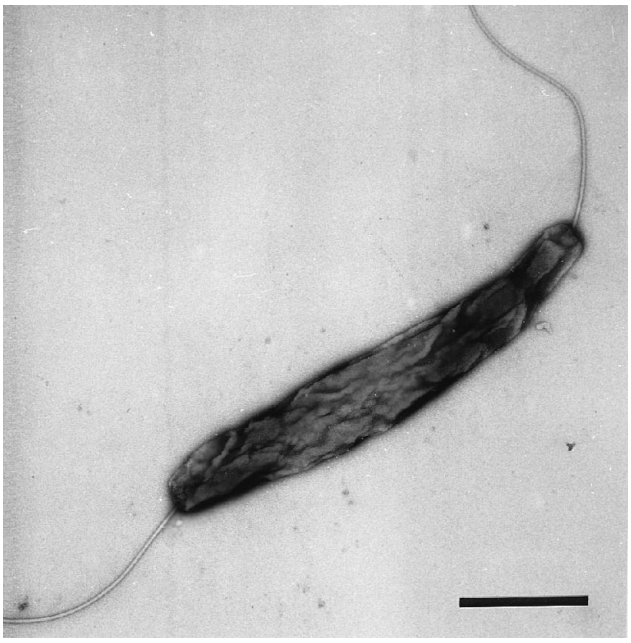

FIG. 1. Transmission electron micrograph of negatively stained *C. jejuni* PG836. The bar equals 1 μm.

Immunoblotting experiments showed the presence of high-M_r material for serostrain O:10 and isolate PG836 (Fig. 3). The materials in lanes 1 and 2 were not clearly resolved into discrete bands, and repeated attempts to produce clearer resolution were unsuccessful. This was an unusual and unexpected result that suggested an atypical O-chain polymer in the high-M_r LPS from strains of serotype O:10. Of importance, however, was the observation that the O:10 antiserum did produce visualization, although weakly, of the high-M_r mate-

FIG. 2. Silver-stained SDS-polyacrylamide gels of proteinase K-digested whole cells of *C. jejuni*. Lanes: 1, serotype O:10 serostrain; 2, serotype O:10 isolate PG836; 3, serotype O:19 isolate OH4382; 4, serotype O:19 isolate OH4384; 5, serotype O:19 serostrain.

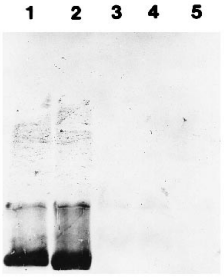

FIG. 3. Immunoblots of proteinase K-digested whole cells of *C. jejuni*, using the O:10 serotyping antiserum. Lanes: 1, serotype O:10 serostrain; 2, serotype O:10 isolate PG836; 3, serotype O:19 isolate OH4382; 4, serotype O:19 isolate OH4384; 5, serotype O:19 serostrain.

rial from the O:10 serostrain and the PG836 isolate but produced no detectable reaction with high-M_r material from strains of serotype O:19 (Fig. 3, lanes 3 to and 5). The O:19 antiserum reacted with LPS from the O:19 serostrain and the two O:19 isolates from GBS patients but not with high-M_r LPS from the O:10 serostrain or the PG836 isolate (Fig. 4). The results of the immunoblotting experiments confirmed the results of serotyping and provided evidence confirming that the isolate PG836 was serotypically different from the previously studied isolates *C. jejuni* OH4382 and *C. jejuni* OH4384 and that serotype differentiation is based on the high-M_r LPS.

Composition and structure of LPS from *C. jejuni* PG836. Analysis of the material from the water-insoluble gel revealed LPS molecules consistent with core OS. The *C. jejuni* PG836 core OS, like the previously described OSs of *C. jejuni* OH4382 and *C. jejuni* OH4384, has a terminal trisaccharide consisting of Neu5Acα2→8Neu5Acα2→3β-D-Gal (1). The *C. jejuni* PG836 core OS resembles most closely the OS of *C. jejuni* OH4384 except for branching on the outer heptose residue with an attached glucose residue and the absence of a laterally attached Neu5Ac on the inner galactose (Fig. 5).

From the aqueous supernatant solution, we recovered high-M_r molecules that contain residues of two previously unknown 7-deoxyheptoses together with *N*-acetylglucosamine and a hexose (probably glucose) as constituents in a branched structure. No structures resembling hyaluronic acid derivatives previously identified in the O:19 strains (1) were detected. The complete details of the chemical characterization of the *C. jejuni* PG836 LPS will be published elsewhere.

DISCUSSION

C. jejuni is a major cause of human enteritis that is usually limited to about 5 days of acute diarrhea, but as early as 1984

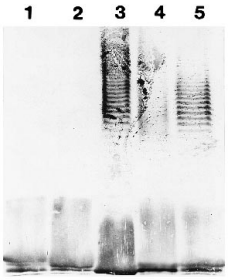

FIG. 4. Immunoblots of proteinase K-digested whole cells of *C. jejuni*, using antiserum against serostrain O:19. Lanes: 1, serotype O:10 serostrain; 2, serotype O:10 isolate PG836; 3, serotype O:19 isolate OH4382; 4, serotype O:19 isolate OH4384; 5, serotype O:19 serostrain.

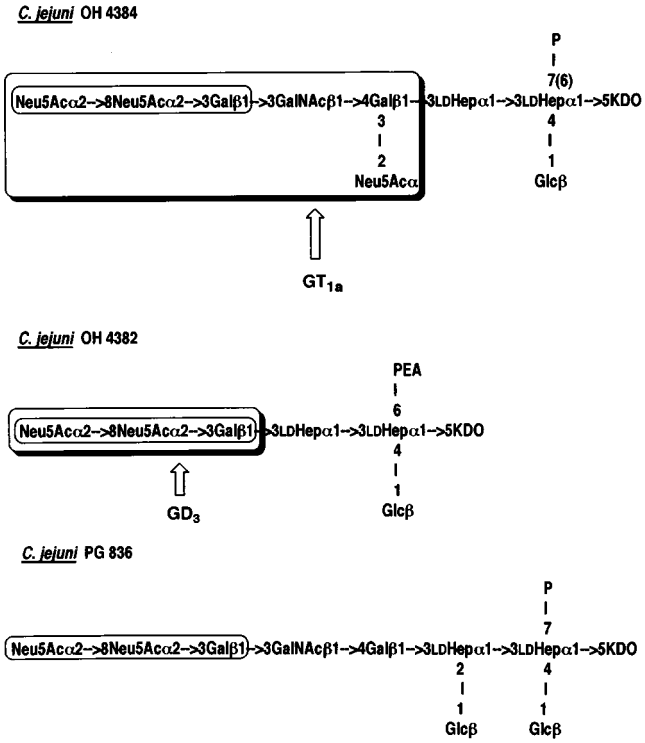

FIG. 5. Molecular structures of LPS core OSs of *C. jejuni* strains. The structures for OSs of *C. jejuni* OH4384 and *C. jejuni* OH4382 were reported previously (1, 2) and are presented here for comparison with the OS structure of *C. jejuni* PG836. KDO, 3-deoxy-D-manno-octosonic acid; LDHep, L-*glycero*-D-*manno*-heptose; P, phosphate; PEA, *O*-phosphoethanolamine.

an association of *C. jejuni* infections with subsequent development of GBS was recognized by Kaldor and Speed (9). Although infections with *C. jejuni* are very common causes of enteritis, the subsequent development of neurological disease is relatively rare (5). The extent to which *C. jejuni* is involved as a precipitating factor in the etiology of neurological diseases is unknown, but the frequency of association of prior infections with GBS has been reported as probably higher than 26% by Rees et al. (17) and as high as 66% by Ho et al. (8), and thus an antecedent infection with this species is now regarded as the single most important predisposing factor (5, 8, 17).

Prompted by the observation that a large proportion of GBS cases in Japan followed a bout of diarrhea caused by *C. jejuni* of serotype O:19 (6, 11, 12), we undertook to investigate the structure of LPS from this serotype in an earlier study (1, 2). The serostrain for the O:19 serotype and two isolates from patients who developed GBS were examined. Each yielded low-M_r LPS corresponding to core OS, and high-M_r molecules that were found to be polymers of repeating units of an amidated hyaluronic acid derivative. However, the core OSs were of greater interest. The O:19 serostrain was found to have a core OS essentially identical to the OS of the O:4 serostrain (1, 2, 4). Low-M_r LPS of both serostrains yielded two types of OS molecules, one with the terminal region mimicking in structure the human ganglioside G_{M1} (a monosialoganglioside) and the other with the terminal region mimicking G_{D1a} (a disialoganglioside), but the two strains differ in the relative proportions of the ganglioside-like structures. Although the frequency of enteritis cases due to infections with isolates of serotype O:4 is approximately nine times as great as the number of cases reported to be due to serotype O:19 isolates (15), it is infec-

tions with isolates of the latter serotype that have been associated with GBS, thus bringing into question the significance of the molecular mimicry of the G_{M1} and G_{D1a} gangliosides in pathogenesis.

Unexpected but most interesting was the finding that the O:19 isolates from GBS patients differed not only from each other but also from the O:4 and O:19 serostrains in their core OS structures (Fig. 5). The GBS isolates *C. jejuni* OH4384 and *C. jejuni* OH4382 have core OSs with terminal regions mimicking those of the gangliosides G_{T1a} and G_{D3}, respectively. Both gangliosides have a terminal trisaccharide (Neu5Acα2→8Neu5Acα2→3β-D-Gal), a structural feature common to the patient isolates but not found, at this time, in any of the nonneuropathogenic strains.

In the present study, the *C. jejuni* PG836 isolate from a patient with MFS was examined. Antibodies against two different preparations of LPS extracted from the isolate were observed in two different patient serum samples, providing evidence for infection with this isolate. Electron microscopy showed curved rods with single flagella at one or at both ends, consistent with the cellular morphology of the species (14). Serotyping as previously described (15) identified the isolate as O:10, a serotype previously not known to be associated with neurological disease. SDS-PAGE and immunoblotting of electrophoresed extracts provided evidence confirming its serotypic classification and that both high-M_r and low-M_r LPSs from this isolate differed in structure and immunological specificity from LPSs of GBS patient isolates of *C. jejuni* OH4384 and *C. jejuni* OH4382. Chemical structures of the O chains of the high-M_r LPS are not completely characterized, but from the available results, it is clear that no structures resembling the hyaluronic acid derivatives found in the O:19 serotype are present. Of considerable significance, however, is the finding that although the low-M_r core OS of *C. jejuni* PG836 differs from those of the OSs of *C. jejuni* OH4384 and *C. jejuni* OH4382, the three isolates possess the same terminal trisaccharide epitope. Hence, the presence of the trisaccharide in the core OS is the single common feature of the three neuropathy-associated patient isolates. The fact that the isolates belong to two different serotypes demonstrates that the trisaccharide is not limited to a particular serotype and may occur in isolates of serotypes other than O:19 and O:10. Furthermore, it is noteworthy that the serostrains that have been examined for core oligosaccharide structure (O:1, O:2, O:4, O:19, O:23, and O:36) were not known to be associated with neuropathics. These six nonneuropathic strains all contained sialic acid residues, but in none was evidence found for the presence of the trisaccharide.

It should be noted that since the trisaccharide occurs on the short incomplete core of *C. jejuni* OH4382, its expression would have as a minimum requirement a core OS structure with a terminal β-D-galactose able to accept the transfer of a Neu5Ac residue at the O-3 position along with the enzyme specific for this acceptor site and, in addition, the linking of a second Neu5Ac residue that would require a separate Neu5Ac2→8 transferase (α-2,8 sialyltransferase). With these requirements met, the assembly of the complete trisaccharide would appear to be independent of the rest of the core OS structure and of the O-serotype classification.

From these results, it is evident that the key factor which distinguishes the neuropathogenic strains, including those of serotype O:2 described by Yuki et al. (26), from those which have not been associated with neuropathies of the GBS and MFS types may be the presence in the bacterium of an active α-2,8 sialyltransferase. The enzyme, therefore, is hypothesized to be an important marker for differentiating neuropathogenic microbes from those that are not neuropathogenic, and it is expected also that information on its activation or acquisition and regulation could significantly advance our understanding of a potentially important mechanism of pathogenesis.

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