

## Humoral immune response against *Campylobacter jejuni* lipopolysaccharides in Guillain-Barré and Miller Fisher syndrome

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### Abstract

In this study we characterized the IgG antibodies against lipopolysaccharides (LPS) of *Campylobacter jejuni* in serum from patients with Guillain-Barré syndrome (GBS), Miller Fisher syndrome (MFS), *C. jejuni* enteritis and normal controls. In patients with GBS and MFS long-lasting titers of IgG1 and IgG3 antibodies against LPS from GBS and MFS associated *C. jejuni* were found. The subclass and course of these antibodies were highly associated with those of antibodies against GM1 and GQ1b in GBS and MFS patients. However, in *C. jejuni* enteritis and normal controls anti-LPS antibodies were predominantly IgG2. Antibody binding with LPS was reduced after treatment with cholera toxin and sialidases, suggesting that the ganglioside-like epitopes in LPS are immunodominant. These results further indicate that antecedent *C. jejuni* infections determine the specificity and isotype of anti-ganglioside antibodies in GBS and MFS patients. © 1997 Elsevier Science B.V.

**Keywords:** Guillain-Barré syndrome; Miller Fisher syndrome; *Campylobacter jejuni*; Lipopolysaccharides; GM1; GQ1b

### 1. Introduction

The Guillain-Barré syndrome (GBS) is an acute monophasic polyneuropathy leading to a heterogeneous spectrum of motor and sensory deficits (Van der Meché and Van Doorn, 1995). The Miller Fisher syndrome (MFS), characterized by ophthalmoplegia, areflexia and ataxia, is considered to be one of its variants. GBS and MFS may result from a transient immune response against peripheral nerves, triggered by an infection which often precedes the neurological onset.

*Campylobacter jejuni* is a frequent cause of antecedent infections in GBS (Rees et al., 1995a), and MFS (Yuki et al., 1994; Jacobs et al., 1995). These infections are associated with the presence of antibodies against gangliosides in patients with GBS (Walsh et al., 1991; Rees et al., 1995b; Jacobs et al., 1996) and MFS (Yuki et al., 1994; Jacobs et al., 1995). Anti-GM1 antibodies are associated with severe

motor involvement in GBS (Jacobs et al., 1995) and anti-GQ1b antibodies with MFS and oculomotor involvement in GBS (Chiba et al., 1993). The high concentration of GM1 in myelin of motor nerves (Ogawa-Goto et al., 1992) and of GQ1b in oculomotor nerves (Chiba et al., 1993) reflects these clinical manifestations. These antibodies may be induced by the infection, since ganglioside-like epitopes are found in lipopolysaccharides (LPS) of *C. jejuni* isolates from patients with GBS (Yuki et al., 1993) and MFS (Salloway et al., 1996). Furthermore, anti-GM1 IgG antibodies from GBS patients cross-react with GBS associated *C. jejuni* (Oomes et al., 1995), and anti-GQ1b IgG antibodies with MFS associated isolates (Yuki et al., 1994; Jacobs et al., 1995).

Two issues considering the cross-reactivity of anti-LPS antibodies in GBS need to be clarified. Firstly, in approximately half of the GBS patients with *C. jejuni* infections no antibodies against gangliosides are found (Walsh et al., 1991; Rees et al., 1995b; Jacobs et al., 1996). These patients may have LPS induced antibodies which cross-react with other peripheral nerve antigens. So far, antibodies against *C. jejuni* LPS were investigated only in small

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groups of GBS patients with anti-GM1 antibodies, but not in GBS patients without these antibodies.

Secondly, ganglioside-like epitopes are also demonstrated in *C. jejuni* isolates from enteritis patients without GBS or MFS (Aspinall et al., 1993; Yuki et al., 1994; Jacobs et al., 1995). This indicates that, in addition to the fine-specificity, other features of the cross-reactive antibodies determine their possible pathogenicity. The subclass may be important since it determines the effector functions of the antibodies. In general, IgG antibodies against bacterial polysaccharides are predominantly of the IgG2 subclass (Hammerström and Smith, 1986). Antibodies against gangliosides in GBS and MFS patients, however, are predominantly IgG1 and IgG3 (Willison and Veitch, 1994; Ogino et al., 1995; Yuki et al., 1995). So far, a comparative study on the IgG subclasses of anti-LPS antibodies in patients with GBS, and in *C. jejuni* enteritis without GBS has not been performed.

To further characterize the humoral immune response against *C. jejuni* LPS, we studied the presence, course, subclass distribution and specificity of serum IgG antibodies against *C. jejuni* LPS in a large and heterogeneous group of GBS and MFS patients, compared to patients with *C. jejuni* enteritis and normal controls.

## 2. Patients and methods

### 2.1. *C. jejuni* isolates and lipopolysaccharides

*C. jejuni* was isolated from 3 patients with MFS (A, B and C), 3 patients with GBS (D, E and F) and 3 enteritis controls without neurological involvement (1, 2 and 3). Patients A–E and 1–3 and their *C. jejuni* isolates were described previously (Jacobs et al., 1995; Jacobs et al., in press). Patients A, B and C suffered from ophthalmoplegia, ataxia and areflexia without limb weakness, and patients D, E and F fulfilled the diagnostic criteria for GBS (Asbury and Cornblath, 1990). Serum anti-ganglioside antibodies were found in MFS patient A and C (GQ1b, GD1b, GD3), B (GQ1b) and GBS patient D (GM1, GD1b), but

not in GBS patient E. Serum from GBS patient F and enteritis controls 1–3 was not available. The *C. jejuni* isolates were serotyped according to the Penner classification system, which is based on the bacterial LPS, as O:4,50 (A), non-typable (B and F), O:23 (C and 1), O:1 (D), O:24 (E), O:22 (2) and O:41 (3). *C. jejuni* was grown for 48 h on blood agar plates in a microaerobic atmosphere, inactivated in 1% formaldehyde and washed in phosphate-buffered saline solution (PBS) (pH 7.4). The LPS fractions from *C. jejuni* were isolated by phenol–water extraction (Westphal and Jann, 1965) and were demonstrated to contain less than 1% protein.

### 2.2. Sera from GBS, MFS and control patients

Serum samples from 41 GBS patients, 3 MFS patients, 12 *C. jejuni* enteritis controls (CJC) without neurological involvement, and 12 normal controls (NC) were tested for IgG antibodies against LPS. The patients were subdivided into 6 groups depending on the presence of antibodies against GM1 or GQ1b and antecedent *C. jejuni* infection (Table 1). Recent *C. jejuni* infections were serologically defined as the presence of IgM, IgA, or high titers of IgG antibodies against *C. jejuni* in ELISA, as previously described (Jacobs et al., 1996). The CJC had diarrhea and a positive *C. jejuni* serology. The samples from the GBS and MFS patients were obtained within 2 weeks of neurological symptoms and before treatment. All sera were tested for antibodies against the gangliosides GM1, GM3, GD1a, GD1b, GD3, GT1b and GQ1b, by enzyme linked immunoadsorbent assay (ELISA) and thin-layer chromatography overlay, according to methods described previously (Jacobs et al., 1996).

### 2.3. Detection of antibodies against lipopolysaccharides

Serum antibodies against LPS were tested in ELISA, by coating 96-well polystyrene microtiter trays (Immuno Maxisorb, Roskilde) with 1  $\mu$ g of LPS in 50  $\mu$ l PBS per well overnight at 37°C. Unspecific binding sites were blocked for 4 h with PBS containing 1% bovine serum

Table 1  
Anti-ganglioside antibodies and *C. jejuni* infections in patients tested for antibodies against *C. jejuni* lipopolysaccharides

Group	Patients		IgG antibodies		<i>C. jejuni</i> infection <sup>a</sup>	Including patient
	number	diagnosis	GM1	GQ1b		
1	12	GBS	12	0	9 (75%)	D
2	8	GBS/MFS	0	8	5 (63%)	A, B and C
3	12	GBS	0	0	12 (100%)	E
4	12	GBS	0	0	0	
5	12	CJC	0	0	12 (100%)	
6	12	NC	0	0	0	
Total	68		12	8	38	

GBS, Guillain-Barré syndrome; MFS, Miller Fisher syndrome; CJC, *C. jejuni* enteritis control without neurological involvement; NC, normal control.

<sup>a</sup> Defined as positive *C. jejuni* serology (Jacobs et al., 1996).

albumine (BSA) (Sigma, St. Louis, MO). After blocking, the plates were incubated overnight at 4°C with serum diluted 1:1000 in PBS–1% BSA. After washing with PBS, the plates were incubated with peroxidase-conjugated goat antihuman IgG ( $\gamma$ -chain specific) (Sigma), diluted 1:2500 in PBS–1% BSA, for 90 min at room temperature. After washing with PBS, the plates were developed with *O*-phenyl diamine (Sigma) in citrate buffer (pH 5.0) and the optical densities (ODs) were read at 490 nm. All samples were assayed in duplicate. The serum was tested in both LPS-coated and blank wells, allowing the OD values to be calculated after subtraction of the blank well OD from the coated-well OD. Serum samples with ODs of more than 3 SD above the mean value of 12 NC sera were considered to be of high titer, and were further tested to determine the IgG subclasses. These samples were diluted 1:250 in PBS–1% BSA and tested according to the same protocol. Subclasses were identified by using peroxidase-conjugated monoclonal goat antihuman IgG1 (clone MH161-1), IgG2 (clone HP6014), IgG3 (clone MH163-1) and IgG4 (clone MH164-4) (CLB, Amsterdam) diluted 1:100, 1:200, 1:200 and 1:200 in PBS–1% BSA, respectively. All serum samples were also tested for IgG1 and IgG2 antibodies against Pneumovax (Merck, Sharp and Dohme, Haarlem), a mixture of capsular polysaccharides from 23 different serotypes of *Streptococcus pneumoniae*, by routine techniques.

#### 2.4. Sialidase treatment of lipopolysaccharides

The LPS fractions were incubated with 0.05 U/ml of sialidase from *Clostridium perfringens* (CP) (Sigma) or

*Arthrobacter ureafaciens* (AU) (Sigma) in 50 mM of sodium acetate buffer (pH 5.5) overnight at 37°C. After incubation, the LPS were incubated at 80°C for 30 min to inactivate the sialidases. In control studies, the LPS were incubated in the same conditions without sialidases. Antibodies against the desialylated LPS were further tested according to the method described in Section 2.3.

#### 2.5. Cholera toxin treatment of lipopolysaccharides

The LPS fractions were coated according to the protocol described in 2.3, and incubated with 25  $\mu$ g/ml of B subunit of cholera toxin (CT) (Sigma) in PBS–1% BSA overnight at 4°C. The plates were emptied before further testing. In control studies, the LPS were incubated in the same conditions without CT. Antibodies against the CT blocked LPS were further tested according to the method described in Section 2.3.

### 3. Results

#### 3.1. IgG antibodies against LPS

In the anti-GM1 positive patients (group 1), 9 (75%) of 12 patients had high IgG activity against LPS of *C. jejuni* isolates from MFS patient A, GBS patients D, E and F, or control 3 (Fig. 1). Six patients had IgG antibodies against all these isolates, 2 only against the 3 GBS associated isolates and 1 only against GBS related isolate F. All these patients with anti-LPS antibodies had a recent *C. jejuni*

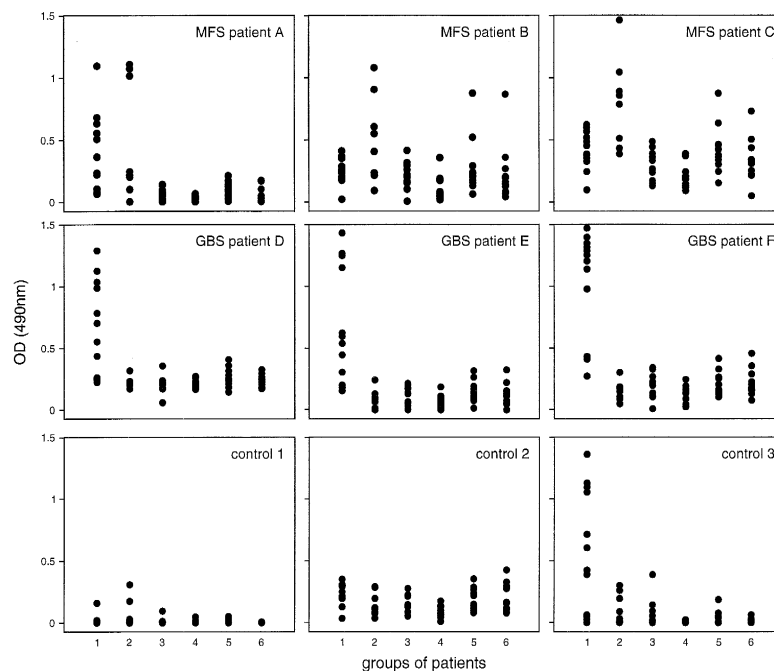


Fig. 1. Serum IgG activity against LPS from *C. jejuni* isolates from MFS patients (A, B, C), GBS patients (D, E, F), and enteritis controls without neurological involvement (1, 2, 3). The patients tested for IgG activity were subdivided into 6 groups according to diagnosis, presence of anti-ganglioside infections and antecedent *C. jejuni* infections (see Table 1).

infection. The 3 patients without anti-LPS activity had no *C. jejuni* infection and 2 had lower anti-GM1 IgG titers than 1:1000. No evident difference in additional IgG activity against other gangliosides was found in the patients with anti-LPS IgG antibodies compared to those without. However, the 6 patients with IgG against the *C. jejuni* isolate A all had additional anti-GD1b antibodies (data not shown).

In the anti-GQ1b positive patients (group 2), 6 (75%) of 8 patients had high IgG activity against LPS of *C. jejuni* isolates from MFS patients A, B or C (Fig. 1). IgG activity against at least 2 of these isolates was found in 5 patients. Four of the patients with anti-LPS antibodies had a recent *C. jejuni* infection. No difference was found in anti-GQ1b IgG titer or antibodies against other gangliosides between patients with anti-LPS antibodies compared to those without. In the anti-GM1/GQ1b negative GBS patients, high IgG activity against LPS was found only in 1 (8%) of the 12 patients with *C. jejuni* infection (group 3), which was to LPS from control isolate 3, and in none of the 12 patients without this infection (group 4) (Fig. 1). Two (17%) of the 12 *C. jejuni* infected controls without GBS or MFS (group 5), and 1 (8%) of the 12 normal controls (group 6) had high anti-LPS IgG activity. The IgG in these patients reacted with LPS from the MFS associated isolates B and C (Fig. 1).

In GBS patients with high anti-LPS activity follow-up samples obtained at 3 or 6 months were also tested. The anti-LPS IgG activity in these samples was lower than in pretreatment samples, although the activity was still higher than in normal controls. The antibody activity against LPS showed a similar course as the activity against gangliosides in individual patients (Fig. 2).

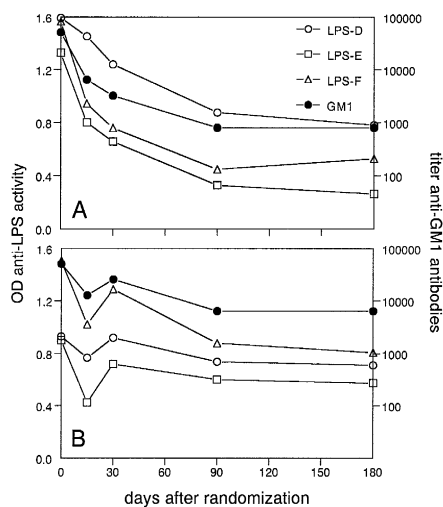


Fig. 2. Follow-up of IgG antibody activity against LPS of 3 GBS associated *C. jejuni* isolates (D, E, F) (expressed as extinctions) and of anti-GM1 IgG activity (expressed as titers) in two GBS patients with *C. jejuni* infection, one treated with intravenous immunoglobulins (A), and one with plasma exchange (B).

Table 2

Subclasses of IgG antibodies against GM1, GQ1b and *C. jejuni* LPS in serum from patients with GBS, MFS, and CJC and NC with high titers of anti-LPS antibodies

Group	Patient	GM1	GQ1b	Lipopolysaccharides <sup>a</sup>						
				MFS			GBS			Control
				A	B	C	D	E	F	3
GBS	1	1	—	—	—	—	1	1	1, 3	1
	2	1	—	1	—	—	1	1	1	1
	3	3, 1	—	3, 1	—	—	3, 1	3, 1	3, 1	3, 1
	4	1	—	1	—	—	1	1	1	1
	5	1	—	1	—	—	1	1	1	1
	6	1	—	—	—	—	1	1	1	1
	7	1, 3	—	1	—	—	1	1	1, 3	1
	8	1	—	1	—	—	1	1	1, 3	1
	9	1	—	—	—	—	—	—	1	—
	10	—	—	—	—	—	—	—	—	1
GBS/MFS	11	—	3, 1	3, 1	—	3, 1	—	—	—	—
	12	—	1	1	—	—	—	—	—	—
	13	—	3	—	3	3	—	—	—	—
	14	—	3, 1	3, 1	3, 1	3, 1	—	—	—	—
	15	—	3	—	1	3, 1	—	—	—	—
	16	—	3	—	3, 1	3, 1	—	—	—	—
CJC	17	—	—	—	2	2	—	—	—	—
	18	—	—	—	2	2	—	—	—	—
NC	19	—	—	—	2, 1	2	—	—	—	—

GBS, Guillain-Barré syndrome; MFS, Miller Fisher syndrome; CJC, *C. jejuni* enteritis control without neurological involvement; NC, normal control.

<sup>a</sup> LPS from *C. jejuni* isolates from MFS patients (A, B, C), GBS patients (D, E, F) and an enteritis control without neurological involvement (3). Numbers 1 to 4 refer to the presence of subclasses IgG1 to IgG4, respectively, with the first mentioned being the predominant subclass.

Axonal degeneration, as indicated by the presence of denervation potentials, was found in 5 (50%) of 10 patients in group 1, 2 (25%) of 8 patients in group 2, 2 (20%) of 10 patients in group 3, and 1 (9%) of 11 patients in group 4. No clear association between the presence of anti-LPS antibodies and axonal degeneration could be demonstrated.

### 3.2. Subclasses of IgG antibodies against gangliosides and LPS

The subclass of the IgG antibodies against GM1, GQ1b and LPS were determined in all patients with high IgG activity against LPS, and are given in Table 2. IgG antibodies against GM1 or GQ1b were all of the IgG1 or IgG3 subclasses. In most patients the predominant isotype of anti-GM1 antibodies was IgG1, and of anti-GQ1b antibodies was IgG3. In GBS and MFS patients the IgG antibodies against LPS were also of the IgG1 or IgG3 subclasses and generally corresponded with the isotype of the anti-ganglioside antibodies in individual patients (Table 2). However, in serum from CJC and NC, antibodies against LPS from *C. jejuni* isolates B and C were predominantly IgG2. Control studies demonstrated that IgG2 antibodies against

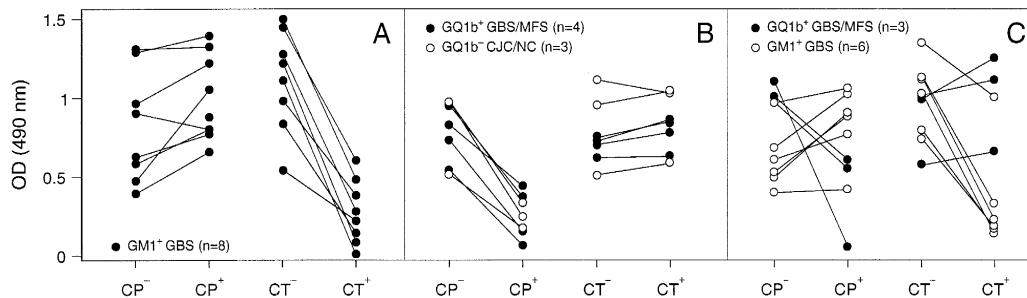


Fig. 3. Serum IgG activity against LPS preincubated with neuraminidase from *Clostridium perfringens* in 50 mM sodiumacetate buffer (CP +), 50 mM sodiumacetate buffer only (CP -), cholera toxin in PBS-1% BSA (CT +), or PBS-1% BSA only (CT -). These experiments were performed with LPS of *C. jejuni* from GBS patient E (A), MFS patient B (B), and MFS patient A (C). Serum was tested from all patients with antibodies against these LPS.

pneumococcal polysaccharides were present in all patients and controls (data not shown).

### 3.3. Specificity of IgG antibodies against LPS

To further characterize the specificity of the anti-LPS antibodies, we incubated the LPS with sialidases and CT. Binding of IgG to *C. jejuni* isolate E in serum from anti-GM1 positive patients was reduced to baseline after blocking the LPS with CT (Fig. 3A). Treatment of the LPS with sialidase CP had no or an increasing effect on the antibody binding (Fig. 3A). The same results were found with LPS from *C. jejuni* isolates D, F and 3 (data not shown).

Treatment of LPS from *C. jejuni* isolate B with sialidase CP reduced the binding of IgG to LPS in serum from the anti-GQ1b positive patients, CJC and NC (Fig. 3B). The reactivity was not influenced by blocking the LPS with CT. The same results were found with LPS from *C. jejuni* isolate C (data not shown). In LPS from *C. jejuni* isolate A a combination of effects was found (Fig. 3C). After blocking with CT the IgG binding to LPS was reduced in anti-GM1 positive patients, but not in anti-GQ1b positive patients. After treatment with sialidase CP, the IgG binding was reduced in anti-GQ1b positive patients but not in anti-GM1 positive patients. Sialidases from CP and AU gave similar results in all experiments.

## 4. Discussion

The present study demonstrates that the IgG responses against *C. jejuni* LPS and gangliosides are closely associated in patients with GBS and MFS. Firstly, high IgG activity against LPS from GBS related *C. jejuni* isolates was found in anti-GM1 positive GBS patients. Anti-GQ1b positive GBS and MFS patients had high titers of IgG against LPS from the MFS related strains. Secondly, the titer course and subclass of the IgG antibodies against LPS corresponded with those of the antibodies against GM1 and GQ1b. Such a similarity in subclass distribution was also found by others (Yuki et al., 1995). Thirdly, the

antibody binding with LPS in sera with anti-GM1 or anti-GQ1b antibodies was reduced to baseline after CT and sialidase treatment of the LPS, respectively. These findings suggest that most of the anti-LPS IgG activity in GBS and MFS patients is due to antibodies against ganglioside-like epitopes in LPS, indicating that these epitopes are immunodominant in a humoral immune response against LPS. This further supports the hypothesis that antecedent *C. jejuni* infections in GBS and MFS trigger the production of cross-reactive antibodies against gangliosides.

Molecular mimicry between gangliosides and LPS has been demonstrated with *C. jejuni* isolates from GBS and MFS patients (Yuki et al., 1993; Salloway et al., 1996). Previously we found that anti-GQ1b antibodies cross-react with LPS of *C. jejuni* from MFS patient A, B and C, and anti-GM1 antibodies with *C. jejuni* from GBS patient E and control 3 (Jacobs et al., 1997). In the present study we demonstrate that anti-LPS antibodies in *C. jejuni* infected GBS patients without anti-ganglioside antibodies are rare. This indicates that, except for these ganglioside-like epitopes, other LPS structures do not induce cross-reactive antibodies. However, *C. jejuni* infection in these patients may induce cross-reactive antibodies against bacterial structures other than LPS. This is supported by the finding that antibodies against peripheral nerve proteins are induced by immunization with *C. jejuni* proteins in animals (Fujimoto and Amako, 1990; Kaldor et al., 1992).

In some patients with anti-ganglioside antibodies we did not find high IgG activity against LPS. This may result from relatively low titers or different fine-specificities of the anti-ganglioside antibodies in these patients. In previous studies we found no binding of anti-GM1 antibodies with LPS of *C. jejuni* from MFS patient A and GBS patient D (Jacobs et al., 1997). In the present study we demonstrated antibodies against these isolates in a subgroup of the anti-GM1 positive patients. These patients all had additional antibody activity against GD1b, suggesting that the antibodies recognize the shared Gal( $\beta$ 1-3)GalNAc-moiety on GM1 and GD1b. The antibodies may also react with this moiety in LPS, since the antibody binding was insensitive to sialidases and could be blocked by CT. This is in accordance with the finding that CT also

binds with Gal( $\beta$ 1–3)GalNAc-containing glycolipids other than GM1 (Schwerer et al., 1995).

In the present study we found antibodies against LPS from MFS associated *C. jejuni* in serum from enteritis and normal controls too. These antibodies are probably directed against ganglioside-like epitopes as well, since sialidase treatment of the LPS reduced the antibody binding. Accordingly, ganglioside-like structures were also demonstrated in *C. jejuni* from patients without neurological involvement (Aspinall et al., 1993). Our study suggests that these structures may also be immunodominant in uncomplicated *C. jejuni* infections. However, these epitopes induce antibodies with other specificities than in GBS and MFS patients, as indicated by the lack of activity against GM1 and GQ1b in controls.

The anti-LPS antibodies further differ with respect to their subclasses in GBS and MFS patients compared to controls. We found high titers of anti-LPS IgG antibodies in serum from some enteritis patients and normal controls, which were predominantly IgG2. The presence of IgG1 and IgG3 antibodies against LPS in GBS and MFS patients, suggests that in these patients humoral immune responses against polysaccharides differ from others. The presence of IgG2 antibodies against pneumococcal capsular polysaccharides in all GBS and MFS patients excludes an IgG2 subclass deficiency, and may indicate that the IgG1 and IgG3 response are restricted to LPS from specific *C. jejuni* strains.

Long-lasting, high titers of IgG1 and IgG3 antibodies are characteristic for T cell dependent immune responses (Papadea and Check, 1989). The role of T cells in the in vivo production of antibodies against LPS and gangliosides in humans, has not yet been elucidated. In vitro studies indicate that the production of antibodies against both bacterial polysaccharides and gangliosides are stimulated in the presence of activated T cells (Ambrosino et al., 1990; Heidenreich et al., 1994). In the immune response against LPS, *C. jejuni* may induce T cell activation by the production of a protein that binds to LPS, serving as a carrier protein for T cell help (Willison and Kennedy, 1993). A candidate for this protein may be enterotoxins, which specifically bind with gangliosides and are produced by specific *C. jejuni* strains (Klipstein and Engert, 1984; Suzuki et al., 1994). If one *C. jejuni* strain expresses both this enterotoxin and ganglioside-like LPS, the enterotoxin may bind to LPS, providing an epitope for T cells. These activated T cells may skew B cells against LPS to switch to IgG1 and IgG3 production.

This subclass switch may be crucial for the pathogenicity of anti-ganglioside antibodies in GBS and MFS. IgG1 and IgG3 antibodies are more effective than IgG2 in activating the classical complement pathway (Feinstein et al., 1986). Interestingly, the suppression of sodium current in sciatic nerve by rabbit anti-GM1 antibodies was found to be complement-dependent (Takigawa et al., 1995). In addition, IgG1 and IgG3 have a higher affinity for Fc

receptors which play a role in opsonization and phagocytosis (Van de Winkel and Capel, 1993).

In conclusion, our study gives further support to the hypothesis that infections with specific *C. jejuni* strains induce cross-reactive IgG1 and IgG3 antibodies against LPS and gangliosides in GBS and MFS patients. Further research is necessary to elucidate the mechanism by which *C. jejuni* determines the specificity and isotypes of the anti-ganglioside antibodies.

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