Anti-GM_1 IgG Antibodies and *Campylobacter* Bacteria in Guillain-Barré Syndrome: Evidence of Molecular Mimicry

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In Guillain-Barré syndrome antibodies to GM_1 and the presence of an antecedent *Campylobacter jejuni* infection are correlated with a more severe course of the disease. From a group of 137 consecutive GBS patients, 11 sera had elevated titers of anti-GM_1 IgG antibodies during the acute stage of disease. Each serum sample was preincubated with three different Penner serotypes of whole *C. jejuni* (PEN O:4/59, PEN O:41) and *Campylobacter coli* (PEN O:22) bacteria. The PEN O:4/59 serotype, isolated from the stools of a Guillain-Barré syndrome patient, inhibited 63 to 93% of the anti-GM_1 activity in 6 of 11 patients. The PEN O:41 inhibited 63 to 100% of the anti-GM_1 antibody activity in 9 of 11 patients. The PEN O:22 inhibited anti-GM_1 antibody activity in only 2 of 11 patients (80 and 86%). Two Guillain-Barré syndrome patients did not show antibody absorption by any of the *Campylobacter* serotypes tested, although this does not exclude the involvement of other serotypes. An *Escherichia coli* control strain did not significantly absorb anti-GM_1 antibodies. The results of this study indicate that anti-GM_1 IgG antibodies in Guillain-Barré syndrome sera recognize surface epitopes on whole *Campylobacter* bacteria and that this recognition is strain-specific. This provides evidence for molecular mimicry in the pathogenesis of Guillain-Barré syndrome.

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Elevated antibody (Ab) titers against various types of gangliosides and other glycolipids have been found during the acute stage of Guillain-Barré syndrome (GBS) [1–4]. We found high anti-GM_1 IgM and IgG Ab titers in 41 (30%) of 137 GBS patients [4, 5]. Elevated anti-GM_1 Ab activity was significantly correlated with a more severe disease course [5]. Serological evidence of an antecedent *Campylobacter jejuni* infection correlated significantly with the presence of anti-GM_1 Abs [5], in accordance with other reports [6, 7]. This association led to the hypothesis that molecular mimicry between antigens on *Campylobacter* bacteria and GM_1 or other glycoconjugates on peripheral nerve tissue could be an important autoimmune mechanism in the pathogenesis in a subgroup of GBS patients. We tested this hypothesis in all 11 GBS patients with high IgG Ab titers against GM_1 by measuring the inhibition of anti-GM_1 Ab activity in absorption experiments with different strains of *C. jejuni* and *Campylobacter coli* bacteria.

Patients and Methods

Patients and Healthy Control Subjects

A group of 147 consecutive GBS patients involved in the Dutch Guillain-Barré trial [5] was routinely screened for serum anti-GM_1 IgG and IgM Abs. All 11 GBS patients with high anti-GM_1 IgG Ab titers in the acute phase of the disease and prior to treatment were selected for this study. In addition, sera from 25 patients with *C. jejuni* infection without neurological involvement and 50 age- and sex-matched healthy blood donors were tested for anti-GM_1 Abs.

Campylobacter *Serology and Serotyping*

Serum IgM, IgG, and IgA antibodies against *Campylobacter* bacteria were determined by an enzyme-linked immunosorbent assay (ELISA) [8]. *C. jejuni* isolates from the stools of 25 patients without neurological involvement and from 1 GBS patient were serotyped according to the Penner classification system [9]. This method is based on the passive hemagglutination technique, which detects soluble heat-stable antigens that have been identified as lipopolysaccharides (LPSs).

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Bacteria

Three different serotypes of *Campylobacter* bacteria and one *Escherichia coli* (*E. coli*) were used in this absorption study. A *C. jejuni* PEN O:4/59 serotype was isolated from the stools of a GBS patient. In addition we used another *C. jejuni* (PEN O:41) and a *C. coli* (PEN O:22). An *E. coli* isolated from the stools of a healthy control subject was used as a gram-negative control. The *Campylobacter* serotypes were grown on chocolate agar plates at 42°C in an anaerobic jar containing a CampyPak gas generator envelope (BBL). *E. coli* was grown on blood agar plates at 37°C. After 18 hours the bacteria were harvested in phosphate-buffered saline (PBS) solution with 1% formaldehyde, centrifuged at 10,000*g*, washed three times in PBS solution, and suspended in PBS solution. After the dry-weight concentration was determined, suspensions were aliquoted and kept at −20°C until use.

Enzyme-Linked Immunosorbent Assay and Absorption Experiments

All serum samples were examined within the same batch of 96-well ELISA plates (Immunoplate II 96F, Nunc, Denmark). All measurements were performed on at least two separate occasions and showed a good reproducibility. Serum samples were diluted out from 1:25 to at least 1:3,200 in PBS buffer with 1% bovine serum albumin (BSA). To each serum dilution an identical volume of a 0.135-mg/ml (dry-weight) suspension of whole bacteria (*E. coli*, *C. jejuni* PEN O:4/59, O:41, or O:22) in PBS–1% BSA was added. Thus, the final serum dilutions ranged from 1:50 to at least 1:6,400 and the bacterial concentration in each sample was 0.0675 mg/ml. The samples were then incubated for 2 hours at 37°C followed by centrifugation for 10 minutes at 10,000*g*. In the supernatant the residual anti-GM_1 IgG activity was measured in an ELISA and compared with the Ab activity in unabsorbed serum. The 96-well plates were coated with 100 μl of ethanol containing 2 μg of bovine GM_1 ganglioside (Sigma, St. Louis, MO) per well. After overnight ethanol evaporation, the plates were washed five times in 0.01 M PBS solution. Remaining aspecific binding sites were blocked by incubating with PBS–1% BSA for 2 hours at 20°C. The plates were incubated with the supernatants of the serum-bacterial solutions and the serum-PBS control. Wells were also incubated with control sera from healthy blood donors and with PBS–1% BSA alone. After 4 hours the plates were washed three times with PBS solution containing 0.05% Tween 20. As second Ab, horse radish peroxidase (HRP)-labeled antihuman IgG was used in a dilution of 1:2,500 (Sigma). After a 1.5-hour incubation the plates were washed five times and developed with 0.05% *O*-phenylenediamine (Sigma) and 0.012% hydrogen peroxide in 0.1 M citrate buffer. After 5 minutes the reaction was stopped with 4 M sulfuric acid and the optical densities were read at 492 nm with a Titertek Multiscan MCC (Labsystems, Finland). Ab activity against other glycolipids (asialo-GM_1 [AGM_1], GD_{1b}, GD_{1a}, and GT_{1b}) was also tested in the ELISA. All antigens were purchased from Sigma.

Other Control Experiments

The possibility of aspecific (Fc-mediated) binding of IgG to the bacteria led to absorption studies being performed using sera from patients with systemic lupus erythematosus (SLE) with high levels of anticardiolipin IgG Abs. Anticardiolipin Abs were measured using an ELISA as described previously [10]. In addition, on various bacterial pathogens, including *C. jejuni*, adhesins for mucosal membranes have been identified [11]. Some of these adhesins, for instance, those present on *Campylobacter pylori*, bind to acidic glycoconjugates [12]. Thus, free adhesins, if present in the supernatant of the centrifuged serum–*C. jejuni* mixture, could theoretically bind with GM_1 on the ELISA plate and inhibit anti-GM_1 IgG binding. This would give a false-positive absorption of anti-GM_1 IgG. To rule out this possibility, we also performed the absorption ELISA protocol using the bacterial solutions without adding patient serum. Here, the ELISA plates were preincubated for 1 hour with supernatant from bacterial solutions. After patient serum was washed five times in PBS–1% BSA, it was added as a second step followed by the procedure as mentioned.

Calculation of the Antibody Titer and Percent Absorption

Log-logit transformation was performed on the sigmoidal dilution curve data, giving a linear progression over a sufficiently large range of serum dilutions:

$$\text{Logit } Y = \ln [(OD/ODmax)/(1 - OD/ODmax)]$$

OD is the measured optical density at a given dilution and ODmax, the maximal absorption value of the serum-PBS range. Standard linear regression was performed on the logit Y versus the log serum dilution. The serum dilution at which the logit Y was equal to 0 (= 50% of maximal optical density) was taken as anti-GM_1 Ab titer. The anti-GM_1 IgG Ab activity following preincubation of patient serum with the different bacteria was compared with the Ab activity of PBS-incubated serum. A decrease of anti-GM_1 IgG Ab activity after preincubation with one of the serotypes of *Campylobacter* was considered to be caused by specific antigen-antibody recognition on the bacterial surface only if the preincubation with *E. coli* did not show such a decrease in titer. The inhibition percentage of anti-GM_1 IgG Ab activity was used as a measure of specific antibody binding to bacterial surface antigens and was calculated as:

$$\text{Percentage inhibition} = [\text{Ab titer (serum without bacteria)} - \text{Ab titer (serum preincubated with bacteria)}]/\text{Ab titer (serum without bacteria)} \times 100\%$$

Results

Disease Severity and Antecedent Infections

Clinical data for the 11 GBS patients positive for anti-GM_1 IgG are given in Table 1. In 9 of the 11 patients tested there was serological evidence of an antecedent *C. jejuni* infection. No serological evidence of other bacterial or viral infections was found. Five of the 11 patients were still not able to walk unaided (F = 2, modified Hughes scale [13]) after 6 months. One patient died of cardiovascular complications. Six of 7 patients tested electromyographically showed moderate to severe denervation activity at 2 or 4 weeks after

Table 1. Clinical Parameters of the 11 Anti-GM_1 IgG–Positive GBS Patients in This Study

Patient No.	Age (yr)	Sex	Treatment	Anti-GM_1 Ab IgG	Anti-GM_1 Ab IgM	Antecedent Infection	UR or GI Disease	Sensory Deficit	Time until F = 2 (days)	Artificial Respiration	Denervation Activity
1	61	M	IgIV	+	−	CJ	−	−	69	−	+
2	74	M	PE	+	−	CJ	Diarrhea	+	>181	+	+
3	57	F	IgIV	+	−	CJ	Diarrhea	−	Dead	−	NT
4	9	M	IgIV	+	+	CJ	UR	−	4	−	NT
5	47	M	IgIV	+	+	CJ	Diarrhea	+	>181	+	+ +
6	15	M	IgIV	+	+	CJ	−	−	20	−	+
7	47	M	IgIV	+	−	−	UR	+	20	−	−
8	77	F	IgIV	+	−	CJ	Diarrhea	+	>181	−	+ +
9	67	F	PE	+	+	CJ	Diarrhea	−	>181	−	+
10	73	F	PE	+	−	−	UR	−	55	+	NT
11	19	M	PE	+	+	CJ	Diarrhea	−	>181	−	NT

IgIV = immunoglobulin intravenous; PE = plasma exchange; Ab = antibody; CJ = *Campylobacter jejuni;* UR = upper respiratory; GI = gastrointestinal; "F = 2" = walking unaided > 10 m (Hughes score); NT = not tested; denervation activity: + = moderate; + + = severe.

Table 2. Presence or Absence of IgG Antibodies to GM_1, GD_{1a}, GD_{1b}, GT_{1b}, and Asialo-GM_1 (AGM_1) in 11 GBS Patients

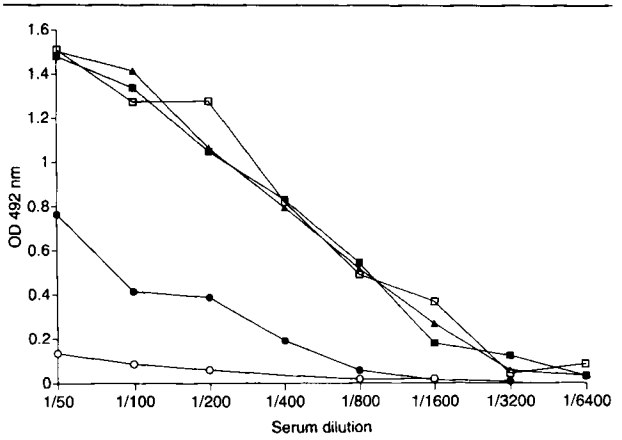
Patient No.	IgG Antibodies GM_1	GD_{1a}	GD_{1b}	GT_{1b}	AGM_1
1	+	−	+	−	+
2	+	−	−	−	+
3	+	−	−	−	−
4	+	−	−	−	+
5	+	−	+	−	+
6	+	−	+	−	+
7	+	−	−	−	−
8	+	−	+	−	+
9	+	+	−	−	−
10	+	−	−	−	−
11	+	−	+	−	+

disease onset that was suggestive of axonal degeneration.

IgG Antibodies to GM_1, GD_{1a}, GD_{1b}, GT_{1b}, and Asialo-GM_1

The anti-GM_1 IgG titers of the GBS sera ranged from 1:210 to 1:4,410, compared to titers from 0 to 1:50 in 50 healthy age- and sex-matched control subjects. No high serum anti-GM_1 Ab titers were found in 25 patients with a *C. jejuni* infection without neurological involvement. In these patients 12 different serotypes were found, including the PEN O:4/59, used in this study, and the PEN O:19 serotype, which was reported to be highly correlated with anti-GM_1 Abs by others [14, 15]. The other serotypes were PEN O:2, O:2/44, O:15, O:18, O:21, O:24, O:30, O:37, O:53, and O:nontypable.

Results (presence or absence) for Ab activity against GD_{1a}, GD_{1b}, GT_{1b}, and AGM_1 in the 11 anti-GM_1 IgG–positive GBS sera are given in Table 2. In 5 of the 11 patients, additional IgG Ab binding to GD_{1b} and AGM_1 was found. In 2 of the 11 patients, additional binding was found to AGM_1 and in 1 patient to GD_{1a}.



Example of the enzyme-linked immunosorbent assay (ELISA) results of Patient 6. Anti-GM_1 IgG activity was measured at different serum dilutions following serum preincubation in phosphate-buffered saline solution without bacteria (open squares), *and with* E. coli (closed squares) *and three* Campylobacter *serotypes: PEN 0:4/59* (closed circles), *0:41* (open circles), *and 0:22* (triangles). *Preincubation with 0:4/59 and 0:41 inhibited 93 and 100% of the serum anti-GM_1 IgG activity, respectively, whereas preincubation with* E. coli *and 0:22 did not result in any decrease in antibody titer.*

Absorption Experiments

An example of the ELISA results after preincubation of patient serum containing anti-GM_1 Abs with different bacteria is given for Patient 6 in the Figure. With the *C. jejuni* PEN O:4/59 and PEN O:41 serotypes, an evident inhibition of the anti-GM_1 activity was found, whereas preincubation with *E. coli* and *C. coli* PEN

Table 3. Inhibition Percentages of Anti-GM_1 IgG Activity in 11 GBS Sera Following Incubation with E. coli *and Different* Campylobacter *Strains*

Patient No.	Anti-GM_1 IgG Titer ($\times 10^2$)	% Inhibition by *E. coli*	% Inhibition by O:4/59	% Inhibition by O:41	% Inhibition by O:22
1	3.1	47	76 (+)	63 (+)	80 (+)
2	9.9	0	0	82 (+)	37
3	3.1	9	0	38	2
4	5.1	2	63 (+)	100 (+)	16
5	2.1	0	71 (+)	75 (+)	0
6	12.0	7	93 (+)	100 (+)	20
7	3.2	0	33	23	37
8	31.0	8	80 (+)	92 (+)	0
9	44.1	0	31	83 (+)	0
10	2.1	29	74 (+)	89 (+)	86 (+)
11	21.9	0	1	86 (+)	0

(+) = specific inhibition of anti-GM_1 IgG activity (for definition see Results).

O:22 did not give any inhibition of the signal when compared with the results of serum incubated with PBS solution. The percentages of inhibition of anti-GM_1 IgG activity in the sera from 11 GBS patients by the four bacterial strains are given in Table 3. The inhibition percentages showed a bimodal distribution that defined a group with low (0–50%) and a group with high (60–100%) inhibition. Since all the *E. coli* results fell in the first group, we interpreted this low inhibition as caused by aspecific Ab absorption, and the high inhibition values as a result of specific antigen-antibody binding. This cutoff point between low and high inhibition equals the mean inhibition plus 3 standard deviations (54.9%) in the *E. coli* group. With this cutoff point, the *C. jejuni* serotype PEN O:41 demonstrated a 63 to 100% (mean, 75.6%) inhibition of anti-GM_1 IgG activity in 9 of 11 patients; the PEN O:4/59, a 63 to 93% (mean, 47.5%) inhibition in 6 of 11 patients; and the PEN O:22, an 80 and 86% inhibition in 2 of 11 patients. No significant inhibition was obtained with the *E. coli* strain. Table 3 shows that all 6 patient sera showing anti-GM_1 IgG absorption with PEN O:4/59 were also inhibited by PEN O:41. Of these, 2 demonstrated absorption with the PEN O:22 serotype as well. In 3 patient sera anti-GM_1 IgG was inhibited exclusively by the PEN O:41 serotype. Only in Patients 3 and 7 was no absorption with any of the three *Campylobacter* serotypes found.

Anticardiolipin Assay

To assess the possibility of Fc-mediated or other aspecific IgG absorption by *C. jejuni* bacteria, we tested sera of 3 SLE patients with high levels of anticardiolipin IgG Abs. Preincubation of these sera with the *C. jejuni* and *E. coli* strains did not decrease the anticardiolipin titers measured by ELISA (data not shown).

Adhesins

Interference due to the presence of free adhesins was ruled out since no inhibition of anti-GM_1 Ab activity in patient sera was found using GM_1-coated ELISA plates preincubated with supernatant from the bacterial solutions (data not shown).

Discussion

This study demonstrated that anti-GM_1 IgG Ab activity in 9 of 11 GBS patients could be inhibited by 63 to 100%, by preincubation with whole cells from at least one of three *Campylobacter* serotypes (PEN O:4/59, PEN O:41, and PEN O:22). This indicates the presence of surface epitopes on *Campylobacter* that cross-react with GM_1. Others identified oligosaccharide structures in purified LPS fractions from *C. jejuni* serotypes PEN O:4 and PEN O:19 [16–19] and showed that monoclonal anti-GM_1 IgM Abs from patients with chronic motor neuropathies cross-react with LPS extracts from PEN O:19, PEN O:4, and PEN O:50 [20]. The results of our study using whole *Campylobacter* bacteria strongly suggest that these cross-reactive LPS antigens are indeed expressed on the bacterial surface and are thus potentially able to elicit an immune response. We were not able to inhibit IgM Abs to GM_1 under the conditions of our absorption assay. This could be due to the lower titer of serum IgM Abs compared to IgG in our GBS patients, or to the lesser affinity of these polyclonal IgM Abs, compared to the monoclonal anti-GM_1 Abs used by Wirguin and coworkers [20]. However, other as yet unknown factors related to the assay method may be involved.

The serotype-specific pattern of anti-GM_1 IgG Ab absorption in the 11 GBS sera by the two *C. jejuni* and one *C. coli* serotypes and the absence in all patients

of a strong Ab absorption by the *E. coli,* which also has a LPS capsule, illustrate the specific nature of the cross-reacting surface epitope(s). In addition these same serotypes did not absorb anticardiolipin IgG Abs, which also excludes aspecific, false-positive binding.

Assessment of Ab reactivity to other gangliosides and AGM_1 demonstrated differences in binding patterns between the 11 patient sera. Five patient sera showed binding to GD_{1b} and AGM_1, suggesting cross-reaction to the carbohydrate structure Gal(β1-3) GalNAc. Two sera reacted also with AGM_1, 1 with GD_{1a}, and 3 others only to GM_1 (see Table 2). These binding patterns do not discriminate between cross-reactive Abs and the presence of several different Ab specificities in the same sample. Therefore, we cannot make definite conclusions as to the fine specificity of these Abs. In agreement with the findings of Wirguin and coworkers [20], we did not find a clear association between the pattern of antiglycolipid binding and the absorption data. For example, of the 5 patient sera with Ab reactivity to GM_1, GD_{1b}, and AGM_1, anti-GM_1 activity was strongly inhibited by preincubation with PEN O:4/59 in 4 patients, while no inhibition at all with this serotype occurred in 1 patient. This clearly indicates the existence of additional fine specificity of Ab binding due to as yet undetermined factors.

The LPS fraction from bacteria is a potent polyclonal B- and T-cell stimulator. Naturally occurring B and T cells recognizing GM_1 could therefore be stimulated aspecifically during a *C. jejuni* infection, giving high anti-GM_1 Ab titers without pathogenic relevance. However, the serotype-specific Ab absorption in this study, the normal concentrations of total IgG and IgM in the sera (data not shown), and in contrast to another study [21], the absence of anti-GM_1 Abs in 25 patients with *C. jejuni* without neurological involvement argue against such a mechanism.

The PEN O:19 serotype has been isolated from the stools of GBS patients [14, 15] and it was suggested that this rare serotype could be the pathogen responsible for GBS. However, we were able to isolate a *C. jejuni* PEN O:4/59 from the stools of a GBS patient, which is a common *C. jejuni* serotype in the Netherlands. Thus, GBS is also preceded by infections with other *Campylobacter* serotypes than the PEN O:19 and common serotypes may certainly be involved. Furthermore, we did not find high anti-GM_1 Ab titers in 2 patients with diarrhea due to serotypes PEN O:19 and O:4/59 infection without GBS. This means that the infrequent occurrence of GBS following a *C. jejuni* infection is probably more due to host factors than to exclusivity of the involved serotype.

In conclusion this study provided experimental evidence for the observed epidemiological correlation between the presence of anti-GM_1 Abs and *C. jejuni.* Anti-GM_1 antibodies in sera from GBS patients were shown to bind specific epitopes on *Campylobacter* bacteria, supporting the hypothesis of molecular mimicry as a possible pathogenic mechanism in GBS.

Addendum

In a recent report [22] we described anti-GQ_{1b} IgG Abs that recognized epitopes on *C. jejuni* in patients with Miller Fisher syndrome, using a similar assay method.

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