

Structure of *Campylobacter jejuni* Lipopolysaccharides Determines Antiganglioside Specificity and Clinical Features of Guillain-Barré and Miller Fisher Patients

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Ganglioside mimicry in the lipopolysaccharide (LPS) fraction of *Campylobacter jejuni* isolated from Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) patients was compared with isolates from patients with an uncomplicated enteritis. The antibody response to *C. jejuni* LPS and gangliosides in neuropathy patients and controls was compared as well. LPS from GBS and MFS-associated isolates more frequently contained ganglioside-like epitopes compared to control isolates. Almost all neuropathy patients showed a strong antibody response against LPS and multiple gangliosides in contrast to enteritis patients. Isolates from GBS patients more frequently had a GM1-like epitope than isolates from MFS patients. GQ1b-like epitopes were present in all MFS-associated isolates and was associated with anti-GQ1b antibody reactivity and the presence of oculomotor symptoms. These results demonstrate that the expression of ganglioside mimics is a risk factor for the development of post-*Campylobacter* neuropathy. This study provides additional evidence for the hypothesis that the LPS fraction determines the antiganglioside specificity and clinical features in post-*Campylobacter* neuropathy patients.

Campylobacter jejuni infection is the most frequent triggering event in postinfectious neuropathies such as the Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) (13, 23, 29). The exact pathogenesis of post-*Campylobacter* neuropathy is unknown, but molecular mimicry between bacterial glycoconjugates and peripheral nerve gangliosides has been implicated (32). Cross-reactive antibodies between *C. jejuni* lipopolysaccharides (LPS) and gangliosides have been identified in GBS and MFS patients (12, 20). The specificity of this cross-reactive antiganglioside-LPS response is associated with different disease profiles. Antibody reactivity against GM1, GM1b, and GalNAc-GD1a is associated with pure motor GBS (2, 33), and anti-GQ1b antibody reactivity has a strong association with oculomotor symptoms and ataxia (15).

Ganglioside mimics have been identified in *C. jejuni* LPS by biochemical and serological methods (17) (Fig. 1). Mass spectrometry revealed the presence of GM1- and GD1a-like structures in LPS from GBS patients (22, 36) and GD3-like structures in MFS-associated LPS (25). Serological studies with ganglioside-binding toxins and monoclonal antiganglioside antibodies confirmed these findings (26, 37). Most *C. jejuni* isolates from GBS and MFS patients that were investigated by biochemical and/or serological methods exhibit ganglioside mimicry, but this may have been caused by selection bias. Little information is available concerning the expression of ganglioside mimics in isolates from uncomplicated *C. jejuni* enteritis

patients compared to neuropathy-associated isolates (26). Furthermore, detailed studies on antibody responses to glycolipid antigens such as LPS and gangliosides in *C. jejuni* enteritis patients have been limited.

The aim of the present study was to investigate whether there is a difference in the expression of ganglioside mimics in unrelated *C. jejuni* isolates from GBS and MFS patients compared to *C. jejuni* isolates from uncomplicated enteritis patients. In addition, we studied the antibody response to LPS and gangliosides in culture-proven *Campylobacter*-associated neuropathy patients and control enteritis patients to investigate host-related differences in the immune response to glycolipid antigens.

MATERIALS AND METHODS

Patients and isolates. We collected 18 GBS- or MFS-related *C. jejuni* isolates from patients in The Netherlands and Belgium between 1991 and 1998 (7). Two isolates were cultured from the diarrheal stools of two family members of a GBS patient. The GBS patient remained culture negative but showed a serological response highly suggestive of a recent *C. jejuni* infection (1). Only one of these strains was included in the analysis, leaving the total number of neuropathy associated strains at 17. Acute-phase serum samples obtained within one week of the onset of symptoms, clinical information on oculomotor symptoms, and ataxia were available for 16 patients. All GBS patients fulfilled the diagnostic criteria (4), and all MFS patients had oculomotor symptoms and ataxia in the absence of limb weakness. Fourteen of the GBS and MFS patients had suffered a period of diarrhea during the weeks before the development of neurological symptoms; four patients did not report a gastrointestinal illness. Two GBS patients had oculomotor symptoms in addition to their limb weakness. All but two neuropathy patients had immunoglobulin A (IgA) and/or IgM serum antibodies against a protein extract of *C. jejuni*, indicative of a recent infection (9). One of the remaining two patients had high-titer IgG anti-*C. jejuni* protein antibodies and a history of diarrhea. Twenty-three control *C. jejuni* isolates were obtained from patients with an uncomplicated *C. jejuni* enteritis without neurological symptoms. Of 15 of these control patients, serum samples taken in the first week of the

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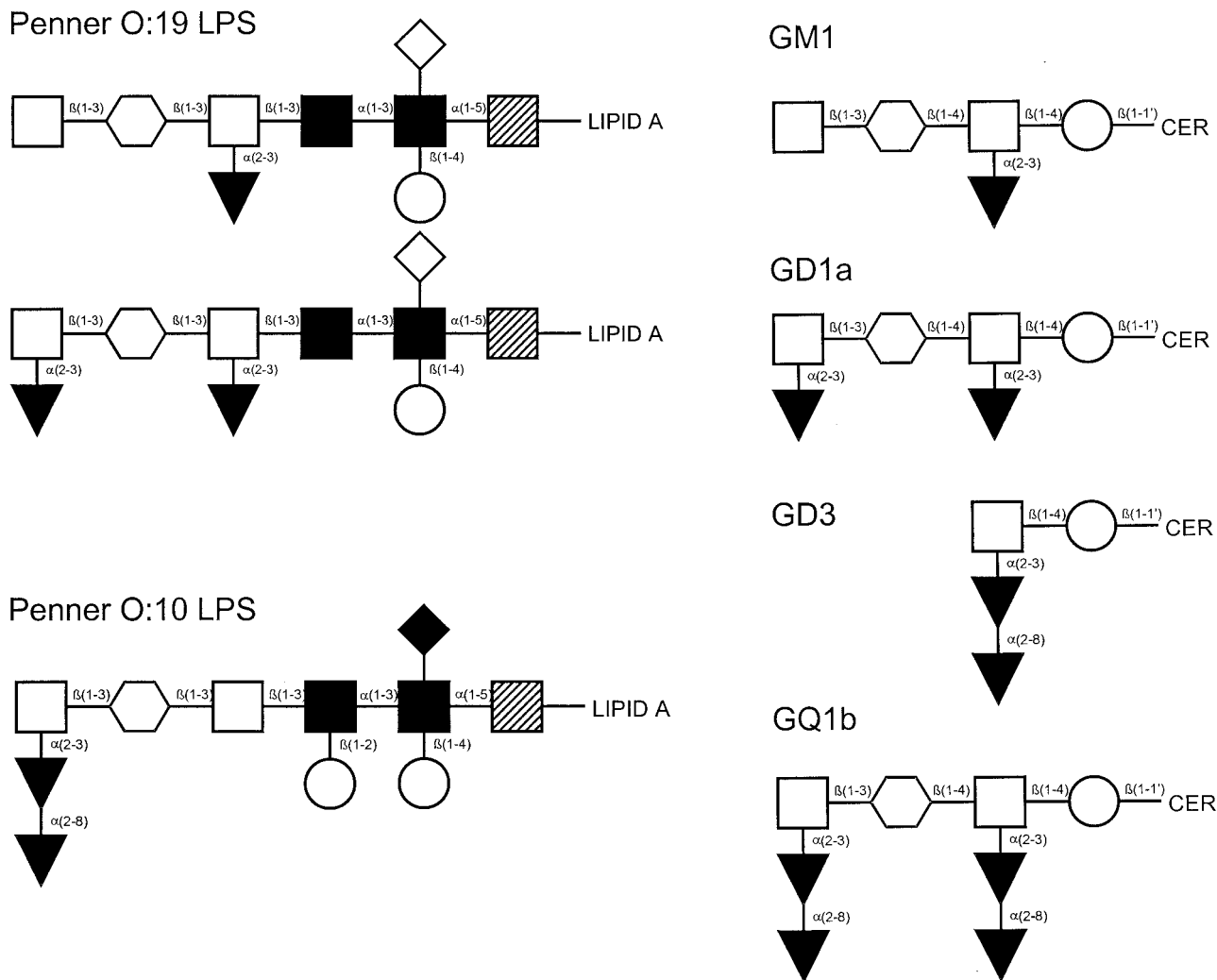


FIG. 1. Structure of *C. jejuni* LPS types and the corresponding ganglioside mimics. Symbols: ○, *N*-acetylgalactosamine; □, galactose; ○, glucose; ▼, sialic acid; ◇, (phosphoryl)ethanolamine; ●, *N*-acetylglucosamine; ■, heptose; ▨, 3-deoxy-*D*-manno-octulosonic acid; ●, dideoxyglucose; ◆, 2-aminoethyl phosphate.

disease were available. All control isolates were collected between 1990 and 1999 from sporadic enteritis patients. All control enteritis patients had suffered from diarrhea. Based on a limited number of patients, there were no differences between the neuropathy and the control enteritis patients with respect to the duration of diarrhea. The seasonal distribution of neuropathy associated and control isolates was the same with a broad summer peak. As additional controls for serotyping of *C. jejuni* isolates, we used reference strains from which the core oligosaccharides have been shown to contain a GM1 mimic (Penner O:19, CCUG 10950) (5), a GD3 mimic (Penner O:10, CCUG 10943) (19), or no ganglioside mimic (Penner O:3, CCUG 10937) (6) (Fig. 1).

Serology. Acute-phase serum samples were tested for IgM and IgG antibody reactivity against asialo-GM1 (GA1), GM1, GM2, GD1a, GD1b, GD3, and GQ1b by enzyme-linked immunosorbent assay (ELISA) and confirmed by thin-layer chromatography as described previously (10). All glycolipids were obtained from Sigma (St. Louis, Mo.), except GQ1b (Biocarb, Lund, Sweden). LPS fractions from all isolates were isolated with the hot-phenol-water method and processed as described previously (12). All LPS fractions showed a dense band migrating at 8 to 15 kDa after electrophoresis on a polyacrylamide gel and silver staining (Novex, San Diego, Calif.), indicating the presence of LPS (5). For the detection of anti-LPS reactivity, serum samples were tested by ELISA at dilutions of 1:100 and 1:1,000 as described previously (11). Samples were considered positive when the corrected optical density value was >3 standard deviations of

the mean optical density for a group of 12 healthy controls without serological evidence of a recent *C. jejuni* infection.

To assess serum samples for cross-reactive antibodies between LPS and gangliosides, serum was incubated with *C. jejuni* LPS conjugated to Octyl-Sepharose CL4B beads. Then, 250 μg of LPS was added to 1 ml of Octyl-Sepharose CL4B in methanol-water (1:1 [vol/vol]) containing 0.1 M KCl and mixed for 1.5 h. After several washes with phosphate-buffered saline (pH 7.8; PBS), serum samples were incubated with LPS-Sepharose conjugates for 5 h at 4°C. After centrifugation, absorbed serum samples were tested for anti-LPS and antiglycolipid reactivity as described above. Serum samples that were incubated with beads coupled to LPS from the Penner O:3 serostrain that does not contain a ganglioside mimic (6) or uncoupled beads served as controls.

Serological typing of *C. jejuni* LPS. (i) Polyclonal serum panel. Purified LPS was tested in an ELISA-based system for the presence of ganglioside-like epitopes with a panel of ganglioside-reactive sera divided into four groups. Some, but not all, of the samples were from culture-positive GBS or MFS patients. The first group (GM1) consisted of 10 serum samples with IgG anti-GM1 reactivity. The second group (GQ1b) consisted of 10 serum samples with IgG anti-GQ1b reactivity. The third group (Other) consisted of four serum samples with anti-ganglioside reactivity against GM2 and GD1a. This group was included to detect ganglioside mimics that could not be defined with sera from the GM1 and GQ1b group. The fourth group (HC) consisted of 12 healthy controls without serolog-

TABLE 1. Antiglycolipid titers and anti-LPS reactivity in GBS and MFS patients

Condition and patient	IgG titer ^a								IgM titer ^a							
	GA1	GM1	GM2	GD1a	GD1b	GD3	GQ1b	LPS	GA1	GM1	GM2	GD1a	GD1b	GD3	GQ1b	LPS
GBS																
GB1	1,600	800	—	—	—	—	—	+	1,600	400	—	—	100	—	—	+
GB2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
GB3	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
GB4	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
GB5	400	—	100	—	—	—	—	+	400	—	—	—	—	—	—	+
GB11	200	800	—	—	3,200	—	—	+	—	—	—	—	—	—	—	+
GB13/14	1,600	400	—	—	—	—	—	+	800	200	—	—	200	—	—	+
GB15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
GB17	25,600	100	—	—	—	100	—	+	200	—	—	—	—	—	—	+
GB18	3,200	2,800	—	—	1,600	—	—	+	—	200	—	—	—	—	—	+
GB21	800	100	—	6,400	—	—	—	+	—	—	—	—	—	—	—	+
GBS oculomotor symptoms																
GB16	200	—	—	—	—	3,200	>102,400	+	100	—	—	—	—	—	—	+
GB19	—	—	—	—	—	100	>12,800	+	100	—	—	—	—	—	—	+
MFS																
MF6	—	—	—	—	100	200	1,600	+	—	—	—	—	—	—	—	—
MF7	—	—	—	—	—	—	400	+	—	—	—	—	—	—	200	+
MF8	—	—	—	—	200	200	800	+	—	—	—	—	200	—	—	+
MF20	1,600	—	—	—	—	—	100	—	400	—	—	—	—	—	—	+

^a NT, not tested; +, titer >100; —, titer <100.

ical evidence for a recent *C. jejuni* infection. An LPS was considered to have a GM1-like epitope when at least 3 of 10 serum samples from the GM1 group had an optical density of >3 standard deviations above the mean of the healthy controls, with a minimum optical density of 0.300 (to avoid false-positive samples due to the low levels of anti-LPS reactivity seen in healthy control samples). The criteria for LPS bearing a GQ1b-like epitope were similar with the GQ1b panel of sera. An LPS was considered to have an undefined ganglioside-like epitope, which could not be designated GM1-like or GQ1b-like, when the LPS showed reactivity with only one or two serum samples in either the GM1 or the GQ1b group or reactivity with one or more serum samples from the "other" group.

(ii) **Toxins, lectin, and MAbs.** The specificity of the binding of cholera toxin B subunit (CT), tetanus toxin fragment C (TT), peanut agglutinin (PNA), and the monoclonal antiganglioside antibodies (MAbs) Sm1 (31), Wo1 (21), Ha1rbc (30), CGM3 (8), and EG1 (8) was validated by binding these ligands to GA1, GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, or GQ1b or to bacterial LPS. Plates (96 well; Nunc Immunosorb, Roskilde, Denmark) were coated with glycolipids as described previously (11). Wells without glycolipids served as controls. Peroxidase-conjugated CT (List Biologicals, Campbell, Calif.; Sigma), biotin-conjugated CT (List Biologicals), peroxidase-conjugated TT (List Biologicals), peroxidase-conjugated PNA (Sigma), or the MAbs diluted in PBS-bovine serum albumin were added for 1 h at room temperature (toxins) or overnight at 4°C (MAbs). After being washed with PBS, plates containing peroxidase-conjugated ligands were developed with *o*-phenyldiamine. Biotin-conjugated CT was incubated with peroxidase-conjugated avidin (ABC Kit; Vector, Burlingame, Calif.) for 1 h at room temperature, followed by washing and development. Plates containing MAbs were incubated with peroxidase-conjugated anti-human or anti-mouse antibodies and washed with PBS before development. Specificity of binding to purified glycolipids was confirmed with a thin-layer chromatography overlay (14).

Statistical analysis. Statistical analysis was performed with Stata version 6.0 (Stata, College Station, Tex.). Differences in proportions were tested with the Fisher exact test. Binding of toxins, lectin, and MAbs was evaluated with the Mann-Whitney U test. All tests were two-sided. A *P* value of <0.05 was considered significant.

RESULTS

Patient serology. All but one of the GBS and all MFS patients had IgM and/or IgG antibody reactivity against purified *C. jejuni* LPS (Table 1). Most serum samples contained reactivity not only against LPS from their autologous isolate but also from other *C. jejuni* isolates. Of 16 neuropathy patients, 13 showed antibody reactivity to a variety of peripheral nerve glycolipids (Table 2). All MFS patients had anti-GQ1b reactivity. Two GBS patients displayed no reactivity against the purified glycolipids, although they both had IgG anti-LPS reactivity. Depletion experiments with LPS conjugated to Sepharose beads demonstrated that the antiglycolipid and anti-LPS antibodies were cross-reactive, not only in patients with anti-GM1 and anti-GQ1b antibodies but also in patients with anti-GD1a and high-titer anti-GA1 antibodies (data not shown).

Serum from neuropathy patients more frequently contained antibody reactivity against any of the panel of purified neural

TABLE 2. LPS structure and diagnosis of patients with *C. jejuni* enteritis

LPS structure	No. of patients (%) with diagnosis of:			<i>P</i>	
	GBS (<i>n</i> = 13)	MFS (<i>n</i> = 4)	Enteritis (<i>n</i> = 23)	GBS or MFS vs enteritis	GBS vs MFS
GM1-like	11 (85)	2 (50)	13 (57)	0.32	0.22
GQ1b-like	2 (15)	4 (100)	2 (9)	0.053	0.006 ^a
Other ganglioside	8 (62)	4 (100)	12 (52)	0.33	0.26
No ganglioside	1 (8)	0	8 (35)	0.054	1.00

^a Statistically significant.

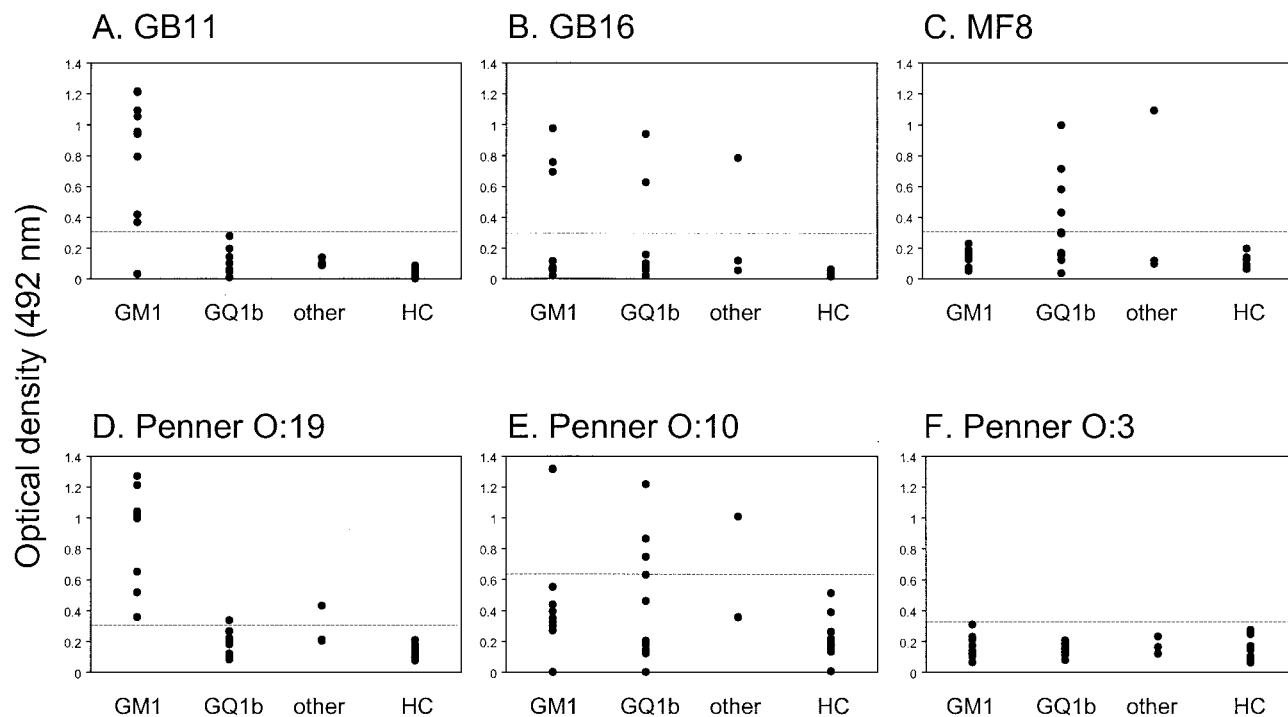


FIG. 2. *C. jejuni* LPS reacts differentially with antiganglioside antisera. Purified LPSs were tested for reactivity against a panel of serum samples containing antiganglioside reactivity against GM1 and GA1 (GM1), GQ1b and GD3 (GQ1b), or other gangliosides and/or a combination of GA1 and GQ1b ("other"). (A and B) LPS from GBS patients GB11 and GB16 reacted with sera of the GM1 group. In panel B, LPS from GBS patient GB16, who has an overlap of GBS and MFS, reacted with the sera of the GM1, GQ1b, and "other" groups. (C) LPS from MFS patient MF8 reacted predominantly with GQ1b sera. (D) LPS from the O:19 serostrain reacted with sera of the GM1 group but not with those of the GQ1b group, whereas LPS from the O:10 serostrain reacted with the sera from the GQ1b group. (F) LPS from the O:3 serostrain, which does not contain a GM1- or GQ1b-like structure, did not react with any of the sera. None of the LPS fractions reacted with sera from the healthy controls (HC). Dashed lines indicate the cutoff values.

glycolipids or *C. jejuni* LPS (15 of 16) than control enteritis patients (6 of 15) ($P = 0.001$). In the enteritis group, only one patient had low-titer IgG anti-GM1. Two other patients had anti-GA1 IgG antibodies. Antibody reactivity to gangliosides with two or more sialic acids could not be detected in any of the enteritis patients. Anti-LPS antibodies occurred in three enteritis patients, and one of them also had antibody reactivity against GA1.

Serological typing of *C. jejuni* isolates with GBS or MFS patient sera. Examples of LPS reactivity with antiganglioside antibody containing serum samples and validation of the serum panel with LPS with a known biochemical structure are shown in Fig. 2. The antiganglioside serum panel reacted with all but one LPS from the GBS and MFS isolates, indicating the presence of ganglioside mimics in almost all neuropathy-associated isolates. LPS from most isolates reacted with serum samples from more than one group, suggesting the presence of multiple ganglioside mimics. The Penner serotype was not related to the expression of ganglioside-like epitopes on the LPS. The frequency of GM1-like epitopes was higher in GBS-associated isolates than in MFS or control isolates (Table 2). GQ1b-like epitopes occurred more frequently in MFS-associated isolates than in GBS or control isolates ($P = 0.006$, Table 2). Of the control enteritis isolates, 35% did not react with any of the antiganglioside sera, indicating the absence of ganglioside mimics in these isolates.

To investigate whether the LPS structure predicts the antiganglioside specificity and clinical features in the neuropathy patients, we correlated the results of the LPS typing with these features. In general, the type of ganglioside mimicry in *C. jejuni* LPS concurred with the antiganglioside specificity and clinical features in the GBS and MFS patients (Table 3).

Serological typing of *C. jejuni* isolates with toxins, lectin, and MABs. All ligands reacted with purified LPS fractions. None of the toxins, lectin, or MABs reacted with LPS from the Penner O:3 strain (data not shown). Validation of CT binding, which in its unconjugated form binds highly selectively to GM1 (3), showed differential binding specificity of CT, depending on the conjugated molecule and independent from the concentration of glycolipids (Table 4). Binding of CT and the two anti-GA1 and anti-GM1 MABs correlated well with the serum panel typing system for GM1-like epitopes. CT binding showed a clear difference between GBS- and MFS-related isolates ($P = 0.03$, Fig. 3A), although not all GBS-related isolates bound CT and CT binding was not restricted to GBS patients with anti-GM1 antibodies. In addition, the level of CT binding was higher in neuropathy-associated isolates than in control isolates ($P = 0.02$). The antidisialosyl MABs clearly distinguished MFS isolates from GBS isolates (Fig. 3B). PNA and TT binding did not have additional value in distinguishing GBS from MFS strains (data not shown).

TABLE 3. LPS structure, antiganglioside reactivity, and clinical features of GBS and MFS patients

Parameter	No. of patients (%) positive for GM1-like LPS		<i>P</i> ^a	No. of patients (%) positive for GQ1b-like LPS		<i>P</i> ^a
	Yes (<i>n</i> = 12)	No (<i>n</i> = 4)		Yes (<i>n</i> = 6)	No (<i>n</i> = 10)	
Antiganglioside reactivity						
Anti-GA1	9 (75)	1 (25)	NS*	3 (50)	7 (70)	NS*
Anti-GM1	6 (50)	0	NS*	1 (17)	5 (50)	NS*
Anti-GD3	4 (33)	1 (25)	NS*	4 (67)	1 (10)	0.04*
Anti-GQ1b	4 (33)	2 (50)	NS*	5 (83)	1 (10)	0.008*
Clinical features						
Oculomotor symptoms	4 (33)	2 (50)	NS†	5 (83)	1 (10)	0.008*
Ataxia	3 (25)	2 (50)	NS†	4 (67)	1 (10)	0.04*

^a NS, not significant. *, Compared to patients without these antibodies; †, compared to patients without these symptoms.

DISCUSSION

The present study demonstrates that *C. jejuni* isolates from GBS and MFS patients more frequently express ganglioside mimics in their LPS than isolates from uncomplicated enteritis patients. Cross-reactive anti-LPS-antiglycolipid responses can be detected in almost all culture proven cases of *C. jejuni*-related GBS and MFS but not in serum from patients with an uncomplicated enteritis. Differences in LPS structure determine the specificity of the antibody response and thereby the clinical features of the patients.

The GBS patients were highly diverse in their antiganglioside specificity, but almost all had antibody reactivity against the LPS from the isolate with which they had been infected. This serves as further evidence that the antiganglioside antibodies in GBS and MFS patients result from the previous infection with *C. jejuni*. The presence of strong anti-LPS antibody reactivity in patients without reactivity against a panel of gangliosides indicates the presence of antibody reactivity against unknown gangliosides. In contrast to the findings in neuropathy patients, the response to bacterial and neural glycolipids was significantly lower in patients with an uncomplicated *C. jejuni* enteritis, despite the presence of ganglioside mimics in the LPS of some enteritis isolates. This finding underscores the influence of host-related factors such as polymorphisms in immune-response genes, in addition to bacterium-related factors, in the development of neurological symptoms after an infection with *C. jejuni* (18, 26). This may

either depend on differential expression of ganglioside mimics in *C. jejuni* LPS, due to phase variation in genes encoding glycosyltransferases (16), or to the influence of other immunomodulating factors (28).

For the present study we developed a method with serum samples containing antiganglioside antibody reactivity to detect and define ganglioside-like epitopes on *C. jejuni* LPS. Serum samples containing antibody reactivity to gangliosides other than GM1 or GQ1b reacted strongly with LPS from several isolates, which indicates the presence of other, as-yet-undefined ganglioside mimics. In addition, most LPS fractions reacted with serum samples from more than one serum group, suggesting the presence of multiple ganglioside epitopes on one isolate (34). The data obtained by the serum panel method were confirmed with MAbs and toxins, thus demonstrating the validity of the method.

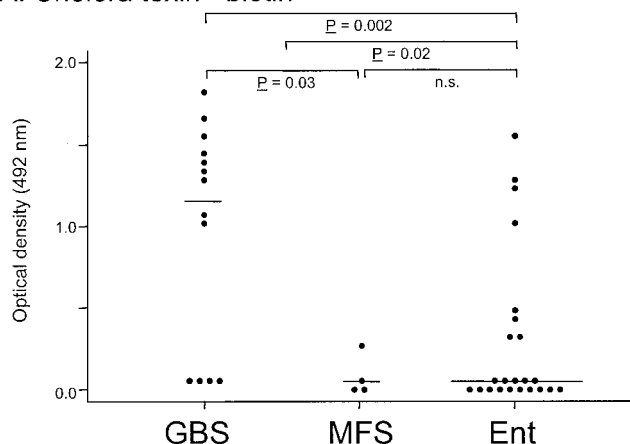
The lack of CT binding of all MFS-related isolates and some GBS-related isolates is important since CT-binding assays are currently being used to identify isolates that have a higher potential to induce neurological symptoms (18, 24). The anti-GA1 and anti-GM1 MAbs reacted selectively with a limited number of GBS-related isolates. These results indicate that the use of very specific ligands may not be sufficient to detect the wide variety of ganglioside mimics in *C. jejuni* LPS. Consequently, GBS patients may have antiganglioside antibodies that are directed against GM1-like gangliosides (e.g., GM1b or GalNAc-GD1a) (2, 35). Together, these results indicate that

TABLE 4. Reactivity of toxins, lectin, and MAbs to purified gangliosides and LPSs from *C. jejuni* serostrains

Toxin, lectin, or MAb	Reactivity ^a of:											
	Ganglioside									LPS		
	GA1	GM1	GM2	GM3	GD1a	GD1b	GT1b	GD3	GQ1b	Pen O:3	Pen O:10	Pen O:19
CT biotin	–	+++	–	–	–	–	–	–	–	–	–	+
CT peroxidase	++	+++	++	+	++	+++	–	–	–	–	–	+++
PNA-peroxidase	+++	–	–	–	–	–	–	–	–	–	–	–
TT-peroxidase	–	+	–	–	+	+++	+++	–	++	–	+	–
MAb Sm1	–	+++	–	–	–	–	–	–	–	–	–	+++
MAb Wo1	+++	+++	–	–	–	++	–	–	–	–	–	+++
MAb Ha1rbc	–	–	–	–	++	+++	–	+++	+++	–	+	+
MAb EG1	–	–	–	–	–	–	–	+	++	–	+	–
MAb CGM3	–	–	–	–	–	–	–	++	++	–	++	–

^a –, No binding; +, weak binding; ++, moderate binding; +++, strong binding.

A. Cholera toxin - biotin



B. Ha1rbc

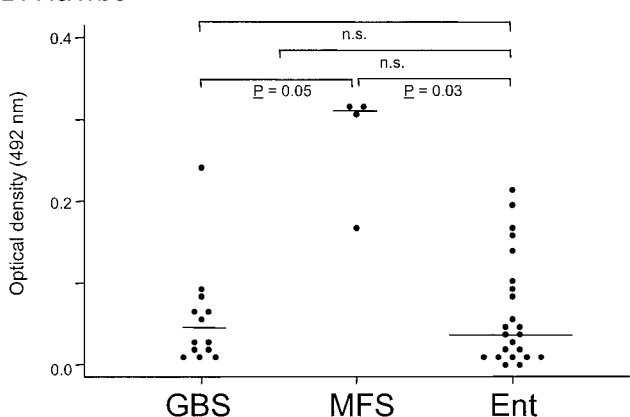


FIG. 3. Differential binding of CT and MAb Ha1rbc to *C. jejuni* LPS from patients with GBS, MFS, and uncomplicated enteritis (Ent). Purified *C. jejuni* LPS was coated onto 96-well plates and incubated with CT (biotin labeled) (A) or Ha1rbc (B). The differences in binding were compared between GBS and MFS isolates and between neuropathy-associated and uncomplicated enteritis isolates.

CT and the GM1 panel may recognize GM1-like immunoreactive epitopes on *C. jejuni* LPS and not necessarily an exact structural mimic of the GM1 oligosaccharide. Furthermore, our results indicate that approaches with the serum panel, toxins and lectins, and monoclonal antibodies all have their advantages and drawbacks. The observed good correlation between the three methods lead us to conclude that all three methods can be used to define “GM1-like” and “GQ1b-like” epitopes.

In conclusion, our data provide strong support for the hypothesis that molecular mimicry between *C. jejuni* LPS and gangliosides plays a key role in the induction of antiganglioside antibodies and neurological symptoms in patients with GBS or MFS. Furthermore, we have demonstrated that heterogeneity in the LPS structure determines the specificity of the antiglycolipid response and thereby the clinical features in patients with a post-*Campylobacter* neuropathy. The development of serological assays for the determination of ganglioside mimicry in *C. jejuni* LPS may be of great help in determining genetic markers for potentially neuropathic strains (27).

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