ANTECEDENT INFECTIONS AND ANTI-GANGLIOSIDE ANTIBODIES IN GUILLAIN-BARRÉ SYNDROME

their role in pathogenesis and heterogeneity

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ANTECEDENTE INFECTIES EN ANTIGANGLIOSIDE-ANTISTOFFEN
BIJ HET SYNDROOM VAN GUILLAIN-BARRÉ

hun rol in de pathogenese en heterogeniteit

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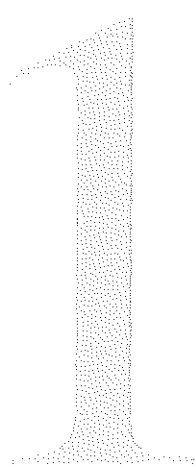


GUILLAIN-BARRÉ SYNDROME (GBS)

CHAPTER 1

GUILLAIN-BARRÉ SYNDROME (GBS)

1.1 INTRODUCTION

The Guillain-Barré syndrome (GBS) is the most common form of acute neuromuscular paralysis in developed countries, but the pathogenesis is still largely unknown. The major clinical features of the syndrome were first united by J-B.O. Landry in 1859 (1). The syndrome was named after G. Guillain and J.A. Barré, two French army neurologists, who in 1916 described, together with A. Strohl, the typical findings in the cerebrospinal fluid (2). This 'dissociation albumino-cytologique', the combination of elevated protein and normal cell count, was in those days an important diagnostic feature that further distinguished the syndrome from other neurological diseases, like poliomyelitis. GBS was early recognized as a postinfectious disorder, although the infectious agents were not identified. Since then, major efforts have been made to elucidate the pathogenesis of GBS. In the last years, research in this field was dominated by the discovery that infections with the Gram negative curved rod *Campylobacter jejuni* frequently precede GBS and by the finding of antibodies against various peripheral nerve gangliosides in serum from GBS patients. This introduction will focus on these infections and antibodies, and will provide a background for understanding the studies described in this thesis.

Definition of GBS

Because the pathogenesis is unknown, 'GBS' is presently a descriptive entity which is defined by a set of clinical, laboratory and electrodiagnostic criteria (Appendix 1). GBS is a polyneuropathy characterized by an acute progressive and symmetrical motor weakness of the extremities with loss of tendon reflexes (3-5). Surveillance studies from widely scattered geographical areas indicate that the annual incidence rate of GBS per 100,000 subjects ranges from 0.6 to 1.9 (6). GBS may affect persons of all ages, but has a higher incidence in elderly (6,7). GBS occurs slightly more often in males than in females (8). It is not associated with occupation and does not occur in families.

Two-third of patients with GBS suffer from a preceding gastrointestinal or respiratory infectious illness from which they mostly recover spontaneously. One to three weeks later the patient may notice numbness or tingling in arms or legs with symmetrical loss of strength in hands or feet. The weakness spreads during the next few hours to weeks and may finally lead to paresis of limb, trunk, extraocular, facial, pharyngeal, and tongue musculature. Due to involvement of respiratory muscles, up to one-third of the patients need artificial ventilation (9). Clinical examination of the patients shows loss of tendon reflexes and often diminished sensibility. The diagnosis is further supported by elevated protein and normal cell count in cerebrospinal fluid and by features

of demyelination and axonal degeneration in electrodiagnostic studies. The progression of weakness ceases by definition within 4 weeks. Spontaneous recovery usually begins 2 to 4 weeks after the cessation of progression, but may be delayed for months. General medical support is of utmost importance in treating GBS patients. Additionally, patients will recover more rapidly from their paresis and have less residual deficits after treatment with plasma exchange (PE) (10,11) or intravenous immunoglobulins (IVIg) (12,14). A pilot study indicated that methyl-prednisolon in addition to IVIg (MP-IgIV) is more effective than IVIg alone (13). The mortality is less than 5%, and mostly due to respiratory or autonomic complications, while persistent and severe functional deficits will occur in approximately 15% of the cases (15,16). GBS has a monophasic course in the majority of patients, but treatment related fluctuations were demonstrated in 12% of patients (17), and about 1% of the patients may have a relapse (16).

Although GBS is accepted as a disease entity, there is a marked patient to patient variation concerning the clinical features. The extent of the motor deficits ranges from minimal weakness of the legs to total paralysis of all extremities and the trunk, with bulbar and facial paralysis, and external ophthalmoplegia. Also the distribution of motor deficits varies between patients as illustrated by the selective weakness of proximal limb muscles in some cases and of distal muscles in others. Sensory involvement may be completely absent, in the case of a 'pure motor GBS', or may be severe, longlasting and include all sensory modalities. Some patients may rapidly recover without residual deficits, even from severe GBS, while others need artificial ventilation or remain bed-bound for years.

The clinical heterogeneity is further illustrated by the presence of distinct variants of GBS (16). The Miller Fisher syndrome (MFS), characterized by ophthalmoplegia, ataxia and areflexia without limb weakness (18), is considered to be a variant of GBS since overlap forms do occur (4,16). Most of the MFS patients recover spontaneously without severe residual disability. Other identified variants are the pharyngeal-cervical-brachial, the pure sensory, the pure ophthalmoplegic, and the lower bulbar variant of GBS (16). Patients with a demyelinating polyneuropathy of unknown cause and a progressive phase of 8 weeks or more by definition suffer from chronic inflammatory demyelinating polyneuropathy (CIDP). The presence of patients with a progressive phase between 4 and 8 weeks, who are considered to suffer from 'subacute GBS' (19), may suggest that the GBS and CIDP are part of a continuous spectrum of demyelinating polyneuropathies.

The heterogeneity of GBS is further indicated by the differences in electrodiagnostic findings between individual patients. In most patients, variable features of demyelination like reduction of nerve conduction velocity, conduction block, prolonged distal latencies or absent F-waves can be demonstrated (20,21). However, different patterns of conduction failure were described in GBS patients, suggesting variable distributions of nerve lesions (22). In addition, electrodiagnostic features of axonal degeneration and sensory nerve deficits are variably found.

In conclusion, the GBS includes patients with a wide spectrum of clinical and electrodiagnostic features. It is presently unknown which factors determine this heterogeneity or whether pathogenic mechanisms in these patients are distinctive.

Histopathology of GBS

The clinical and electrodiagnostic heterogeneity is reflected by the pathological findings reported in biopsy studies and autopsy cases of GBS patients. Classic pathological studies showed prominent multifocal and segmental demyelination with infiltration of mononuclear cells in peripheral nerves (23-26). Therefore, GBS is often regarded as an acute inflammatory demyelinating polyneuropathy (AIDP) (27). The lesions are patchy, and scattered along the whole peripheral nervous system, including the spinal roots and terminal myelinated intramuscular nerve twigs (28). Peripheral nerves of all functional modalities in the whole body can be affected. GBS patients differ from each other with respect to the extent and distribution of the lesions (26). Selective involvement of anterior (29) or posterior (26) roots suggests that the disease may also specifically affect motor or sensory nerves. Autopsy of a patient with the MFS showed that the lesions were most severe in the oculomotor nerves and spinal ganglia (30).

In GBS, the cellular infiltrates in peripheral nerves are mostly perivascularly localized and consist predominantly of macrophages with variable proportions of lymphocytes (25). Electron microscopic studies of nerves from AIDP patients showed that processes of macrophages enter between myelin lamellae and strip the myelin from intact axons (25,28,31). The complement activation marker C3d and the terminal complement neoantigen C5b-9 were identified along the outer surface of the Schwann cells (32). The lymphocytes infiltrated in peripheral nerves were characterized in an immunocytochemistry study as predominantly $CD4^+$ T-cells, although $CD8^+$ T-cells and B-cells were also present, but their relation to demyelination remains unclear (33). Other studies reported patients with profound demyelination in absence of significant infiltrates (26,34), indicating that demyelination may result from different pathogenic pathways.

Involvement of peripheral nerve axons, as indicated by Wallerian-like degeneration, occurs to a variable extent in patients with GBS, and may be secondary to severe inflammation and demyelination (3,29,35). Feasby et al., however, described several patients with severe GBS in whom electrodiagnostic and pathological studies suggested primary axonal degeneration without demyelination (36,37). Others challenged the concept of primary axonal involvement in GBS, arguing that the electrodiagnostic findings reported in these patients (low compound muscle action potentials (CMAP) and inexcitable motor nerves) do not discriminate between axonal degeneration and distal demyelination causing conduction block between the most distal site of stimulation and the target muscle (38-40).

Recently, autopsy studies on Chinese patients with acute flaccid paralysis gave a new impetus to the issue of primary axonal involvement in GBS. In northern China, an endemic form of acute paralysis annually affects children and young adults from rural areas in the summer (41). The patients meet the clinical criteria for the diagnosis of GBS, but have different electrodiagnostical and pathological features compared to AIDP (31,42-44). Autopsy studies performed early in the disease, showed predominant axonal degeneration without significant demyelination. Electron microscopic studies demonstrated macrophages in the periaxonal spaces, between the axolemma and the ensheathing Schwann cell, which engulf the axon while sparing the myelin sheath (31). Complement activation marker C3d was identified on the axolemma of motor

fibres, especially at the nodes of Ranvier (45). These authors suggest to subdivide GBS, and to distinguish the AIDP from this acute motor axonal neuropathy (AMAN), with axonal degeneration restricted to motor nerves, and from the acute motor sensory axonal neuropathy (AMSAN), which they consider to be a severe form of AMAN with extension of axonal changes to sensory nerves (31).

The demonstration of these distinct pathological substrates may partly explain the clinical and electrodiagnostic heterogeneity in GBS. It is not known, however, whether the findings in patients from northern China can be extrapolated to other regions in the world. Furthermore, it is unclear whether the AMAN/AMSAN and AIDP are absolutely distinct entities and whether they result from different pathogenic mechanisms. Alternatively, these entities are extreme forms in a continuous spectrum, in which the clinical and pathological features are determined by the relative contribution of several pathogenic and host factors. The applicability of this subclassification in clinical practice is difficult, since the diagnosis of acute axonal involvement can only be made by biopsy or autopsy. Further classification of GBS will depend on the identification of the antigenic targets and the immunopathological mechanisms in these various patterns.

Immunopathogenesis of GBS

The autopsy studies strongly indicate the involvement of activated complement factors and macrophages in demyelination and axonal degeneration. Specific T-cells and antibodies against axonal or myelin antigens probably guide the complement and macrophages to selectively attack the myelin in AIDP or the axon in AMAN and AMSAN. The axonal degeneration found in some patients with severe AIDP is probably secondary to the demyelination.

Several lines of evidence indicate that T-cells are activated in GBS (46). Circulating T-cells from GBS patients showed an increased expression of class II major histocompatibility complex (MHC) molecules, transferrin receptors, and interleukin-2 receptors (47). In addition, transient high serum concentrations of soluble interleukin-2 receptor, shed from the surface of activated T-cells, were reported in GBS patients and correlated with disease activity (48-50). The interleukin-2 concentration, the signal required for T-cells to proliferate, is also transiently elevated in GBS (51). The mutation frequency in the hypoxanthine guanine phosphoribosyl transferase gene of circulating T-cells, as a measure of T-cell stimulation, is increased during the acute phase of GBS and declines with clinical recovery (52). Although these results clearly indicate that T-cells in GBS are activated, the specificity of these T-cells has not been elucidated. In some patients T-cells against the peripheral nerve antigen P2 were demonstrated, but they were also found in healthy controls (53-55). Therefore, the exact role of activated T-cells in GBS patients remains unclear. They may be involved in (i) providing help to anti-neural B-cells, (ii) activating macrophages, (iii) direct cytotoxic effects on peripheral nerves, or (iv) providing immunosuppression during recovery of the patient. Furthermore, T-cells may be activated because of the antecedent infection and be unrelated to the peripheral nerve damage.

The high concentration or activity of interleukin-6, γ-interferon, and tumor necrosis factor α in serum of GBS patients compared to patients with non-inflammatory neurological diseases and healthy subjects, further indicates that the immune system is activated in the acute phase of GBS (56-58). Transforming growth factor β, an immuno-

suppressive cytokine, peaks before onset of recovery, suggesting that it has a role in terminating the immune response in GBS (59).

The therapeutic effect of plasma exchange in GBS patients suggests that humoral factors may play a role in the pathogenesis. Whether this effect can be attributed to the removal of cytokines, antibodies, complement or other factors, is presently unknown. Several findings indicate that antibodies against peripheral nerves are involved in the pathogenesis of GBS. Firstly, immunohistochemical and electronmicroscopical studies demonstrated deposits of immunoglobulins along the myelin sheath of peripheral nerves in a proportion of GBS patients, although they were also identified in patients without neurological diseases (24,60). However, the presence of activated complement factors in nerves of controls is rare, suggesting that antibody depositions may result in complement activation in GBS patients only (32,45). Secondly, immunoglobulins purified from plasma of MFS patients, but not from normal and disease controls, interfere with neuromuscular transmission, possibly by blocking acetylcholine release (61,62). Immunoglobulin fractions from GBS patients have not been tested so far. Thirdly, antibodies to peripheral nerve myelin (63), many specific myelin glycoproteins, including myelin-associated glycoprotein, P0 and P2 (64,65), and glycolipids, including neutral (66), sulphated (67), and acidic glycolipids (68), were reported in serum of different subgroups of GBS patients. However, most of these antibodies were also found in patients with other diseases and normal controls, and their pathogenicity could not be demonstrated in passive transfer experiments. Recently, antibodies against peripheral nerve gangliosides were identified in a subgroup of GBS patients (reviewed in 1.3). Interestingly, some of these antibodies are associated with clinical manifestations in GBS patients and induce pathophysiological effects in animal models.

Is GBS an autoimmune disease?

The observations in histological and serological studies may support an immune mediated pathogenesis of GBS. This parallels the situation in other disorders like rheumatoid arthritis, multiple sclerosis, and myasthenia gravis, which are generally classified as 'autoimmune diseases'. These diseases are presumed to be characterized by the failure of natural tolerance of the immune system against autoantigens, resulting in an immune response against autoantigens and subsequent tissue damage and functional loss. However, most autoimmune diseases show a strong association with a specific age of onset, female sex, family, HLA haplotype, the presence of other autoimmune diseases, and a relapsing-remitting course of disease, none of which are evident in GBS. Therefore one may ask whether GBS can be considered as an autoimmune disease. This term is not unequivocal since it may refer to distinct afferent and efferent parts of the autoimmune response. The findings reported in the previous two paragraphs may indicate that the efferent part of the immune response, in which components of the immune system selectively damage the tissue, actually takes place in GBS. However, the afferent part of the immune response in GBS patients is probably not primarily induced by the peripheral nervous tissue. This is the case only in an animal model for GBS, the experimental autoimmune neuritis, in which animals are immunized with peripheral nerve extracts and develop an inflammatory polyneuropathy. In GBS patients the immune response inducing peripheral nerve damage is more likely triggered by infections or vaccinations which frequently precede the develop-

ment of GBS, as will be discussed in the next paragraph. According to this hypothesis GBS would be classified as an immune mediated polyneuropathy which is induced by exogenous antigens. One has to keep in mind, however, that definite proof is lacking, and that GBS is a heterogeneous disorder in which the (immuno)pathogenesis may differ between patients.

1.2 *CAMPYLOBACTER JEJUNI* AND OTHER ANTECEDENT INFECTIONS IN GBS

The spectrum of antecedent infections in GBS

GBS is considered to be a prototype of a 'postinfectious disease' since approximately two-third of patients have some form of preceding infectious illness. A remarkable diversity of infectious agents has been identified (27,69). *Campylobacter jejuni* and cytomegalovirus (CMV) are common causes of these infections, and their association with GBS has been established in large case-control studies (70-72). In addition, infections with *Mycoplasma pneumoniae* and Epstein-Barr virus (EBV) are frequently reported in GBS (73,74), although a significant association with GBS could not be demonstrated in a large case-control study (70). GBS has also been described in association with human immunodeficiency virus (HIV) infection (75). Interestingly, GBS is usually encountered in patients with recent seroconversion, but not in patients with manifest immunodeficiency (75). The GBS is distinct from the chronic painful neuropathy which frequently develops in patients with AIDS. Some patients have developed GBS after infections with other agents, like herpes simplex virus, varicella zoster virus, hepatitis A and B virus, measles virus, respiratory syncytial virus, influenza A and B virus, *Haemophilus influenzae 1b*, *Borrelia burgdorferi*, *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Streptococcus pneumoniae*, *Toxoplasma gondii*, and many others (27,69). Most of these micro-organisms were reported in case-reports, indicating that they are rare in GBS and may represent coincidental findings. In general, the incidence of most infections has not been determined in a large group of GBS patients and has not been compared with an adequate control group.

The time interval between the onset of infectious illness and the first neurological manifestations of GBS is one to three weeks in most patients (6,27,70,76). The clinical presentation of the infectious illness in most GBS patients suggests a respiratory or gastrointestinal infection, and is not evidently different from other patients with these infections. The infections are usually self-limiting and most patients have already recovered spontaneously from this disease before the onset of neurological symptoms.

Other antecedent events consistently reported in GBS are vaccinations and surgery (27). The association with surgery may reflect the increased risk of infection during the post-operative period, with wounds, transplantations, and blood transfusions as possible routes of infection. The frequency of infections in GBS may even be underestimated: some infections have a subclinical course, others are difficult to diagnose because they precede GBS by several weeks. Therefore, one could speculate that infections and vaccinations are a general triggering mechanism in the pathogenesis of GBS.

Campylobacter jejuni infections in GBS

Campylobacter jejuni is a micro-aerophilic, Gram-negative, curved rod (77). Since the improvement of selective culture techniques for the isolation of the bacteria from the stools in 1977 (78), *C. jejuni* is identified as the most frequent cause of bacterial gastroenteritis in developed countries (79). Common sources of infection include poultry, raw milk, and contaminated water. *C. jejuni* infections often lead to diarrhoea, abdominal pain, blood and mucus in the stools, and constitutional upset, but may frequently have a subclinical course. Extra-intestinal complications of *C. jejuni* infection such as reactive arthritis, cholecystitis, pancreatitis, conjunctivitis, and carditis are well described (80). The infection is usually self-limiting and most patients show a rapid recovery after treatment with erythromycin or equivalent antibiotics. Stool culture is required for definitive diagnosis of infection. The presence of serum IgM, IgA, and/or high titres of IgG antibodies against *C. jejuni* is strongly supportive for an acute infection (79,81).

In 1982, shortly after the development of adequate culture systems, Rhodes and Tattersfield first reported the isolation of *C. jejuni* from the stools of a patient with GBS (82). Since then, *C. jejuni* infections were frequently identified in GBS patients by culture or serology in case-reports and small series (Table 1). Because the mean period of bacterial excretion after onset of diarrhoea is 16 days (83), stool cultures are often negative at neurological onset of GBS. Therefore, studies based on stool culture alone will underestimate the frequency of previous *C. jejuni* infections in GBS. In the first serological study on a larger series of patients, Kaldor and Speed found evidence for a recent infection in 21 (38%) of 56 GBS patients but in none of 30 normal controls (84). Other culture and serological studies confirmed this high percentage of *C. jejuni* infections in GBS patients compared to controls (Table 1). These studies indicate that *C. jejuni* is a major cause of antecedent infections in GBS worldwide, involving Europe, North-America, Japan, Australia, and northern China. The clinical manifestations of the *C. jejuni* infection in GBS patients were not evidently different from other patients with this infection. Erythromycin treatment of the antecedent *C. jejuni* infection did not prevent the development of GBS, although this was reported only in a small group of patients (82,85-89).

It is presently unknown whether *C. jejuni* infections more frequently lead to GBS than infections with other agents. Alternatively, GBS is not associated with specific infections, indicating that the predominance of *C. jejuni* infections in GBS follows from the high frequency of this infection in the community.

Controversy exists as to whether GBS patients with a *C. jejuni* infection are a clinical and pathological subgroup distinct from GBS patients without this infection (Table 1). Some reports suggest that patients with *C. jejuni* infection suffer from a more severe form of GBS (84,90,91), with less sensory deficit (72,90,92), more extensive axonal degeneration (90,91), or poorer outcome (72,90,91). In the endemic variant of GBS in northern China, *C. jejuni* infections are more frequent in patients with AMAN than AIDP (43). However, several studies on GBS patients from Europe and North-America were unable to demonstrate an association between *C. jejuni* infections and this clinical and electrodiagnostic pattern (76,86,89,93,94).

The issue may be complicated by the possible association of *C. jejuni* infections with more than one clinical subgroup in GBS. *C. jejuni* was also isolated from stool

TABLE 1. Recent *Campylobacter jejuni* infections in GBS patients: association with axonal degeneration, clinical patterns and outcome [a].

Reference	Frequency [b]		Axonal [d]	Clinical association	Poor outcome [e]
Case-reports					
Rhodes, 1982 (82)	1 [c,s]		+	pure motor	+
Molnàr, 1982 (237)	1 [c]		+	nt	+
Constant, 1983 (85)	1 [c]		nt	MFS	—
Pryor, 1984 (86)	1 [c]		—	pure motor	—
	1 [c]		—	MFS	—
Wroe, 1985 (93)	1 [c]		—	MFS	—
De Bont, 1986 (238)	1 [c,s]		+	severe weakness	—
Kohler, 1987 (87)	1 [c]		nt	nt	+
Sovilla, 1988 (89)	3 [c,s]		—	—	—
Ropper, 1988 (88)	1 [c]		+	severe, pure motor	+
	3 [c]		—	—	—
Yuki, 1990 (90)	2 [s]		+	severe, pure motor	+
Kuroki, 1991 (101)	7 [c,s]		—	—	—
Yuki, 1991 (235)	6 [s]		+	pure motor	nt
Duret, 1991 (239)	1 [s]		+	pure motor	—
Yuki, 1992 (107)	13 [c,s]		nt	nt	nt
Fujimoto, 1992 (104)	4 [c]		nt	nt	nt
Frequency studies					
Kaldor, 1984 (84)	21/56	(38%) [s]	nt	severe weakness	—
Speed, 1987 (240)	22/45	(49%) [s]	nt	nt	nt
Winer, 1988 (70)	14/99	(14%) [s]	nt	nt	+
Walsh, 1991 (180)	14/94	(15%) [s]	+	nt	nt
Gruenewald, 1991 (241)	3/17	(18%) [s,c]	nt	nt	nt
Boucquey, 1991 (76)	6/42	(14%) [s]	nt	—	—
Nobile-Orazio, 1992 (179)	3/16	(19%) [s]	nt	nt	nt
Gregson, 1993 (178)	15/42	(36%) [s]	nt	nt	nt
Kuroki, 1993 (105)	14/46	(30%) [c]	nt	nt	nt
Enders, 1993 (94)	15/38	(39%) [s]	—	—	—
Vriesendorp, 1993 (91)	10/58	(17%) [s]	+	severe weakness	+
Mishu, 1993 (71)	43/118	(36%) [s]	nt	nt	nt
Von Wulffen, 1994 (242)	11/42	(26%) [s]	nt	nt	nt
Rees, 1995 (72)	25/96	(26%) [c,s]	+	pure motor	+
	2/7	(29%) [c]	nt	MFS	nt
Ho, 1995 (43)	25/38	(66%) [s]	+	—	—
Jacobs, 1996 (214,243)	46/154	(32%) [s]	nt	severe, pure motor	+ after PE — after IVIg

a. Abbreviations: nt, not tested; +, present; –, absent; MFS, Miller Fisher syndrome; PE, plasma exchange; IVIg, intravenous immunoglobulins.
b. Number of patients in case-reports, or number of *C. jejuni* infected patients per number of tested patients in frequency studies.
c. Determined by stool culture.
d. Association with severe axonal degeneration.
e. Determined at least three months after diagnosis of GBS.
s. Determined by serology.

specimens from patients with MFS (95-99) and a patient with isolated abducens paresis (100). In addition, *C. jejuni* infections were reported in GBS patients with ophthalmoparesis who made a rapid recovery (85,86,101). These findings indicate that *C. jejuni* infections may not only be related to pure motor GBS, but also to the cranial nerve variants.

Some reports suggest that only specific *C. jejuni* serotypes are associated with GBS. *Campylobacter* bacteria are classified according to two serotyping systems. The heat-stable antigen typing of Penner (PEN) is based on the lipopolysaccharide (LPS) portion of the bacteria (102), and the heat-labile antigen typing of Lior (LIO) is based on the flagellar protein (103). In Japan, the majority of isolates from GBS patients have the PEN O:19 serotype (101,104,105), an uncommon serotype in that region (105). Also a family outbreak of GBS with *C. jejuni* PEN O:19 was reported in Japan (106). However, *C. jejuni* with this serotype was rarely isolated from GBS patients from Europe, indicating that there may be geographical differences. Other serotypes that have been reported in GBS patients are PEN O:1, O:2, O:4,59, O:24, and O:64 (72,105, 107-109). *C. jejuni* isolates from patients with MFS had the PEN O:2, O:4/50, O:23 and O:10 serotype (98,99,110). Several isolates from GBS and MFS patients were not classifiable according to the present Penner system (72,98). In addition, some studies indicate that the GBS associated isolates also have a specific Lior serotype. *C. jejuni* isolates from GBS patients in Japan are usually LIO:7, but LIO:27 and in MFS patients LIO:4 were also reported (97,104,107). Antibodies against the outer membrane proteins of the LIO:11 serotype were frequently found in serum from German GBS patients with *C. jejuni* infections (94).

Several mechanisms have been proposed by which *C. jejuni* infections may play a role in the development of GBS. Firstly, nerve damage may be induced by a *C. jejuni* enterotoxin which is homologous with cholera toxin (111,112) and binds with gangliosides in human peripheral nerves (113,114). However, it is unlikely that these toxins have a direct effect on peripheral nerves since they are rarely absorbed into the circulation and GBS does not develop during *C. jejuni* infection, but one to three weeks later. Secondly, *C. jejuni* infections may trigger a transient immune response against peripheral nerve antigens. IgM, IgG and IgA antibodies against *C. jejuni* generally peak at two weeks after infection (79), and possibly reflect the time lag between the infection and the first neurological symptoms. The mechanisms by which *C. jejuni* infections may induce anti-neural antibodies are further discussed in paragraph 1.4.

In conclusion, *C. jejuni* is the predominant cause of antecedent infections in GBS. However, it is presently unclear whether (i) *C. jejuni* infections are specifically associated with GBS or reflect the high frequency in the community, (ii) *C. jejuni* infections are associated with distinct clinical subgroups in GBS, and (iii) by which mechanism *C. jejuni* infections may contribute to the pathogenesis of GBS.

1.3 ANTI-GANGLIOSIDE ANTIBODIES IN GBS

Gangliosides were first identified as peripheral nerve targets for antibodies in patients with paraproteinaemic neuropathy (115-117). These findings stimulated the research on anti-ganglioside antibodies in other neurological and autoimmune dis-

eases. Thus far, the highest titres of anti-ganglioside antibodies were demonstrated in patients with various forms of inflammatory neuropathy.

Gangliosides

Gangliosides are glycosphingolipids consisting of a ceramide moiety and a sialic acid containing oligosaccharide. Since both the ceramide and the oligosaccharide moiety have variable structures, the gangliosides form an extensive family of different molecules (Appendix 2). Gangliosides are predominantly present at the cell surface with the hydrophobic ceramide portion anchored in the external leaflet of the plasma membrane and with the hydrophilic oligosaccharide exposed extracellularly (118). Gangliosides are ubiquitous in plasma membranes of vertebrate organisms but the local density of specific gangliosides highly depends on the tissue, species and age of the organism (119,120). The expression of gangliosides at the cell surface also depends on the functional status of the cells (121).

Gangliosides are particularly abundant in neuronal plasma membranes (122). The predominant gangliosides in human peripheral nerve myelin are LM1, GM3, GD1b, and GD3 (123,124) and in axons GM1, GD1a, GD1b, and GT1b (Appendix 2) (125). Some gangliosides show a preference for specific subsets of nerve fibres. The concentration of GM1 and GD1a is higher in the myelin of human motor nerves than of sensory nerves (124). GQ1b is predominantly found in human oculomotor nerves, which are involved in eye motility (126). In addition to these major gangliosides, many different gangliosides are present in low concentrations in peripheral nerves (121). The structure and tissue distribution of most of these minor gangliosides have not been determined.

Due to the structural diversity, ubiquitous distribution and strategic localization, gangliosides are suggested to play a role in various signal recognition and transduction processes at the plasma membrane (reviewed by Hakomori) (121). Gangliosides are presumed to function as receptors in cell-cell recognitions, including immunomodulation, cell growth and differentiation (121), and in binding of toxins, like cholera toxin, tetanus toxin and botulinum toxin (127). They also appear to serve as membrane anchors for membrane-bound functional proteins, and to modulate their activity. Interestingly, gangliosides stimulate neuronal sprouting, enhance the formation of neuromuscular junctions and induce neuronal regeneration in animal models of peripheral neuropathy (128). The particular function of most gangliosides in peripheral nerves, however, is presently unknown.

Detection of anti-ganglioside antibodies

Many studies investigating the incidence of anti-ganglioside antibodies in serum of different groups of patients have been published in the last decade. These studies report a remarkably wide range of frequencies both in patients with a large diversity of disorders and in healthy subjects. This variability is presumably related to the selection of patients and to differences in the techniques used to detect anti-ganglioside antibodies. These antibodies are usually detected by enzyme-linked immunosorbent assays (ELISA) and thin-layer chromatography (TLC) overlay. The binding characteristics of antibodies to gangliosides in ELISA were shown to be highly influenced by the temperature and by the presence of accessory lipids (129,130). Studies comparing

the ELISA results from different laboratories showed a broad variation with respect to samples with low titres of anti-ganglioside antibodies (131,132). A meta-analysis showed that the reported frequency of anti-GM1 antibodies highly depends on the use of detergents and the incubation time of the serum (133). The frequency of anti-ganglioside antibodies also depends on the definition of the cut-off values used to define positive and negative samples. The detection of anti-ganglioside antibodies is further complicated by contamination of the commercial ganglioside preparations with other glycolipids. To exclude antibody activity against these contaminants it is necessary to confirm the positive ELISA results in TLC overlay. Recently, an international concerted action (INCAT) was undertaken to standardize the assays for the detection of anti-ganglioside antibodies.

Anti-ganglioside antibodies recognize the oligosaccharide portion of the gangliosides. This was demonstrated by the cross-reactivity of anti-ganglioside antibodies with gangliosides and other glyco-conjugates with homologous oligosaccharide moieties. Anti-GM1 antibodies frequently cross-react with GD1b and asialo-GM1, suggesting that they bind to the Gal(β1-3)GalNAc-structure which these glycolipids have in common (see Appendix 2) (134). The Gal(β1-3)GalNAc-structure is also wide-spread in glycoproteins (135), and human monoclonal anti-GM1 antibodies cross-react with glycoproteins in peripheral nerve extracts (136,137). It is therefore unknown whether anti-ganglioside antibodies bind with gangliosides or other target structures in peripheral nerves. Consequently, antibody activity against gangliosides in ELISA and TLC-overlay may not necessarily reflect activity against peripheral nerve antigens.

Anti-ganglioside antibodies in polyneuropathies other than GBS

Antibodies against GM1 and against the disialosyl moiety in gangliosides like GD1b, GD3, GT1b, and GQ1b are consistently associated with specific forms of neuropathy and their role in pathogenesis has been studied by passive transfer experiments and active immunizations.

(1) Antibodies to GM1

High titres of anti-GM1 antibodies are predominantly found in patients with chronic motor neuropathies and lower motor neuron syndromes (138,139). These antibodies may be monoclonal or polyclonal, and are usually of the IgM isotype. Anti-GM1 antibodies are especially common in patients with multifocal motor neuropathy (MMN) (140,141). This chronic neuropathy is characterized by asymmetric limb weakness without sensory deficits. The electrodiagnostic hallmark of MMN is multifocal motor conduction block, indicating multifocal demyelination of motor nerves. The anti-GM1 antibodies are found in approximately half of the patients with MMN (139).

The clinical manifestations in patients with anti-GM1 antibodies partly reflect the tissue distribution of GM1 in human peripheral nerves. The concentration of GM1 is higher in the myelin of motor nerves compared to sensory nerves (124). In addition, human anti-GM1 antibodies bind with motor neurons and not with dorsal root ganglion neurons (142). Intraneural injections with cholera toxin, which recognizes GM1, showed specific staining at the nodes of Ranvier (143). Human anti-GM1 antibodies also preferentially bound at the node of Ranvier, to the nodal gap and paranodal myelin (144,145), and at the distal motor terminals at the end plate (145).

The pathogenicity of anti-GM1 antibodies has been investigated in experimental animal studies. Intraneural injections of anti-GM1 antibodies from patients with MMN in rat sciatic nerves induced a complement dependent demyelination with conduction block (146), although this was not confirmed by others (147). In the mouse phrenic nerve/diaphragm preparation it was demonstrated that sera from MMN patients induce distal motor nerve conduction block in the absence of complement (148). Furthermore, rabbit anti-GM1 antisera without active complement interfered with voltage sensitive potassium current in rat nerve fibres (149). In presence of active complement these antisera decreased sodium current, and caused a progressive increase of non-specific leakage current (149). Rabbits immunized with GM1 developed anti-GM1 antibodies which also bind at the nodes of Ranvier (150,151). These rabbits developed a subclinical neuropathy characterized by impairment of nerve conduction and mild axonal changes (150,151). Together, these data indicate that anti-GM1 antibodies are likely to play a role in failure of motor nerve conduction in MMN, presumably by binding at the nodes of Ranvier. Their mechanism of action is unknown, but they may induce subtle structural alterations in axons or paranodal myelin, or prevent generation of action potentials by interfering with the function of sodium channels which are also concentrated at the nodes of Ranvier (152).

The site of action of the anti-GM1 antibodies will be determined by their fine-specificity and by the distribution of the targets in peripheral nerves. However, sera from patients with MMN contained heterogeneous populations of anti-GM1 antibodies with different fine-specificities to GM1 and structurally related molecules (130,141). O'Hanlon et al. reported that monoclonal anti-GM1 antibodies from MMN patients showed a broad and diverse binding pattern with the rodent peripheral nerve system (153). This heterogeneity in fine-specificity and subsequent localization of the binding to peripheral nerves may account for the clinical differences found in patients with anti-GM1 antibodies.

(2) Antibodies to GQ1b and other disialylated gangliosides

Monoclonal IgM antibodies against gangliosides with the NeuNAc(α2-8)NeuNAc disialosyl moiety, like GD1b, GD3, GT1a, GT1b, and GQ1b (see Appendix 2), are found in patients with a chronic large fibre sensory neuropathy and paraproteinaemia (154-160). These patients are characterized by profound ataxia, loss of proprioception and vibration sense, and a remarkable preservation of muscle strength. The lesions are located in the dorsal root ganglia, resulting in loss of the large ganglionic neurons and axonal degeneration of large sensory nerves (161). In addition, the patients variably suffer from ophthalmoplegia, and may have cold agglutinins due to activity of the monoclonal antibodies against sialylated structures on red blood cells. Therefore, the syndrome has recently been reported under the acronym CANOMAD (chronic ataxic neuropathy with ophthalmoplegia, M-protein, agglutination and disialosyl antibodies) (162).

The clinical and pathological features in these patients are partly in accordance with the tissue distribution of the disialylated gangliosides. GD1b is present on the cell surface of human dorsal root ganglion neurons (163), and GQ1b is predominantly found in the myelin of human oculomotor nerves (126). Immunohistochemical studies with the anti-disialosyl paraprotein from these patients show that the antibodies bind

with rodent dorsal root ganglia, but also with motor nerve terminals, muscle spindles, myelinated axons, and nodes of Ranvier (162). The selective involvement of dorsal root ganglia in patients will therefore depend on other factors, like the blood-nerve barrier (BNB) which is naturally deficient at the dorsal roots (164).

The paraproteins may have a complement independent cytotoxic effect, as was demonstrated by the death of neurons in a rat dorsal root ganglion cell culture after application of the anti-disialosyl paraprotein purified from a patient with sensory ataxic neuropathy (165). In addition, these antibodies impair nerve excitability and the release of neurotransmitter in the mouse phrenic nerve/diaphragm preparation, suggesting that these antibodies may also directly interfere with structures essential for nerve conduction or neuromuscular transmission (162). Rabbits immunized with GD1b produce anti-GD1b antibodies and develop a selective loss of dorsal root ganglionic neurons with clinical manifestations closely resembling those in patients: severe ataxia, but with intact superficial sensation and muscle strength (166). No cellular infiltrations are found in the affected tissues, further supporting the hypothesis that the neural damage is antibody-mediated. Experiments with passive transfer of the antibodies to other animals, however, were not performed.

(3) Antibodies to other gangliosides

High titres of antibodies against several other gangliosides have been reported in patients with many forms of neuropathy, but their association with clinical manifestations is not elucidated. A single study on antibodies against GD1a reports that these antibodies are related to a severe motor neuropathy (167). Interestingly, the highest concentrations of GD1a in human peripheral nerves are found in axons (125), and in myelin of motor fibres (124). Although rabbits immunized with GD1a developed a flaccid paralysis, serum anti-GD1a antibodies were not detected (150). High titres of anti-ganglioside antibodies are also consistently reported in patients with CIDP, although these antibodies are not restricted to one specificity. Instead, antibodies against LM1, GM1, GM2, GM3, GD1a, GD1b and GT1b have all been reported in variable proportions of CIDP patients (68,168-173). The relation between specific anti-ganglioside antibodies and clinical subgroups of CIDP patients, however, is presently unknown.

Together these studies indicate that the clinical manifestations of some neuropathies are related to the fine-specificity of the anti-ganglioside antibodies. The site of action of these antibodies will depend on the local distribution of gangliosides or other targets in the peripheral nervous system. However, gangliosides at the cell surface are frequently shielded and not available for antibody binding (121). The site of action will also depend on the accessibility for the antibodies from the blood. The role of the BNB is presently unclear. The BNB is formed by tight junctions between endothelial cells from blood vessels near the peripheral nerves and prevents the passage of large proteins (164). The BNB is normally deficient at the spinal nerve roots and terminal intramuscular nerve twigs and may also be bypassed by the uptake of immunoglobulins at the peripheral nerve terminals. In addition, the BNB is likely to become more deficient in GBS, as may be indicated by the high albumin concentration in cerebrospinal fluid.

Anti-ganglioside antibodies in GBS

In 1988, Ilyas et al. first reported the presence of antibodies against gangliosides in the serum from patients with GBS (174). Since then, antibodies against a variety of gangliosides like LM1, GM1, GM1b, GalNAc-GM1b, GM2, GM3, GD1a, GalNAc-GD1a, GD1b, GD3, GT1a, GT1b, and GQ1b have been detected in different proportions of GBS patients (Table 2). The variety of anti-ganglioside antibodies in GBS contrasts with the distinct specificities found in patients with MMN and CANOMAD. This may indicate that GBS consists of different pathological subgroups with distinct patterns of anti-ganglioside antibody specificities. Preliminary studies suggest that some anti-ganglioside antibodies are associated with the clinical manifestions in GBS patients.

(1) Antibodies to GM1

The first evidence for the clinical relevance of anti-ganglioside antibodies in GBS came from reports on anti-GM1 antibodies. Many studies have demonstrated anti-GM1 antibodies in the serum of GBS patients, although the reported frequency is highly variable (0-78%) (Table 2). The presence of anti-GM1 antibodies was found to be associated with a more severe (90, 134,172,175,176), pure motor variant of GBS (90,92,175-178), with more extensive axonal degeneration (90,175,176,178,179) and poor recovery (68,90,134,172,175,178,180-182) (Table 3). The prominent motor involvement is also found in other forms of neuropathy associated with anti-GM1 antibodies, and is partly in accordance with the distribution of GM1 in the peripheral nervous system (as discussed in the previous paragraph). The same clinical pattern is associated with *C. jejuni* infections, which show a significant overlap with anti-GM1 antibodies in GBS in some studies (Table 3).

TABLE 2. Spectrum of anti-ganglioside antibodies in serum from GBS patients.

Ganglioside	Frequency [a]		Number tested [a]	References
	Mean	Range		
LM1	26%	4 - 58%	286	43,68,123,168,174,244
GM1	23%	0 - 78%	819	43,68,91,94,123,134,172,174-176,178-182, 213,214,242
GM1b	31%	21 - 40%	203	215,217
GM2	9%	0 - 16%	224	63,68,134,245,246
GM3	3%	-	29	245
GD1a	10%	0 - 30%	412	43,68,167,174,180,181,187,188,245,246
GalNAc-GD1a	13%	12 - 14%	108	215,217
GD1b	21%	2 - 77%	539	43,68,91,123,134,174,180,215,242,245,246
GD3	6%	-	51	246
GT1b	6%	2 - 13%	209	68,123,174,245,246
GQ1b	10%	0 - 21%	289	126,176,183-186,247

a. As determined in published frequency studies (case studies were not included).

TABLE 3. Anti-GM1 antibodies in patients with GBS: association with *C. jejuni* infections, axonal degeneration, clinical patterns and outcome [a]

Reference	Frequency [b]		Isotype	*C. jejuni* [c]	Axonal [d]	Clinical association	Poor outcome [e]
Case-reports							
Yuki, 1990 (90)	2		IgG	+	+	severe, pure motor	+
Gregson, 1991 (177)	1		IgG,IgM	nt	+	pure motor	–
Frequency studies							
Ilyas, 1988 (174)	0/26	(0%)	IgG,IgM	nt	nt	nt	nt
Svennerholm, 1990 (123)	1/50	(2%)	IgG,IgM	nt	nt	nt	nt
Walsh, 1991 (180)	14/95	(15%)	IgG,IgM	+	+	–	+
Van den Berg, 1992 (175)	4/22	(18%)	IgG,IgM,IgA	nt	+	severe, pure motor	+
Ilyas, 1992 (134)	15/53	(28%)	IgA	nt	nt	severe weakness	+
Ilyas, 1992 (68)	10/53	(19%)	IgG,IgM	nt	nt	–	+
Nobile-Orazio, 1992 (179)	5/16	(31%)	IgG,IgM	+	+	severe, more acute	–
Simone, 1993 (172)	10/23	(43%)	IgG,IgM	nt	nt	severe weakness	+
Willison, 1993 (176)	2/20	(10%)	IgG	nt	+	severe weakness	nt
Yuki, 1993 (181)	8/37	(22%)	IgG	nt	nt	–	+
	4/37	(11%)	IgM	nt	nt	–	–
Gregson, 1993 (178)	12/42	(29%)	IgG,IgM	+	+	pure motor	+
Vriesendorp, 1993 (91)	5/58	(9%)	IgM	–	–	–	–
Enders, 1993 (94)	6/32	(19%)	IgG,IgM,IgA	–	–	–	–
Seiser, 1994 (182)	4/15	(27%)	IgG,IgM,IgA	nt	nt	–	+
Von Wulffen, 1994 (242)	33/42	(78%)	IgG	+	nt	nt	nt
	20/42	(48%)	IgM	+	nt	nt	nt
	24/42	(56%)	IgA	+	nt	nt	nt
Ho, 1995 (43)	16/38	(42%)	IgG	–	–	–	–
Rees, 1995 (213)	24/96	(25%)	IgG,IgM,IgA	+	–	motor	–
Jacobs, 1996 (214), 1997(247)	31/154	(20%)	IgG,IgM,IgA	+	partly	acute, severe, pure motor, no cranial nerve involvement	+ after PE – after IVIg

a. Abbreviations: nt, not tested; +, present; –, absent; PE, plasma exchange; IVIg, intravenous immunoglobulins.
b. Number of patients in case-reports, or number of patients with anti-GM1 antibodies per number of tested patients in frequency studies.
c. Association with recent *C. jejuni* infection.
d. Association with severe axonal degeneration.
e. Determined at least 3 months after diagnosis of GBS.

However, recent reports from others were unable to demonstrate an association between anti-GM1 antibodies, *C. jejuni* infections and clinical and electrodiagnostic subgroups (Table 3) (43,91,94). The controversy is probably related to the small number and selection of patients, differences in techniques used to detect anti-ganglioside antibodies, time of serum sampling, and accuracy of the clinical and electrodiagnostic data. Furthermore, anti-ganglioside antibodies may differ with respect to the titre, isotype, and fine-specificity, which were not defined in most of these studies.

(2) Antibodies to GQ1b

The presence of anti-GQ1b antibodies is highly associated with the MFS variant of GBS (126,176,183-186). In 117 (96%) of the 122 reported MFS patients anti-GQ1b antibodies were demonstrated (Table 4). Anti-GQ1b antibodies were also reported in a proportion of GBS patients and were found to be associated with ophthalmoplegia (Table 4) (126,185). The association between ophthalmoplegia and anti-GQ1b antibo-

TABLE 4. Anti-GQ1b antibodies in patients with GBS-variants and GBS: association with clinical patterns [a].

Reference	Frequency [b]	Isotype	Clinical association
GBS-variant patients			
Chiba, 1992 (183)	6	IgG	MFS
Yuki, 1993 (184)	13/16 (81%)	IgG,IgM	severity of ataxia in MFS
Willison, 1993 (176)	4	IgG,IgM	MFS
Chiba, 1993 (126)	18/19 (95%)	IgG	MFS
	5	IgG	acute ophthalmoparesis
Willison, 1994 (129)	9	IgG1/3,IgM,IgA	MFS
Roberts, 1994 (61)	4	nt	MFS
Hammers, 1994 (248)	1	nt	MFS
Jacobs, 1995 (98)	3	IgG,IgM	MFS
Buchwald, 1995 (62)	1	IgG,IgM	MFS
Yuki, 1996 (185)	45/46 (98%)	IgG1/3,IgM,IgA	MFS
	8	IgG1/3,IgM	acute ophthalmoparesis
O'Leary, 1996 (186)	13/13 (100%)	IgG1/3	MFS
	3	IgG1/3	acute oropharyngeal palsy
GBS patients			
Chiba, 1992 (183)	0/16 (0%)	IgG	nt
Yuki, 1993 (184)	1/15 (7%)	IgG,IgM	abducens paresis
Willison, 1993 (176)	2/20 (10%)	IgG	nt
Chiba, 1993 (126)	5/29 (17%)	IgG	ophthalmoplegia
Yuki, 1996 (185)	16/85 (21%)	IgG1/3,IgM,IgA	ophthalmoplegia
Jacobs, 1997 (247)	5/124 (4%)	IgG,IgM,IgA	ophthalmoplegia, inexcitable sensory nerves

a. Abbreviations: nt, not tested; MFS, Miller Fisher syndrome.
b. Number of patients in case-reports or number of patients with anti-GQ1b antibodies per number of tested patients in frequency studies.

dies is further strengthened by the presence of these antibodies in patients with acute ophthalmoparesis without areflexia, ataxia and myasthenia gravis (126,185). However, anti-GQ1b antibodies were also recently reported in patients with ataxia, areflexia, and lower cranial nerve involvement without ophthalmoparesis (186).

Most anti-GQ1b positive patients with GBS and MFS have additional antibody activity against GT1a, while half of the patients have additional antibody activity against GD1b and GD3. The specificity of these antibodies partly overlaps with those in patients with CANOMAD, which react with all disialylated gangliosides. In addition, patients with MFS and CANOMAD both have ophthalmoplegia and ataxia. The clinical difference between these forms of neuropathy may be related to the difference in isotype, titre course and fine specificities of the anti-GQ1b antibodies.

Recently it was shown that serum with anti-GQ1b antibodies from MFS patients interferes with neuromuscular transmission in the mouse phrenic nerve/diaphragm preparation, probably by blocking the acetylcholine release at the motor nerve terminals (61,62). The same results were found with immunoglobulin fractions from these patients, although it is presently unknown if the effects can be attributed to the anti-GQ1b antibodies. Further studies have demonstrated that the presence of complement is essential to induce these pathophysiological effects (dr. J.J. Plomp, personal communication).

(3) Antibodies to other gangliosides

Yuki et al. reported that anti-GD1a antibodies in GBS are related to a more severe, motor variant with axonal involvement and poor prognosis (181,187), but this was not confirmed by others (43,94). One patient with anti-GD1a and anti-GT1a antibodies was reported to have selective involvement of the pharyngeal-cervical-brachial motor nerves (188). Antibodies against many other gangliosides in patients with GBS were reported, but their clinical relevance could not be demonstrated or was not investigated.

In conclusion, these recent studies give some support to the hypothesis that anti-ganglioside antibodies partly determine the clinical and electrodiagnostic manifestations in GBS. The clinical and pathological heterogeneity in GBS may therefore partly be explained by the spectrum of anti-ganglioside antibodies. However, the data so far are preliminary and inconclusive since they were obtained in selected and small groups of patients which were tested for antibodies against one or a limited number of gangliosides. Detailed studies on the clinical relevance of antibodies against most abundant peripheral nerve gangliosides, like LM1 and GD1b, are presently not available.

1.4 INDUCTION OF ANTI-GANGLIOSIDE ANTIBODIES BY ANTECEDENT INFECTIONS IN GBS

Naturally occurring anti-ganglioside antibodies

Serum anti-ganglioside antibodies are not restricted to patients with GBS and other neuropathies. Instead, antibodies against various gangliosides, like LM1, GM1, GM2, GD1a, and GD1b, have also been reported in healthy subjects (68,123,168,189-192). Normal peripheral blood B-cells, including naive lymphocytes from umbilical cord,

could be stimulated to secrete anti-GM1 antibodies *in vitro* (193-195). Antibodies against a wide range of other autoantigens are also present in healthy persons (196,197), and anti-ganglioside antibodies probably belong to this normal repertoire of autoantibodies. Most naturally occurring autoantibodies are of the IgM isotype, have low titres, appear to have low affinity and are polyreactive with a variety of self and microbial antigens (198). These antibodies are predominantly produced by $CD5^+$ B-cells which generally have a germline configuration of genes coding for the immunoglobulin hypervariable regions (198). This indicates that these B-cells were not triggered by specific antigens and have not undergone affinity maturation. These naturally occurring autoantibodies are presumably not pathogenic. Instead, they have been suggested to play a role in homeostasis by (i) disposing degraded autoantigens and senile cells, (ii) serving as a first line of defense against infectious agents, and (iii) modulating an ongoing specific antibody response (reviewed by Schwartz) (199).

Anti-ganglioside antibodies in patients with GBS and other neuropathies

Anti-ganglioside antibodies in patients with GBS, MFS, and other neuropathies differ from these naturally occurring autoantibodies in several aspects (Table 5). These anti-ganglioside antibodies have a higher titre, and show a restrictive reactivity, as indicated by the selective binding with specific gangliosides. In addition, anti-ganglioside antibodies in GBS patients are predominantly of the IgG1, IgG3 and IgA class, although IgM antibodies also occur. Anti-ganglioside antibodies in patients with CANOMAD and MMN are IgM, but V-gene sequencing studies of anti-GM1 B-cell lines, cloned from peripheral blood of these patients, show a high degree of somatic mutations in genes coding for the hypervariable regions of the immunoglobulins (162,200,201). The somatic mutations in CANOMAD and MMN, and the isotype-switch in GBS and MFS suggest that anti-ganglioside antibodies in patients with these neuropathies are induced by antigen-driven mechanisms with consequent affinity maturation. Serum anti-ganglioside antibodies from patients with MFS, CANOMAD, and MMN

TABLE 5. Differences between anti-ganglioside antibodies in normal controls and patients with MMN, CANOMAD, MFS and GBS [a].

Antibody characteristics	Normal	MMN	CANOMAD	MFS	GBS
Titre	low	high	high	high	high
Titre course	?	chronic	chronic	transient	transient
Predominant isotype(s)	IgM	IgM	IgM	IgG1/3	IgG1/3,IgA
Ganglioside specificity	many	GM1,GD1b	disialylated	GQ1b,GT1a	many
Clonality B-cells	poly	poly/mono	mono	poly	poly
Somatic mutations B-cells	?	+	+	?	+
Acute infection related	–	–	–	+	+
Pathophysiologic effect [b]	–	+	+	+	?

a. Abbreviations: MMN, multifocal motor neuropathy; CANOMAD, chronic ataxic neuropathy with ophthalmoplegia, M-protein, agglutination, and antibody activity against dislalylated gangliosides; MFS, Miller Fisher syndrome; GBS, Guillain-Barré syndrome; ?, unknown; poly, polyclonal; mono, monoclonal; +, present; –, absent.

b. As determined in the mouse phrenic nerve/diaphragm preparation.

have a pathophysiological effect on neuromuscular transmission and nerve conduction (61,62,148,162). This effect was not found with serum from healthy subjects. The pathogenicity of the anti-ganglioside antibodies may therefore be determined by the titre, isotype, and affinity of the antibodies, which all depend on antigen-driven mechanisms.

The antigens and immunological mechanisms responsible for the formation of long-lasting, high titres of IgG and IgA anti-ganglioside antibodies in patients with GBS, are presently unknown. Polysaccharide antigens are generally regarded as T-cell-independent antigens which directly stimulate B-cells by polyclonal activation, or specifically, by cross-linking the surface membrane immunoglobulins on B-cells (202). These triggers will stimulate B-cells to proliferate and differentiate into IgM secreting plasma cells without, or with very limited, switching to other immunoglobulin classes. Long-lasting high titres of IgG and IgA antibodies, on the other hand, are characteristic of a T-cell-dependent immune response. *In vitro* studies have shown that mitogen activated T-cells can stimulate the production of anti-ganglioside antibodies by B-cells derived from peripheral blood of GBS patients (195). T-cells may give help to B-cells by cognate interaction, whereby the antigen is captured by the surface membrane immunoglobulins, endocytosed, processed, and presented in association with the class II MHC molecules on the B-cell surface to helper T-cells (202). After recognition of this processed antigen-MHC complex by the T-cell receptor, T-cells will be activated and provide the contact and cytokine-mediated help necessary for B-cell class switching and affinity maturation. However, carbohydrate antigens presumably can not be intracellularly processed and presented by class II MHC molecules (203). Therefore, T-cells can not be activated in an MHC-restricted manner by pure gangliosides, but only by antigens with additional epitopes that can be expressed by the MHC and recognized by the T-cell receptor. These epitopes may be provided by glycoproteins with sialylated oligosaccharides, or by a carrier protein which binds to gangliosides (204). Alternatively, T-cells are activated in an MHC-unrestricted manner, as will be further discussed in Chapter 5.

Several antigens have been proposed to trigger the production of anti-ganglioside antibodies in GBS (Table 6). Some patients were reported who developed GBS within 2 weeks after parenteral administration of purified gangliosides from bovine brain (145,179,205-210). These gangliosides were used in the treatment of patients with various neurological diseases, based on their *in vitro* potential to promote nerve repair by enhancing nerve sprouting (128,211). Gangliosides were supposed to be not immunogenic in humans since daily parenteral injections to 12 patients for 3 months did not induce anti-ganglioside antibodies or neuropathy (123). However, in subsequent reports on patients who received ganglioside injections and developed GBS, high titres of IgM and IgG antibodies against gangliosides were demonstrated (145,179,206). Interestingly, these anti-ganglioside antibodies were not found in ganglioside treated patients who did not develop GBS (145). Epidemiological surveys found no significantly higher incidence of GBS in ganglioside treated subjects compared to controls (212), but the studied population was probably too small to demonstrate an association with this relatively rare disease. Therefore, it is presently unknown if parenteral injections of gangliosides are coincidentally associated with GBS or induce anti-ganglioside antibodies and trigger GBS in a small proportion of susceptible subjects.

TABLE 6. Possible mechanisms leading to high titre of anti-ganglioside antibodies in GBS.

Aspecific increase of naturally occurring autoantibodies	
Parenteral administration of gangliosides	
Peripheral nerve damage	
Antecedent infections or vaccinations	
aspecifically:	polyclonal B-cell activation
specifically:	molecular mimicry with gangliosides
	production of ganglioside-binding carrier proteins

Anti-ganglioside antibodies in GBS may also be formed secondarily by autoimmunization against gangliosides released from damaged peripheral nerves. Autoantibodies are frequently induced by non-immune mediated tissue damage, like the antibodies against myocardial antigens in patients with myocardial infarction (249). However, serum anti-ganglioside antibodies are already present on the first day of neurological symptoms and the titres invariably decline during the course of disease suggesting that they are produced before the neural damage. In addition, isotype switching occurs before neurological onset and not during follow-up. Moreover, high titres of IgG and IgA anti-ganglioside antibodies are not found in patients with peripheral nerve damage due to hereditary motor sensory neuropathy, trauma, or metabolic disorders like diabetes mellitus.

Antecedent infections may provide the antigenic stimulus for the production of anti-ganglioside antibodies in GBS, as was indicated by the significant association between *C. jejuni* infections and anti-GM1 antibodies (180,213,214). In addition, antibodies against other gangliosides like GM1b, GalNAc-GD1a, and GalNAc-GD1b are also more frequently present in GBS patients with *C. jejuni* infections than in those without (215-217). The antibody production against *C. jejuni* peaks one week after infection, which coincides with the delay between infection and neurological onset of GBS. It is known that infections stimulate the production of antibodies against many autoantigens, due to aspecific bystander activation of B-cells. However, *C. jejuni* infections are not associated with increased immunoglobulin levels or antibodies against other glycolipid antigens, suggesting that the immune response is specific and predominantly driven by *C. jejuni* antigens. *C. jejuni* infections may specifically induce anti-ganglioside antibodies by at least two possible mechanisms. Firsly, *C. jejuni* bacteria produce surface receptors or soluable toxins which bind to host gangliosides, and may function as carrier proteins (204). Secondly, these bacteria may express antigens which mimic neural structures. This molecular mimicry will lead to a cross-reaction of the immune response against bacterial antigens with similar antigens on peripheral nerves. This molecular mimicry hypothesis has obtained much support since the discovery of ganglioside-like structures on particular *C. jejuni* strains.

Molecular mimicry with gangliosides

The concept of molecular mimicry as a possible mechanism by which infections may induce pathogenic immune responses, has been proposed in many immune mediated diseases (218,219). The paradigmatic example is rheumatic carditis, in which the immune response against polysaccharides from streptococci cross-reacts with

glycopeptides in cardiac tissue (220, 221). The role of molecular mimicry between microbial and autologous antigens in producing a cross-reactive immune response can be established on different levels: (i) homology in biochemical structure or linear amino acid sequence, (ii) cross-reactivity of antibodies with both structures, (iii) induction of cross-reactive antibodies by immunization with the microbial antigen, and (iv) induction of cross-reactive antibodies which induce dysfunction or tissue damage. Two conditions need to be fulfilled to induce a cross-reactive immune response against autologous antigens by molecular mimicry. Firstly, the microbial and autologous antigens need to be sufficiently similar to induce an immune response that can cross-react. Secondly, the microbial antigen needs to be sufficiently different from the autoantigen to break the tolerance of the immune system to 'self', and induce a specific immune response, possibly with affinity maturation and isotype switch.

There is some circumstantial evidence that anti-ganglioside antibodies in some patients with GBS are induced by lipopolysaccharides (LPS) from particular *C. jejuni* strains. LPS are cell-wall constituents present in most Gram-negative bacteria. High-molecular weight smooth-form LPS consists of a lipid (the lipid A) which anchors the molecule in the outer membrane of the bacteria, and a polysaccharide which consists of a core oligosaccharide and a polymer of repeating oligosaccharide units (the O-specific chain) (Figure).

Sialic acids, which are characteristic for gangliosides, were demonstrated in the core oligosaccharide portion of the LPS from various *C. jejuni* strains, but not in other enteric bacteria (222,223). Detailed biochemical analysis of these core oligosaccharides showed a similarity in structure with gangliosides of human peripheral nerve (Table 7). This molecular mimicry has been demonstrated in *C. jejuni* isolates from several patients with GBS, a single patient with MFS, and several patients without a neuropathy (Table 7) (99,224-227). Some authors suggest that the ganglioside-like structures in LPS are related to the Penner serotype, which would explain the predominance of certain Penner serotypes in GBS (223,228). Interestingly, LPS from serostrain PEN O:3 is not sialylated and this Penner serotype is not associated with GBS (229). However, different ganglioside-like epitopes were found in isolates with the same Penner serotype, as was demonstrated for the PEN O:19 (227) (Table 7.)

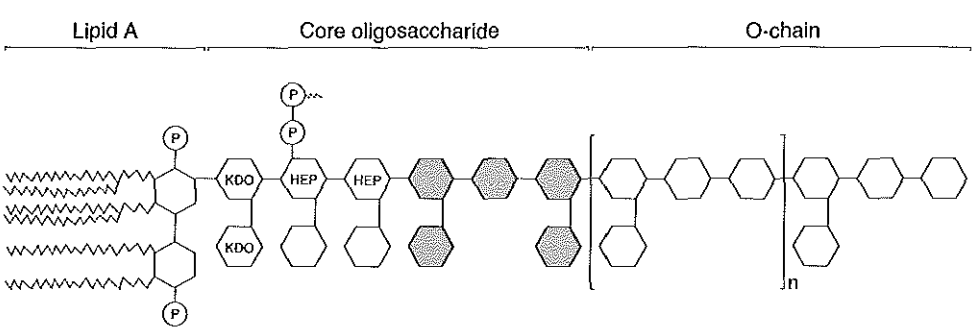
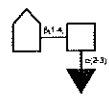
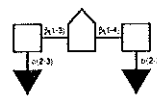
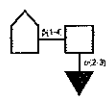
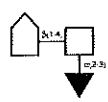
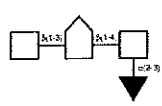
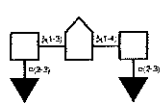
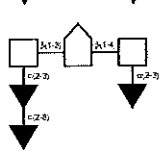
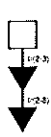
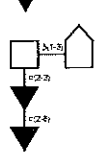


Figure. Schematic figure of the chemical structure of lipopolysaccharide. Ganglioside-like moieties are present in the outer core oligosaccharide (shaded structures). Abbreviations: P, phosphate; KDO, 3-deoxy-D-*manno*-octulosonic acid; HEP, L-*glycero*-D-*manno*-heptose; ~~~~, long-chain (hydroxy) fatty acid.

TABLE 7. Molecular mimicry between *C. jejuni* lipopolysaccharides and gangliosides[a].

Reference	Origin isolate	Penner serotype	Ganglioside[c]	Shared structure
Aspinall et al., 1993 (224)	serostrain[b]	O:1	GM2	
	serostrain	O:4	GD1a	
	serostrain	O:23	GM2	
	serostrain	O:36	GM2	
Aspinall et al., 1993 (225)	serostrain	O:2	GM3	
Yuki et al., 1993 (226)	GBS patient	O:19	GM1	
Aspinall et al., 1994 (227)	serostrain	O:19	GD1a	
	GBS patient	O:19	GT1a	
	GBS patient	O:19	GD3	
Salloway et al., 1996 (99)	MFS patient	O:10	GD3/GQ1b	

a. Abbreviations: MFS, Miller Fisher syndrome; GBS, Guillain-Barré syndrome; for symbols shared structures see Appendix 2.
b. Serostrains are bacteria used as reference strain in serotyping according to the Penner classification. These bacteria were isolated from patients without neuropathy.
c. Ganglioside which shows most similarity with lipopolysaccharide.

Moreover, the LPS O-chains, and not the core oligosaccharides, were later identified as the predominant antigenic determinants for the Penner classification (230). Although the biochemical analysis of *C. jejuni* LPS clearly identified ganglioside-like structures, it remains to be clarified whether these structures can induce anti-ganglioside antibodies with the specificity found in patients with GBS and MFS. A single study showed the cross-reactivity of serum anti-GM1 antibodies from a GBS patient with the LPS from the autologous *C. jejuni* isolate (228). In the other biochemical studies the presence of serum anti-ganglioside antibodies and their cross-reactivity with LPS was not investigated. Because ganglioside-like structures in LPS and the specificity of the anti-ganglioside antibodies are highly variable, it will be necessary to investigate the cross-reactivity with isolates and antibodies from the GBS and MFS patients themselves.

Other factors involved in cross-reactive immune responses

If *C. jejuni* is involved in the pathogenesis of GBS, how can such a common infection, with an estimated incidence of 1 per 100 per year, be followed by such a rare disease as GBS, with an incidence of 1 to 2 per 100,000 per year? Assuming that one third of GBS patients has an antecedent *C. jejuni* infection, one may calculate that approximately 1 in 2,000 to 5,000 subjects with *C. jejuni* infection will subsequently develop GBS (231). The first restrictive factor is the presence of adequate ganglioside-like structures on *C. jejuni* which have the capacity to induce anti-ganglioside antibodies with the specificity found in GBS. Such a molecular mimicry may be rare among *C. jejuni* strains. However, ganglioside-like structures were also demonstrated in *C. jejuni* isolates from patients with uncomplicated enteritis (Table 7), although the exact configuration of these structures may differ from those in GBS and MFS isolates. In addition, the immunogenicity of these structures may depend on other bacterial factors, like carrier proteins and other structures in LPS. One candidate is the enterotoxin that is produced by a subgroup of *C. jejuni* which binds to gangliosides and may function as a carrier protein to provide adequate T-cell help (204). Another candidate is the O-chain in the LPS, which appears to be expressed at a higher level in isolates from GBS patients than from controls (232), and which has an important adjuvant activity in antibody responses against LPS (233). The possible contribution of the O-chain in the immunogenicity may explain the predominance of particular Penner serotypes in GBS.

Host factors also play an important role in the production of anti-ganglioside antibodies and the development of GBS, since epidemics of *C. jejuni* associated GBS do not occur. An outbreak of *C. jejuni* of serotype PEN O:19 was followed by only one case of GBS (234). Two sisters were reported who developed GBS after infection with PEN O:19 (106), indicating that the disease susceptibility may depend on the immunogenetic background of the host. Moreover, in GBS patients an association was found between *C. jejuni* infections and human leukocyte antigen (HLA) B35 in Japan (235), and HLA-DQB1*03 in the United Kingdom (236). These HLA associations may further indicate that T-cells are also involved in patients with *C. jejuni* infections. However, the HLA associations reported so far are weak and only just significant, and should be confirmed in further studies.

Most likely, a combination of bacterial and host factors will explain why *C. jejuni* infection leads to GBS only in a minority of cases. Further research is needed to iden-

tify these factors and to comprehend the mechanism by which they lead to GBS.

1.5 SCOPE OF THE THESIS

The present knowledge, as described in the previous sections (Chapter 1.1 to 1.4), gives some support to the hypothesis that GBS results from an antecedent infection which induces a transient immune response to peripheral nerves. This immune response may recognize various targets with a different distribution and function in peripheral nerves, resulting in a variety of clinical and pathological manifestations. This thesis focuses on peripheral nerve gangliosides as possible targets for the anti-neural immune response in GBS patients. Microbial antigens mimicking gangliosides may induce anti-ganglioside antibodies during antecedent infection and thereby determine the specificity of these antibodies. The scope of this thesis is (i) to identify the spectrum of antecedent infections and anti-ganglioside antibodies in patients with GBS, (ii) to establish their relation to clinical and electrodiagnostic manifestations and prognosis, and (iii) to determine the role of antecedent infections and molecular mimicry in generating anti-ganglioside antibodies in GBS and MFS.

To investigate the relation between infections, anti-ganglioside antibodies and clinical presentation, we had the opportunity to use pretreatment serum samples from 154 GBS patients, who participated in two therapeutic studies, conducted by the Dutch GBS Study Group. These patients were carefully evaluated according to a previously established protocol with respect to a detailed array of clinical and electrodiagnostic features during a follow-up of six months. The serum samples were obtained within two weeks of neurological onset and before treatment, and were used to determine the presence of antibodies against eight major peripheral nerve gangliosides by ELISA and thin-layer chromatography overlay. In addition, these samples where used to determine the serology of acute infections with 16 agents. With respect to the role of antecedent infections in inducing anti-ganglioside antibodies we focused on the cross-reactivity of anti-ganglioside antibodies with *C. jejuni*, the most common infection in GBS. We had the opportunity to investigate the cross-reactivity with *C. jejuni* isolated from patients with GBS. *C. jejuni* isolates from patients with the MFS were also used. In these investigations it is crucial to use serum and autologous *C. jejuni* isolates from GBS and MFS patients themselves since both the specificity of anti-ganglioside antibodies and the ganglioside-like structures on *C. jejuni* are highly heterogeneous.

The following chapters of the thesis describe the studies concerning antecedent infections (Chapter 2), anti-ganglioside antibodies (Chapter 3), and the cross-reactivity of anti-ganglioside antibodies with *C. jejuni* isolates (Chapter 4) in patients with GBS and MFS.

Chapter 2 describes the frequency of the acute infections in the group of GBS patients compared to a group of sex-, and age-matched control patients with other neurological diseases. Also, the association between antecedent infections and specific clinical patterns was investigated.

Chapter 3 is a compilation of a series of studies concerning the presence of anti-ganglioside antibodies in GBS patients, and their relation with antecedent infections and clinical presentation. We identified several subgroups of GBS patients with distinct recent infections, anti-ganglioside antibodies and clinical presentations. The prognosis of these subgroups after different forms of treatment and the relevance of determining infection serology and anti-ganglioside antibodies in optimalizing treatment of individual patients are discussed. The relation between particular anti-ganglioside antibodies and involvement of distinct subsets of nerve fibres was further supported by the electrodiagnostic findings in these patients. The possible mechanisms by which anti-ganglioside antibodies interfere with nerve function are discussed.

Chapter 4 describes the cross-reactivity of anti-ganglioside antibodies with *C. jejuni* isolates from patients with GBS, MFS, and enteritis without neuropathy. The cross-reactivity of anti-ganglioside antibodies from GBS and MFS patients was determined by inhibition ELISA. The cross-reactive structures on *C. jejuni* were localized in the LPS fraction of the bacteria, and further characterized by specific sialidases and cholera toxin. In addition, the molecular mimicry between LPS and peripheral nerve antigens was demonstrated by using a monoclonal anti-ganglioside antibody purified from a patient with paraproteinaemic neuropathy. The titre course, isotype, and specificity of the antibody formation against *C. jejuni* LPS in patients with GBS and MFS was investigated, and compared with the response in subjects with uncomplicated *C. jejuni* enteritis.

Chapter 5 discusses the significance of the results described in Chapters 2 to 4. These findings and data recently reported in the literature are integrated in a model on the role of infections and anti-ganglioside antibodies in the pathogenicity and heterogeneity of GBS.

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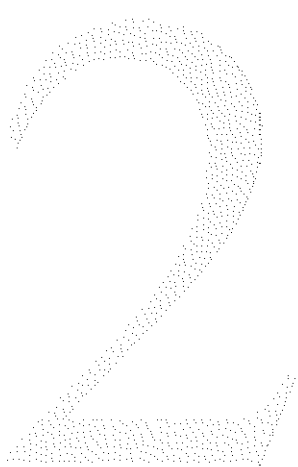
ANTECEDENT INFECTIONS IN GBS



CHAPTER 2

THE SPECTRUM OF ANTECEDENT INFECTIONS IN GUILLAIN-BARRÉ SYNDROME: A CASE-CONTROL STUDY

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ABSTRACT

The Guillain-Barré syndrome (GBS) is an acute polyneuropathy which is usually preceded by an infection. Many infectious agents have been reported in GBS patients but their relation to GBS is unclear. To investigate which infections are specifically associated with GBS, we conducted a serological study for 16 infectious agents in 154 GBS patients and 154 sex- and age-matched controls with other neurological diseases. The seasonal distribution of serum sampling in the GBS and control group was the same. In GBS patients *Campylobacter jejuni* (32%), cytomegalovirus (13%), Epstein-Barr virus (10%), and *Mycoplasma pneumoniae* (5%) were the most common causes of recent infections. These infections were all significantly more frequent in GBS patients than in the controls. Infections with *Haemophilus influenzae* (1%), parainfluenza 1 virus (1%), influenza A virus (1%), influenza B virus (1%), adenovirus (1%), herpes simplex virus (1%) and varicella zoster virus (1%) were also demonstrated in GBS patients, but not more frequently than in controls. *C. jejuni* and CMV infections were respectively associated with anti-GM1 and anti-GM2 antibodies, and with pure motor and severe motor-sensory variants of GBS, as reported previously. Infections with EBV and *M. pneumoniae* were not associated with specific anti-ganglioside antibodies and neurological patterns. This study indicates that many infectious agents may precede GBS, but that certain infectious agents are specifically related to GBS.

INTRODUCTION

The Guillain-Barré syndrome (GBS) is an acute polyneuropathy which may lead to a variety of motor and sensory deficits. It is considered to be a postinfectious disease since approximately two-third of patients report some form of preceding infectious illness. These infections may effect an immune response against peripheral nerve antigens, as suggested by the interval of 1 to 4 weeks between antecedent infection and onset of weakness. A remarkable diversity of infectious agents has been reported in patients with GBS (1). The association between GBS and infections with *Campylobacter jejuni* and cytomegalovirus (CMV) has been demonstrated in case-control studies (2-4). Furthermore, *C. jejuni* infections are associated with antibodies against the ganglioside GM1, and a severe, pure motor variant of GBS with a poor prognosis after plasma exchange (5,6). GBS patients with a CMV infection more frequently have antibodies against the ganglioside GM2 (7-8), and suffer from severe sensory deficits and cranial nerve involvement (9). These findings strongly indicate that *C. jejuni* and CMV infections determine the specificity of the immune response against peripheral nerves leading to distinct clinical variants of GBS. The variety of reported infectious agents in GBS may therefore underlay the immunological and clinical heterogeneity in GBS.

Controversy exists whether infections with other frequently reported agents, like Epstein-Barr virus (EBV) and *Mycoplasma pneumoniae*, are more often present in GBS patients compared to controls (10,11), or not (2). The majority of the 'GBS-related infections' were reported only in small and selected groups of GBS patients, without age-, sex-, and season-matched controls, indicating that they may represent coincidental findings. In addition, many of these infections were clinically defined without serological confirmation, or with serology performed on serum samples obtained a long time after neurological onset or after treatment. Moreover, in most studies the possible association between these infections, anti-ganglioside antibodies, and clinical presentation was not investigated. Therefore, the role of infectious agents in GBS, other than *C. jejuni* and CMV, is presently not established.

The aim of the present study was to determine the spectrum of antecedent infections in a large, unselected group of GBS patients and to investigate whether these infections are more common in GBS than in a matched control group. In addition, we investigated whether these infections in GBS patients are associated with specific anti-ganglioside antibodies and distinct clinical presentations.

PATIENTS AND METHODS

Patients

The GBS patients in this study participated in the Dutch Guillain-Barré trial comparing the therapeutic effect of plasma exchange (PE) and intravenous immunoglobulins (IVIg) (12), or in the pilot study evaluating the therapeutic effect of methyl-prednisolon in addition to IVIg (MP-IVIg) (13). The 147 patients in the PE/IVIg-trial entered the study from June 1986 to December 1989, and the 25 patients in the MP-IVIg pilot study from September 1990 to September 1992. All patients fulfilled the diagnostic criteria for GBS (14), were unable to walk 10 m independently and were admitted within 2 weeks of onset of weakness. All patients were evaluated with respect to the presence of paresthesias, sensory deficits (two-point discrimination, position

sense, and tactile function in hands and feet), cranial nerve involvement, functional score, and Medical Research Council (MRC) sumscore, at study entry and subsequently at 16 time points according to a previously established protocol during a follow-up period of 6 months. At entry, all patients were asked if they suffered from infectious illness in the preceding 4 weeks. Pre-treatment serum samples obtained within 2 weeks of neurological onset were available from 154 (90%) of the 172 patients included in these therapeutic studies. The 18 excluded cases did not differ from the other patients regarding their antecedent illnesses, neurological manifestations and course of disease.

Controls

As a control group for the serology studies we used serum samples from 154 sex and age (± 4 yr) matched control patients with other neurological diseases (OND) than GBS. The time of serum sampling in the control group and the group of GBS patients had the same seasonal distribution. The OND controls suffered from chronic inflammatory demyelinating polyneuropathy (n=21), other forms of neuropathy (n=60), multiple sclerosis (MS) (n=41), cerebrovascular accidents (n=14), motor neuron disease (n=6), myasthenia gravis (n=6), and other neurological disorders (n=6). All samples were obtained at admission in the hospital and before treatment. In addition, serum samples from 50 healthy subjects were tested.

Infection serology

Serum samples from GBS patients were tested to determine recent infections with 16 viruses or bacteria frequently reported in GBS. The assays were performed according to routine techniques with previously established criteria for positivity (Table 1). For EBV serology, sera were preabsorbed with Gull-sorb (Gull Laboratories, Salt Lake City, USA) to remove the IgG antibodies against EBV. This preabsorption increased the sensitivity of the assay and prevented false positivity by binding of IgM rheumatoid factors with IgG anti-EBV antibodies. Because we were interested in infections which were more frequent in GBS patients than controls, we only determined the serology for specific infections in the controls if more than 5 GBS patients were positive for this infection, the minimal number to be significant in the McNemar's test. The frequency of CMV and *C. jejuni* infections in the patients included in the PE/IVIg trial, were reported previously (6,8,9), but without comparison with the matched control group.

Anti-ganglioside antibodies

Serum samples from all GBS patients were tested for IgM, IgG and IgA antibodies against the peripheral nerve gangliosides LM1 (sialosyl paragloboside), GM1, GM2, GM3, GD1a, GD1b, GT1b and GQ1b, by enzyme-linked immunosorbent assay and confirmed in thin-layer chromatography overlay, according to methods described previously (16).

Statistical analysis

Differences in frequency of infections between GBS patients and OND controls were tested with the McNemar's test, and between GBS patients and healthy controls with the Chi-square test without continuity correction or Fisher's exact test. The odds ratio for each infectious agent was also estimated using a multivariate conditional logit model for matched case-control data, with disease status (GBS or control) as dependent variable and the infectious agents as independent variables (15). With respect to the clinical features of GBS patients, differences in proportions were tested with the Chi-square test without continuity correction or Fisher's exact test, and differences in medians with the Wilcoxon-Mann-Whitney *U* test. The time for patients to reach independent locomotion was analysed by the Kaplan-Meier method and the log-rank test. All calculations were performed using STATA 5.0 for Windows 95 (Stata Statistical Software: Release 5.0, College station, TX: Stata Corporation 1997). A p-value <0.05 was considered to be significant.

TABLE 1. Assays and criteria used to define the frequency of recent infections in GBS patients and controls [a].

Infectious agent	Technique	Criterium for recent infection
Campylobacter jejuni	ELISA	IgM, IgA and/or high titre IgG [b]
Haemophilus influenzae	ELISA	IgM, IgA and IgG
Mycoplasma pneumoniae	ELISA	IgM
Cytomegalovirus	ELISA	IgM [c]
Epstein-Barr virus	IF	IgM against viral capsid antigen
Hepatitis A virus	ELISA	IgM
Hepatitis B virus	ELISA	surface antigen
Herpes simplex virus	CF	titre ≥ 64
Varicella zoster virus	CF	titre ≥ 64
Measles virus	CF	titre ≥ 64
Influenza A virus	CF	titre ≥ 64
Influenza B virus	CF	titre ≥ 64
Parainfluenza 1 virus	CF	titre ≥ 64
Parainfluenza 2 virus	CF	titre ≥ 64
Adenovirus	CF	titre ≥ 64
Respiratory syncytial virus	CF	titre ≥ 64

a. Abbreviations: ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; CF, complement fixation.
b. As previously reported (6).
c. As previously reported (8).

RESULTS

Infections and antecedent illness in GBS patients

In the 4 weeks preceding GBS, 105 (68%) of the 154 patients reported some form of infectious illness, as indicated by their clinical symptoms. The most common identified cause of recent infections in patients with GBS was *C. jejuni* (32%) (Table 2). In GBS patients recent infections with CMV (13%), EBV (10%), *Mycoplasma pneumoniae* (5%), and less often with *Haemophilus influenzae* (1%), parainfluenza 1 virus (1%), influenza A virus (1%), influenza B virus (1%), adenovirus (1%), herpes simplex virus (1%) and varicella zoster virus (1%), were also demonstrated (Table 2). The majority of GBS patients with positive serology had clinical symptoms of preceding infectious illness (Table 2). Patients with *C. jejuni* infections had more often diarrhoea compared to patients without this infection ($p<0.001$), while symptoms of upper respiratory tract infection (URTI) were less frequently found in patients with this infection ($p=0.001$). *C. jejuni* infections were also associated with other manifestations of infectious illness than diarrhoea and URTI, like nausea, vomitting, and abdominal pain. Patients with antecedent CMV infection frequently had URTI, and other manifestations of infection as nausea, fever, and exanthema, but none suffered from diarrhoea. Other manifestations of infection were fever and sore throat in EBV infected patients, and pneumonia and pancreatitis in *M. pneumoniae* infected patients. In the 49 patients who did not report symptoms of antecedent infectious illness, a substantial proportion

TABLE 2. Incidence of positive infection serology and symptoms of antecedent infectious illness in GBS patients [a].

Infection serology	N [b] (n=154)	Diarrhoea (n=25)	URTI (n=68)	Other [c] (n=12)	None (n=49)
Campylobacter jejuni	49 (32%)	19 (76%)*	12 (18%)*	4 (33%)	14 (29%)
Cytomegalovirus	20 (13%)	0*	8 (12%)	3 (25%)	9 (18%)
Epstein-Barr virus	16 (10%)	1 (4%)	5 (7%)	2 (17%)	8 (16%)
Mycoplasma pneumoniae	7 (5%)	0	3 (4%)	2 (17%)	2 (4%)
Haemophilus influenzae	2 (1%)	0	2 (3%)	0	0
Parainfluenza 1 virus	2 (1%)	0	2 (3%)	0	0
Influenza A virus	2 (1%)	0	1 (1%)	0	1 (1%)
Influenza B virus	1 (1%)	0	1 (1%)	0	0
Adenovirus	1 (1%)	0	1 (1%)	0	0
Herpes simplex virus	1 (1%)	0	0	0	1 (2%)
Varicella zoster virus	1 (1%)	0	0	0	1 (2%)
> 1 infectious agent	13 (8%)	0	4 (6%)	2 (17%)	7 (14%)
≥ 1 infectious agent	87 (56%)	20 (80%)*	30 (44%)	9 (75%)	28 (57%)

a. Abbreviations: N, number; URTI, upper respiratory tract infection.
b. Number of patients with positive infection serology (percentage of group of 154 patients).
c. Other symptoms than diarrhoea or URTI.
*. $p<0.05$ compared to patients without these symptoms of infection.

had positive serology for infections with *C. jejuni* (29%), CMV (18%), EBV (16%), and *M. pneumoniae* (4%) (Table 2). The high proportion of *C. jejuni* infections in these patients is in accordance with the frequent subclinical course of this infection. Positive serology for recent infection with parainfluenza 2 virus, hepatitis A and B virus, respiratory syncytial virus, or measles virus could not be demonstrated. None of the infections in GBS patients showed a clear seasonal predominance.

Positive serology for more than one infection was found in 13 (8%) of the GBS patients, but significant associations with specific infections were not found. Most of these GBS patients had positive EBV serology, and were additionally positive for *C. jejuni* and CMV (n=2), *C. jejuni* (n=2), *M. pneumoniae* (n=3), CMV (n=2), and *H. influenzae* (n=1). The other patients were positive for CMV and additionally for *C. jejuni* (n=1), *M. pneumoniae* (n=1), and influenza A virus (n=1).

Antecedent infections in GBS patients compared to controls

Serology for infections with *C. jejuni*, CMV, EBV, and *M. pneumoniae* was also performed in the two control groups. Univariate analysis showed that these infections were all significantly more frequent in GBS patients compared to matched OND controls (Table 3). The attributable proportion of these infections, i.e. the proportion of patients that can be attributed to these infections provided they play a causal role in GBS, was calculated as 0.44 (Table 3). Using a multivariate conditional logit model for matched case-control data, only infections with *C. jejuni*, CMV, and EBV appeared to be independently associated with GBS. Acute infections in the OND controls were not

TABLE 4. Association between antecedent infections and anti-ganglioside antibodies in GBS patients [a].

Antibodies to [b]:	N [c] (n=154)	*C. jejuni* (n=49)	CMV (n=20)	EBV (n=16)	*M. pneumoniae* (n=7)	No infection [d] (n=75)
GM1	31 (20%)	20 (41%)*	1 (5%)	1 (6%) [e]	0	10 (13%)
GM2	9 (6%)	3 (6%)	5 (25%)*	0	0	1 (1%)
GM3	2 (1%)	0	0	0	0	2 (3%)
GD1a	5 (3%)	2 (4%)	0	0	0	3 (4%)
GD1b	27 (18%)	14 (29%)*	1 (5%)	0	0	12 (16%)
GT1b	2 (1%)	0	0	0	0	2 (3%)
GQ1b	6 (4%)	2 (4%)	0	0	0	4 (5%)
LM1	9 (6%)	3 (6%)	0	1 (6%)	1 (14%)	4 (5%)

a. Abbreviations: N, number; CMV, cytomegalovirus; EBV, Epstein-Barr virus.
b. IgM, IgG, and/or IgA antibodies to ganglioside.
c. Number of patients with anti-ganglioside antibodies (percentage of group of 154 patients)
d. No serologically defined infection with the agents reported in this table.
e. This patient also had a positive *Campylobacter jejuni* serology.
*. $p<0.05$ compared to patients without this infection.

significantly associated with any of the diagnostic categories. The OND patients with positive serology suffered from MS (n=8), CIDP (n=4), other polyneuropathies (n=7), cerebrovascular accident (n=1), myasthenia gravis (n=1), Friedreich's ataxia (n=1), and commotio cerebri (n=1).

Antecedent infections, anti-ganglioside antibodies, and clinical subgroups

C. jejuni infection was significantly associated with antibodies against GM1, and CMV infections with antibodies against GM2 (Table 4), as previously reported (5-8). Interestingly, the only GBS patient with herpes simplex virus had high titres of IgG antibodies against LM1, GM3, GD1a, GD1b, GT1b, and GQ1b. This patient had a pure motor variant of GBS with ophthalmoplegia and hypoglossal palsy, who needed artificial ventilation and recovered to independent locomotion 69 days after IVIg. Infections with EBV or *M. pneumoniae*, or any of the other agents, were not associated with the tested anti-ganglioside antibodies.

Infections with *C. jejuni* and CMV were each associated with a distinct clinical presentation in GBS, as we previously described (6,9). In addition, the case-control study enabled us to investigate the association between these infections and sex and age. The interaction between sex and *C. jejuni* infection was almost significant; the odds ratio (95% confidence interval) in male GBS patients was 14.4 (3.1 - 68.1) and in female GBS patients 2.9 (3.1 - 7.1) (p=0.078).

The median age of the GBS patients with EBV infection was 29 years, and with *M. pneumoniae* infection 28 years, indicating that they were significantly younger than the other GBS patients (median 50 years) (p=0.002 and p=0.001, respectively). Eleven (69%) of the 16 EBV infected patients had respiratory insufficiency, which was a significantly higher proportion compared to patients with other or no infections (37%)

TABLE 3. Incidence of recent infections in GBS patients compared to matched OND controls and to healthy controls [a].

Infection serology	GBS patients (n=154)	OND controls (n=154)	Odds ratio (95% CI) [b]	p-value [b,c]	Attributable proportion [b,d]	Healthy controls (n=50)	Odds ratio (95% CI) [b]	p-value [e,f]
C. jejuni	49 (32%)	18 (12%)	3.1 (1.7 - 5.9)	0.0001	0.22	4 (8%)	5.4 (1.9 - 15.1)	0.001
Cytomegalovirus	20 (13%)	3 (2%)	9.5 (2.3 - 84)	0.0002	0.12	0	-	0.005
Epstein-Barr virus	16 (10%)	1 (1%)	16.0 (2.5 - 671)	0.0003	0.09	0	-	0.01
M. pneumoniae	7 (5%)	1 (1%)	7.0 (0.9 - 316)	0.03	0.04	0	-	0.20
≥ 1 infectious agent [g]	79 (51%)	23 (15%)	7.2 (3.6 - 16.5)	<0.0001	0.44	4 (8%)	12.1 (4.3 - 33.8)	<0.001

a. Abbreviations: GBS, Guillain-Barré syndrome; OND, other neurological diseases; CI, confidence interval; -, cannot be calculated.
b. GBS patients compared to OND patients.
c. Determined by McNemar's test.
d. Attributable proportion is calculated as (odds ratio - 1)/odds ratio x frequency in GBS patients.
e. GBS patients compared to healthy controls.
f. Determined by Chi-square or Fisher's exact test.
g. Recent infection with at least one of the agents in this table.

(p=0.01). Infections with these or other micro-organisms were not significantly associated with time to reach nadir, MRC sumscore at entry and nadir, distribution of weakness, cranial nerve involvement, sensory loss, paresthesias, or time to recovery. Also, the GBS patients with multiple positive serology did not significantly differ from the other patients with respect to their neurological presentation.

DISCUSSION

In the present study, *C. jejuni*, CMV, EBV, and *M. pneumoniae* were identified as the most common causes of antecedent infections in GBS. These infections were all more frequently found in the GBS patients than in the age- and sex-matched controls with OND. Infections with herpes simplex virus, varicella zoster virus, influenza A and B virus, parainfluenza 1 virus, adenovirus, and *H. influenzae* were also identified, but their frequency was not significantly higher than in controls. These findings confirm the concept that certain infections are specifically related to GBS, although a spectrum of infections may precede the disorder.

Previous case-control studies demonstrated *C. jejuni* infections in 14% to 36% of GBS patients, and in 1% to 10% of controls (2-4), which is in accordance with our findings. The present study justifies the conclusion that *C. jejuni* is the predominant cause of antecedent infection in GBS. However, *C. jejuni* is also the predominant cause of acute bacterial enteritis in The Netherlands, and our study shows that in patients with OND and healthy controls *C. jejuni* occurs more frequently than infections with CMV, EBV and *M. pneumoniae*. Therefore, one may argue that GBS is a postinfectious disease not related to specific infections, in which the predominance of *C. jejuni* infections simply reflects the high frequency of this infection in the community. However, *C. jejuni* infections in GBS patients are related to a distinct severe and pure motor variant of GBS (4,6), suggesting that these bacteria specifically induce an immune response against motor nerve antigens in a subgroup of GBS patients. GM1 may be one of the targets since anti-GM1 antibodies are associated with these infections in GBS (5,6), and higher concentrations of GM1 are present in myelin of motor fibres compared to sensory fibres (16).

CMV was the most common viral cause of antecedent infection, present in 13% of GBS patients. This finding is in accordance with the 11% to 22% reported in other large studies (2,11,17). The presence of IgM antibodies to CMV suggests a recent CMV infection, although reactivation of a latent infection cannot be excluded. According to the report of Dowling and Cook, CMV infections in GBS patients are clustered in time periods of 10 to 16 weeks (11), but this could not be confirmed in our study. The high frequency of sensory and cranial nerve involvement in CMV-associated GBS patients may indicate that CMV infections induce specific antibodies against antigens present in these nerves. Anti-GM2 antibodies are associated with CMV infections in GBS (7,8), but the distribution of GM2 in the human peripheral nerve system is presently unknown. Others reported that CMV infections induce antibodies against sulfatide and other sulfated glycosphingolipids from human peripheral nerves (18). Interestingly, anti-sulfatide antibodies were reported to be related to sensory nerve involvement in GBS and other neuropathies (19). The association between CMV infections

and anti-sulfatide antibodies in GBS patients, however, has not been established.

The present study is the first which demonstrates a significantly higher frequency of EBV and *M. pneumoniae* infections in GBS patients compared to case-matched controls. Provided these infections play a role in the pathogenesis of GBS, 9% of GBS patients can be attributed to EBV infections, and 4% to infections with *M. pneumoniae*. These significant but low attributable proportions may explain why Winer et al. (2), in a smaller case-control study, were not able to demonstrate an association with one of these infections. We found no relation between these infections and antibodies against the major peripheral nerve gangliosides. However, *M. pneumoniae* infections in GBS were reported to be associated with antibodies against galactocerebroside, the predominant glycolipid in human peripheral nerves (20). The ubiquitous distribution of this antigen in the peripheral nervous system may explain why we did not find an association with involvement of specific nerve fibres.

Thirteen (8%) of the GBS patients in our study had a positive serology for more than one infectious agent, as was also found in other serological studies (17). This may indicate that dual-antigen induced immune responses play a role in a subgroup of GBS patients, as was also suggested for other immune mediated neuropathies (21). Because we did not culture the organisms, it is not excluded that some of these patients had a single infection inducing polyclonal B-cell activation, or antibodies against common or cross-reactive structures on different micro-organisms, resulting in a false double positive serology. However, studies based on culture alone will underestimate the frequency of recent infections, since most infections precede GBS for several weeks, and the infectious agents may be cleared at the time of neurological onset. Our findings should be confirmed in prospective large case-control studies using culture, serological and molecular biology techniques to determine recent infections.

The mechanisms underlying the predominance of infections with *C. jejuni*, CMV, EBV, and *M. pneumoniae* in GBS are unknown. Induction of immune responses to glycoconjugates in peripheral nerves is a probable general feature of these agents, as indicated by their association with antibodies against gangliosides and other glycolipids in GBS patients. Moreover, infections with *M. pneumoniae*, CMV, and EBV are associated with cold agglutinins, which bind with carbohydrate antigens with highest affinity at 4°C, a characteristic shared with anti-ganglioside antibodies (22). Interestingly, monoclonal cold agglutinins against disialylated gangliosides from patients with chronic ataxic neuropathy reduced nerve excitability and neurotransmitter release in the mouse phrenic nerve/diaphragm preparation (23). Anti-ganglioside antibodies in GBS patients with *C. jejuni* infection may be induced by molecular mimicry, since ganglioside-like structures were identified in *C. jejuni* lipopolysaccharides (24). In addition, it was demonstrated that anti-GM2 antibodies cross-react with CMV infected cells (7). Alternatively or in addition, these infectious agents express receptors or toxins which bind to carbohydrate antigens and act as carrier proteins. *C. jejuni* produces a toxin which specifically binds to gangliosides (25). *M. pneumoniae* binds with sialylated carbohydrates from the blood group I antigen and induces antibodies against this structure (26). Further research is needed to investigate if molecular mimicry with gangliosides and/or production of glycolipid-reactive carrier proteins explains the predominance of these infections in GBS.

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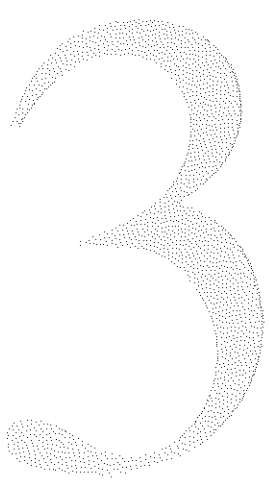
ANTI-GANGLIOSIDE ANTIBODIES IN GBS



ANTI-GANGLIOSIDE ANTIBODIES IN GBS

CHAPTER 3.1

INTRODUCTION

The Guillain-Barré syndrome (GBS) is considered to be an immune mediated polyneuropathy since deposits of complement and immunoglobulins, and infiltration of macrophages are present in peripheral nerves of these patients (reviewed in Chapter 1.1) (1,2). In blood of GBS patients, anti-neural antibodies, inflammatory cytokines and activated T-cells are also frequently found (1). Moreover, GBS patients benefit from treatment with plasma exchange or intravenous immunoglobulins (2). However, the peripheral nerve antigens which are recognized by the immune response, have not been identified.

In 1982, Ilyas et al. first reported the presence of antibodies to gangliosides in serum from a subgroup of GBS patients (3). Anti-ganglioside antibodies have also been reported in patients with other forms of immune mediated neuropathy (reviewed in Chapter 1.3). Interestingly, anti-ganglioside antibodies from some of these patients were found to interfere with nerve conduction and neuromuscular transmission (4-6). Gangliosides form a large family of sialylated glycosphingolipids which slightly differ from each other with respect to the oligosaccharide moiety (see Appendix 2). Gangliosides are found in relatively high concentrations in neural membranes (7), in which they presumably play a role in multiple signal recognition processes (8). Specific gangliosides show a distinct distribution in peripheral nerves: GM1 is concentrated in axons, and in myelin of motor nerve fibres (9,10), while GQ1b is predominantly found in myelin of oculomotor nerves (11). If anti-ganglioside antibodies also induce nerve dysfunction in GBS, one would expect that the neurological deficits in a patient with these antibodies reflect the distribution of the target ganglioside in peripheral nerves. The reported clinical heterogeneity of GBS (reviewed in Chapter 1.1), may partly result from the variety of anti-ganglioside antibodies in GBS patients.

The studies in Chapter 3 investigated (i) which anti-ganglioside antibodies are present in sera from GBS patients, (ii) if the presence of specific anti-ganglioside antibodies was related to distinct clinical symptoms and electrodiagnostic findings reflecting the localization of the target ganglioside in peripheral nerves, (iii) if anti-ganglioside antibodies are associated with antecedent infections, indicating that infection may induce these antibodies in GBS, and (iv) if the classification of GBS patients based on the presence of specific antecedent infections and anti-ganglioside antibodies has prognostic relevance and may give a guideline to optimize treatment.

The anti-ganglioside antibodies were determined in the serum from 154 of the 172 GBS patients, who were enrolled in two therapeutic studies (12,13). These studies investigated the therapeutic effect of plasma exchange, intravenous immunoglobulins, and the combination of methyl-prednisolon and intravenous immunoglobulins.

All patients fulfilled the diagnostic criteria for GBS (14), and were admitted within two weeks of onset of weakness. The patients were evaluated with respect to severity and distribution of weakness, sensory loss, paresthesia, cranial nerve deficiency, and respiratory insufficiency during a follow-up of 6 months.

Serum from each GBS patient was tested for the presence of IgM, IgG and IgA antibodies to the major peripheral nerve gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b and LM1. These anti-ganglioside antibodies were detected by enzyme-linked immunosorbent assay and thin-layer chromatography overlay. In addition, sera from various controls with other neurological diseases, or uncomplicated infections, and from healthy subjects were also tested according to the same methods. The pretreatment serum samples from the same GBS patients were also used to determine the serology for acute infections with 16 agents, as described in Chapter 2.

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CHAPTER 3.2

CAMPYLOBACTER JEJUNI INFECTIONS AND ANTI-GM1 ANTIBODIES IN GUILLAIN-BARRÉ SYNDROME

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ABSTRACT

The group of patients with Guillain-Barré syndrome (GBS) is very heterogenous with regard to antecedent infections, immunological parameters, clinical manifestations and response to treatment. In this study, the presumed pathogenic factors anti-GM1 antibodies and *Campylobacter jejuni* infections were related to the clinical characteristics. Serum from 154 patients with GBS, 63 patients with other neurological diseases (OND) and 50 normal controls (NC) were tested for the presence of antibodies against GM1 and *C. jejuni*. Anti-GM1 antibodies were detected in 31 (20%) GBS patients, 5 (8%) OND patients and in none of the NC. Evidence for a recent *C. jejuni* infection was found in 49 (32%) GBS patients and less often in OND patients (11%) or NC (8%). In GBS patients, the presence of anti-GM1 antibodies was significantly associated with *C. jejuni* infections. The subgroup of GBS patients with anti-GM1 antibodies suffered more often from a rapidly progressive and more severe neuropathy with predominantly distal distribution of weakness, without deficits of cranial nerves or sensory disturbances. The subgroup with *C. jejuni* infection also more often had a severe pure motor variant of GBS. Recovery of the patients with anti-GM1 antibodies and *C. jejuni* infections was not as good after plasma exchange compared with intravenous immunoglobulins.

INTRODUCTION

The Guillain-Barré syndrome (GBS) is a subacute polyradiculoneuropathy resulting in progressive weakness and areflexia (1). Although GBS is accepted as a disease entity, a large heterogeneity exists between individual GBS patients with regard to the severity and distribution of weakness, the degree of sensory deficit, the extent of demyelination and axonal degeneration and the response to treatment (2,3). The clinical and electrophysiological manifestations may, to some extent, be determined by biological factors like age, antecedent infections, and immunological parameters. Therefore, laboratory characteristics added to clinically defined cases may help to delineate specific subgroups (3).

Campylobacter jejuni has recently been identified as a major cause of antecedent infections in GBS patients (4-14). Some reports suggest that GBS patients with *C. jejuni* infections suffer from a more severe form of GBS (4,6,10), with less sensory deficit (6,13,14) and with poorer recovery (6,10,13), although this has not been found by others (8,11).

Antibodies against the ganglioside GM1 have been demonstrated in different proportions (9-78%) of GBS patients (6,7,10-19). The presence of serum anti-GM1 antibodies was found to be associated with a more severe (6,13), pure motor variant of GBS (6,14,15, 19), with more extensive axonal degeneration (6,15,16,19) and worse recovery (6,7,13, 15,17,19), although others have not found these associations (10,11). There is also still controversy on whether GBS patients with anti-GM1 antibodies suffer more frequently from an antecedent *C. jejuni* infection (6,7,12,13) or not (10,11).

In this retrospective study, we determined the presence of antibodies against *C. jejuni* and GM1 in the serum of 154 GBS patients and analyzed whether the presence of these antibodies is related to a subgroup of patients with distinct clinical manifestations and response to treatment.

PATIENTS AND METHODS

Serum samples were obtained from GBS patients who were included either in the Dutch GBS trial, comparing the therapeutic effect of plasma exchange (PE) and intravenous immunoglobulins (IVIg) (20), or in the pilot study, evaluating the effect of methyl prednisolone and IVIg (MP-IVIg) (21). All patients fulfilled the criteria for GBS (1), were unable to walk 10 m independently, and were admitted within 2 weeks of onset of weakness. The functional score and the Medical Research Council (MRC) sum score (22), ranging from 60 (normal) to 0 (tetraparalytic), were determined at study entry and subsequently at 16 time points during a follow-up period of six months. The rapidity of progression was indicated by the number of days from the onset of weakness to the moment of maximal weakness. The severity of weakness was indicated by the lowest MRC sum score. From the 172 GBS patients who participated in these two studies, pretreatment serum samples of 154 patients were available for serological testing. The 18 excluded cases did not differ from the other patients regarding their clinical manifestations and course of disease. Sixty-seven patients were treated with PE, 66 with IVIg and 21 with MP-IVIg. Serum samples from 63 patients with other neurological diseases (OND) and from 50 normal controls (NC) were also tested. The group of OND included patients with chronic inflammatory demyelinating polyneuropathy (CIDP) (16), multiple sclerosis (MS) (17), chronic polyneuropathy (PNP) other than CIDP (15) (PNP and paraproteinaemia (5), hereditary motor and sensory

neuropathy (2), pure sensory PNP (3), pure motor PNP (3), sensory motor PNP (2)) and 15 patients with various other disorders (cardiovascular accident (CVA) (3), myasthenia gravis (3), amyotrophic lateral sclerosis (ALS) (3) and others (6)). All samples were taken from patients within their active phase of disease before treatment was started and were tested without knowledge of the clinical data.

Detection of antibodies against *C. jejuni*

Serum antibodies against *C. jejuni* were determined by an indirect enzyme-linked immunosorbent assay (ELISA) for IgG (23) and by antibody class capture ELISA for IgM and IgA antibodies (24). The presence of anti-*C. jejuni* antibodies was expressed as a ratio of optical densities (OD) between a test sample and the cut-off serum sample, which was included in all tests. A ratio for IgM and/or IgA antibodies higher than 1.0 was considered as evidence for a recent *C. jejuni* infection (26). A ratio of IgG antibodies higher than 7.0, indicating a high titre, was considered suggestive for a recent *C. jejuni* infection (23).

Detection of antibodies against GM1

Enzyme-linked immunosorbent assay. IgM, IgG and IgA antibodies against GM1 were tested in an ELISA as described previously (25). For each isotype a serum sample from a GBS patient with a high titre of anti-GM1 antibodies was used as a positive control in each assay. To correct for inter-assay variations all extinctions were normalized against the positive control serum. Serum samples with an OD of more than 3 standard deviations above the mean value of 50 NC sera were tested in a thin-layer chromatography (TLC) overlay to exclude antibody binding to contaminants in the GM1 preparation. Positive serum samples were tested again in ELISA, using serial dilutions starting at 1:100. The reciprocal of the highest dilution that resulted in an OD higher than the cut-off value was then taken to be the titre.

Thin-layer chromatography overlay. TLC was performed on aluminium-backed Kieselgel 60 $WF_{254}S$ high-performance TLC plates (Merck, Darmstadt, Germany) that were coated with 500 pmol of GM1 and developed in chloroform/methanol/0.25% calcium chloride in water (50:40:10, by volume). After chromatography, the plates were airdried and dipped in a solution of 0.1% polyisobutylmethacrylate in *n*-hexane. The plates were airdried and blocked with phosphate-buffered saline (PBS) with 1% bovine serum albumine (BSA) for 1 hour and incubated with serum diluted 1:100 in PBS-0.1% BSA for 4 hours at 4°C. After washing with PBS, the plates were incubated for 2 hours at 4°C with peroxidase-conjugated goat anti-human IgM (μ-chain specific) or IgG (γ-chain specific) or IgA (α-chain specific) (Sigma) diluted 1:2,500 in PBS-0.1% BSA and washed with PBS. The plates were developed for 10 to 150 seconds using an enhanced chemiluminescence procedure (Amersham, UK).

Statistical analysis

Differences in proportions were tested with the Chi-square test without continuity correction and differences in medians were tested with the Wilcoxon-Mann-Whitney *U* test. The time for patients to reach independent locomotion was analyzed by the Kaplan-Meier method and the log-rank test. A p-value<0.05 was considered to be significant.

RESULTS

IgM and/or IgA antibodies and/or high titres of IgG antibodies against *C. jejuni* were detected in 32% of 154 GBS patients, 11% of 63 OND patients, and 8% of 50 NC (Table 1). According to this criterium, recent *C. jejuni* infections were significantly more often present in GBS patients than in patients with OND and NC. The OND patients with a recent *C. jejuni* infection suffered from CIDP (2), MS (3), pure sensory PNP (1), or CVA (1). Moreover, IgM and/or IgA antibodies, a more specific but less sensitive criterium for recent infection, were also more often found in GBS patients than in OND patients and NC (Table 1).

Elevated titres of anti-GM1 antibodies were detected in the serum of 31 (20%) of the 154 GBS patients. IgM anti-GM1 antibodies were found in 16 (10%), IgG in 22 (14%) and IgA in 11 (7%) GBS patients (Figure 1). Twelve patients had elevated titres of two or three classes and 6 patients of three classes. GBS patients with anti-GM1 IgA antibodies, but without IgM or IgG antibodies, were not found. Anti-GM1 antibodies were found in 5 (8%) of 63 OND patients but not in NC (Figure 1). Anti-GM1 IgM antibodies were present in 1 patient with CIDP and 3 patients with a chronic pure motor PNP other than CIDP and IgA antibodies in another patient with CIDP.

Serological evidence for a recent *C. jejuni* infection was more often found in the GBS patients with anti-GM1 antibodies (65%) than in the patients without anti-GM1 antibodies (24%) ($p<0.001$) (Table 2). Anti-GM1 antibodies of the IgM, IgG and IgA isotype were all associated with *C. jejuni* infection ($p<0.001$) (Figure 1). IgM and/or IgA antibodies against *C. jejuni* were also associated with anti-GM1 antibodies ($p<0.001$). In OND patients, no association was found between anti-GM1 antibodies and *C. jejuni* infections. In additional experiments, it was demonstrated that anti-GM1 antibodies are not absorbed by the *C. jejuni* protein extract used to determine the *C. jejuni* serology (data not shown).

TABLE 1. Prevalence of elevated titres of IgM and IgA and high titres of IgG antibodies against *C. jejuni* in patients with GBS, OND and NC [a].

Anti-*C. jejuni* antibodies	GBS (n=154)	OND (n=63)	p-value [b]	NC (n=50)	p-value [c]
IgM	28 (18%)	5 (8%)	0.056	1 (2%)	0.004
IgA	34 (22%)	4 (6%)	0.006	2 (4%)	0.004
IgG	29 (19%)	2 (3%)	0.003	3 (6%)	0.03
IgM and/or IgA	42 (27%)	7 (11%)	0.01	2 (4%)	0.001
IgM, IgA and/or IgG	49 (32%)	7 (11%)	0.002	4 (8%)	0.001
Two or three isotypes	29 (19%)	4 (6%)	0.02	1 (2%)	0.004

a. Abbreviations: GBS, Guillain-Barré syndrome; OND, other neurological diseases; NC, normal controls.
b. Patients with GBS compared to patients with OND.
c. Patients with GBS compared to NC.

TABLE 2. Clinical characteristics of patients with GBS associated with *C. jejuni* infection and anti-GM1 antibodies [a].

	C. jejuni infections					Anti-GM1 antibodies				
	+ (n=49)		− (n=105)		p-value	+ (n=31)		− (n=123)		p-value
Diarrhoea	19	(39%)	6	(6%)	<0.001	12	(39%)	13	(11%)	<0.001
MRC sum score [b] at entry [c]	36	(0-52)	41	(8-56)	0.049	32	(0-50)	41	(12-56)	0.001
Days to lowest MRC sum score [c]	7	(2-21)	9	(2-21)	0.48	6	(1-18)	9	(3-21)	<0.001
Lowest MRC sum score [c]	30	(0-50)	35	(0-55)	0.03	20	(0-50)	36	(0-55)	0.01
Tetraplegia	13	(27%)	12	(11%)	0.02	10	(32%)	15	(12%)	0.007
Predominantly distal weakness	23/48	(48%)	32/101	(32%)	0.055	19	(61%)	36/118	(31%)	0.002
Cranial nerve impairment	25	(51%)	67	(64%)	0.13	12	(39%)	80	(65%)	0.008
Sensory deficit at entry	23	(47%)	66/100	(66%)	0.03	13/30	(43%)	76/119	(64%)	0.04
Paresthesias	32	(65%)	92	(88%)	0.001	17	(55%)	107	(87%)	<0.001
Anti-GM1 antibodies	20	(41%)	11	(10%)	<0.001	—		—		—
C. jejuni infections	—		—		—	20	(65%)	29	(24%)	<0.001

a. Abbreviations: MRC, Medical Research Council.
b. MRC sum score ranges from 60 (normal) to 0 (tetraparalytic).
c. Median (2.5-97.5% percentile).

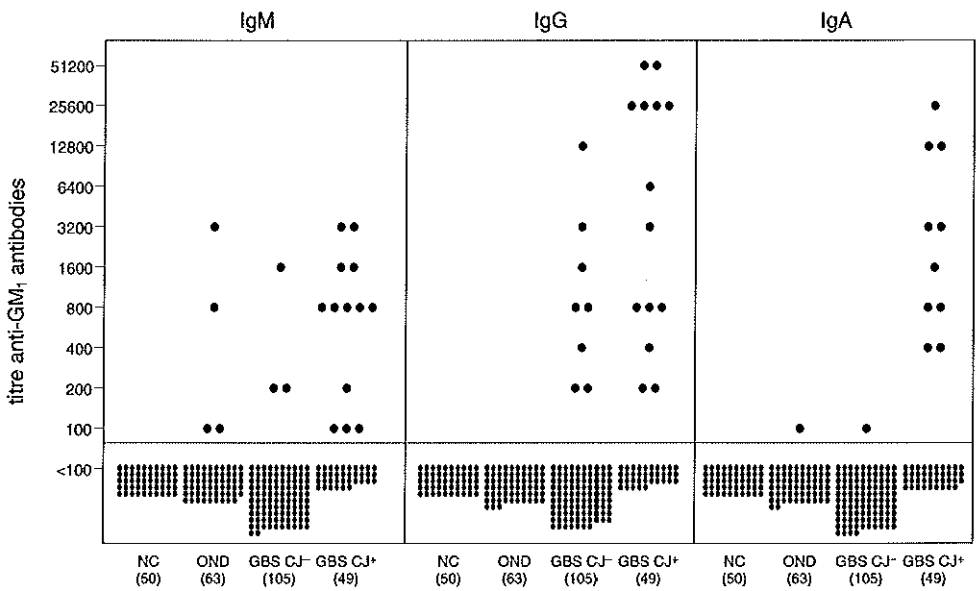

Figure 1. Titre of serum anti-GM1 antibodies in patients with Guillain-Barré syndrome (GBS), other neurological diseases (OND) and normal controls (NC). GBS patients were subdivided in patients with *Campylobacter jejuni* infection (CJ^+) and without (CJ^-).

The clinical characteristics of GBS patients associated with antecedent *C. jejuni* infection and anti-GM1 antibodies are given in Table 2. No differences were found in sex and age between the GBS patients with or without *C. jejuni* infections or anti-GM1 antibodies. The presence of IgM and/or IgA antibodies against *C. jejuni* only was associated with a predominantly distal weakness without cranial nerve impairment in addition to a more severe maximal weakness with less paresthesias and sensory deficits (data not shown).

The clinical manifestations associated with the presence of anti-GM1 antibodies were predominantly related to the IgG and IgA class. GBS patients with anti-GM1 IgM antibodies did not differ from patients without these antibodies with respect to days to peak severity, tetraplegia, distribution of weakness, and cranial and sensory nerve impairment.

In the group of GBS patients with a recent *C. jejuni* infection, 24 patients were treated with PE, 22 with IVIg, and 3 with MP-IVIg. A longer median time to recover in the subgroup with *C. jejuni* infection was only found in the patients treated with PE ($p=0.003$), and not in the patients treated with IVIg or MP-IVIg. The patients with *C. jejuni* infections had a significantly shorter median time to reach independent locomotion after IVIg or MP-IVIg than after PE (Figure 2b). In the patients without *C. jejuni* infections, there was no difference between the treatment modalities (Figure 2a). Patients with only IgM and/or IgA antibodies against *C. jejuni* also had a better prognosis after IVIg or MP-IVIg than after PE (data not shown).

In the group of GBS patients with anti-GM1 antibodies, 10 were treated with PE, 13 with IVIg, and 8 with MP-IVIg. In the subgroup treated with PE, the median time to recover was longer in the patients with anti-GM1 antibodies (>181 days) than in those without (69 days) (p=0.03). In the group of patients treated with IVIg alone, recovery was not associated with the presence of anti-GM1 antibodies. The patients with anti-GM1 antibodies had a significantly shorter median time to recover after IVIg or MP-IVIg than after PE (Figure 2d). In patients without anti-GM1 antibodies, there was no difference between the treatment modalities (Figure 2c). The presence of anti-GM1 antibodies of the IgM, IgG and IgA class were all negative prognostic factors in the patients treated with PE.

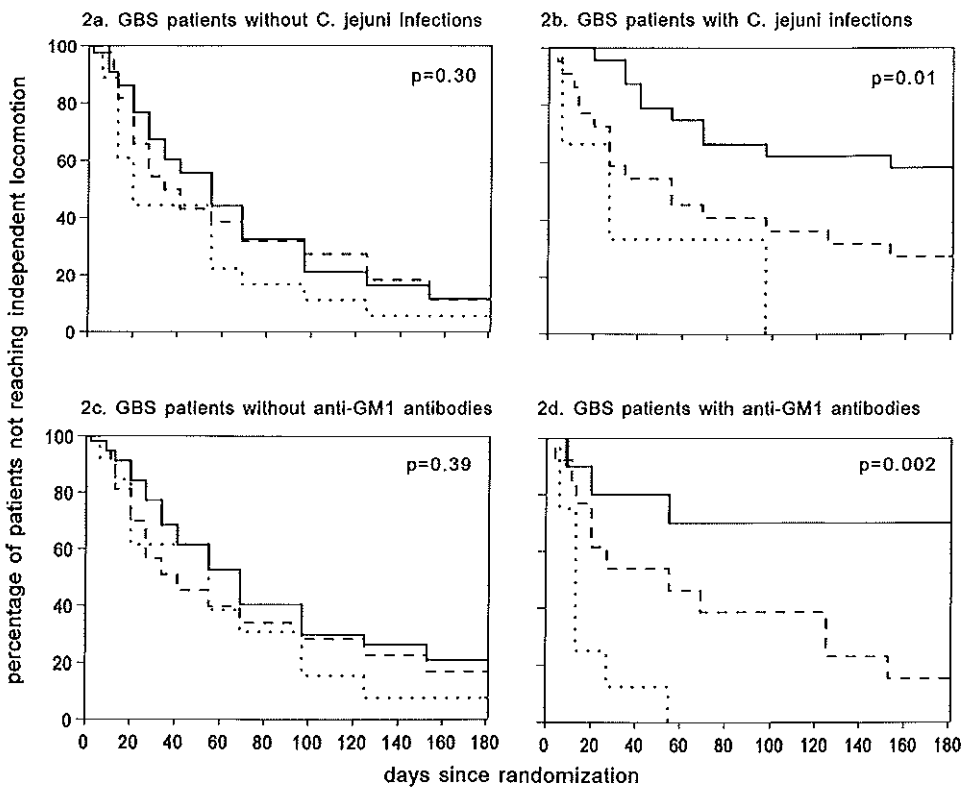

Figure 2. Kaplan-Meier curves indicating the percentage of patients who were not able to walk independently for 10 m. Follow-up during 181 days of patients treated with plasma exchange (——), intravenous immunoglobulins (IVIg) (– –), or methyl-prednisolon and IVIg (·····).

DISCUSSION

In this study on 154 patients with GBS, the presence of *C. jejuni* infections and anti-GM1 antibodies seems to define a clinically distinct subgroup of patients. These patients more often had a severe and predominantly distal weakness without sensory deficits or cranial nerve impairment. This clinical picture resembles, at least in part, multifocal motor neuropathy (MMN) and the acute motor axonal neuropathy (AMAN) in China, two disorders with predominantly distal weakness and, in general, without sensory or cranial nerve involvement that are also associated with the presence of anti-GM1 antibodies (26,27). Such similarity indicates a possible role for anti-GM1 antibodies in the pathogenesis of motor nerve impairment. This is supported by the finding of a higher concentration of GM1 in human motor nerves compared to sensory nerves (28) and by the binding of anti-GM1 antibodies with peripheral nerves at the node of Ranvier (29). In addition, monoclonal antibodies against GM1 from patients with MMN can induce conduction block in a mouse phrenic nerve/diaphragm preparation leading to unresponsiveness (30). However, even in the subgroup of GBS patients with anti-GM1 antibodies, heterogeneity of clinical manifestations exists. This clinical diversity may be related to the heterogeneity of anti-GM1 antibodies regarding the fine specificity, titre, avidity, isotype, and capacity to bind complement.

The presence of *C. jejuni* infections and anti-GM1 antibodies seems to define a distinct subgroup of patients in which PE is less effective than IVIg or MP-IVIg. This parallels MMN, since these patients also do not respond to PE (26) and are claimed to recover after IVIg (31,32). However, our analysis is retrospective and includes only a small group of patients treated with MP-IVIg. Prospective studies are needed to confirm these findings.

In the GBS patients, the presence of anti-GM1 antibodies was significantly associated with antecedent *C. jejuni* infections ($p<0.001$). This finding supports the hypothesis that antibodies against GM1 in GBS patients are induced during the antecedent infection with *C. jejuni.* The high percentage (91%) of recent *C. jejuni* infections in GBS patients with IgA anti-GM1 antibodies further strengthens the relation with enteric infections. Recently, it has been shown that lipopolysaccharides from a *C. jejuni* isolate from a GBS patient with anti-GM1 antibodies express a GM1-like structure (33). Also, specific *C. jejuni* strains are recognized by anti-GM1 antibodies from the serum of GBS patients (34). A similar observation has been made in *C. jejuni* isolates from patients with the Miller Fisher syndrome that bind specifically with anti-GQ1b antibodies (25).

The association between anti-GM1 antibodies and recent *C. jejuni* infections has been demonstrated in some studies (6,7,12,13) but not in others (10,11). In our study, involving a large group of GBS patients, the association is significant but not absolute. This may explain why a significant association was not found in studies investigating smaller groups of GBS patients. Besides, the patients in our study suffered from a relatively severe variant of GBS. Since anti-GM1 antibodies and *C. jejuni* infections are both more frequently found in patients with severe GBS, the association between anti-GM1 antibodies and *C. jejuni* infections could more easily be demonstrated in this group of patients. The association also depends on the sensitivity and specifivity of the assays used to detect the antibodies.

There are several explanations for the finding that not all GBS patients with anti-GM1 antibodies had a *C. jejuni* infection. Firstly, some GBS patients may have the same epiphenomenic or nonpathogenical anti-GM1 antibodies that are also found in low titres in some normal controls. Secondly, it is possible that other infectious agents also express GM1-like structures. Anti-GM1 IgG antibodies have been demonstrated in a patient with a chronic PNP after *Mycoplasma pneumoniae* infection (35). Thirdly, mechanisms other than infections may be involved in the induction of anti-GM1 antibodies.

Some GBS patients have *C. jejuni* infections without having anti-GM1 antibodies. There are several explanations for this. Firstly, some recent *C. jejuni* infections in these patients may be unrelated to GBS, since these were also found in 8% of NC. Secondly, the particular *C. jejuni* may not express a GM1-like structure, since the presence of this epitope is strain specific (34). These GBS patients could have had an infection with a *C. jejuni* strain expressing structures that mimic neural components other than GM1. In these patients, T-lymphocytes or antibodies against other neural epitopes can be involved in the pathogenesis of GBS. This is supported by the finding in animals that antibodies against peripheral nerve proteins are induced after immunization with *C. jejuni* (36,37). Other infections and antibodies against other glycolipids may further delineate the clinical heterogeneity in GBS.

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CHAPTER 3.3

CAMPYLOBACTER JEJUNI INFECTION AND TREATMENT FOR GUILLAIN-BARRÉ SYNDROME

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To the Editor:

Rees et al. (Nov. 23 issue) (1) reported that patients with Guillain-Barré syndrome (GBS) and recent infection with *C. jejuni* have a more severe residual disability after one year than patients without *C. jejuni* infection. In later studies they showed that patients with the combination of *C. jejuni* infection and antibodies against GM1 ganglioside have the worst prognosis (2). The effect of treatment was not included in their analyses, however.

We studied the outcome in 147 patients with GBS six months after their enrollment in a trial comparing plasma exchange (PE) with intravenous immunoglobulins (IVIg) (3). None of the patients could walk independently at the time of randomization. Pretreatment serum from 133 patients were tested serologically for *C. jejuni* infections and anti-GM1 antibodies (4). The patients were classified according to whether *C. jejuni* infection and anti-GM1 antibodies were present. Table 1 summarizes our findings.

Our study confirmed the unfavourable prognosis in the patients with both *C. jejuni* infection and anti-GM1 antibodies. However, the more severe residual disability was found only after treatment with PE, and not after treatment with IVIg. Five of the patients treated with PE were studied again at least one year after randomization, and none could walk independently. In contrast, 8 of 10 patients with *C. jejuni* infection and anti-GM1 antibodies treated with IVIg recovered. In a logistic-regression model, the percentage of recovery among the patients with *C. jejuni* infection and anti-GM1 antibodies was lower after PE (18%) than after treatment with IVIg (82%). The difference in outcome between patients treated with PE and those treated with IVIg could not be explained by other prognostic factors, such as age and severity of diseae at onset. Our data indicate that the combination of *C. jejuni* infection and anti-GM1 antibodies is associated with a poor prognosis in patients with GBS who are treated with PE. Prospective studies are needed to investigate whether such patients should be treated preferentially with IVIg.

TABLE 1. Patients with Guillain-Barré syndrome who could walk independently six months after the start of treatment [a].

Serological subgroups	PE (n=67) [b]	IVIg (n=66) [b]
Positive for anti-GM1 antibodies		
with *C. jejuni* infection	0/7	9/11 (82%)
without *C. jejuni* infection	3/3 (100%)	2/2 (100%)
Negative for anti-GM1 antibodies		
with *C. jejuni* infection	10/17 (59%)	7/11 (64%)
without *C. jejuni* infection	35/40 (88%)	36/42 (86%)

a. Abbreviations: PE, plasma exchange; IVIg, intravenous immunoglobulins.
b. Data are presented as number of patients able to walk /number of patients in subgroup (%).

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CHAPTER 3.4

CYTOMEGALOVIRUS INFECTIONS AND ANTI-GM2 ANTIBODIES IN GUILLAIN-BARRÉ SYNDROME

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ABSTRACT

To investigate whether antecedent cytomegalovirus (CMV) infections in patients with Guillain-Barré syndrome (GBS) are associated with the presence of specific anti-ganglioside antibodies, acute phase serum from 130 patients with GBS and 200 controls was tested. Anti-GM2 IgM antibodies were found more often in GBS patients with CMV infection (22%) than in patients without this infection (2%) (p=0.003). CMV infections may elicit anti-GM2 antibodies in susceptible patients, which may contribute to the pathogenesis of CMV associated GBS.

INTRODUCTION

Antecedent infections and anti-ganglioside antibodies are related to various clinical patterns in the Guillain-Barré syndrome (GBS) (1). *Campylobacter jejuni* infections are associated with anti-GM1 antibodies and a more severe and pure motor variant of GBS (2,3). Some *C. jejuni* strains express GM1-like epitopes and may induce anti-GM1 antibodies (4). Cytomegalovirus infections are the most frequent viral infections preceding GBS (5), and are associated with severe sensory loss, cranial nerve involvement and respiratory insufficiency (6). Recently, three GBS patients were reported with a CMV infection and antibodies against the ganglioside GM2 (7). Such antibodies may contribute to the pathogenesis of GBS, since GM2 is found in peripheral nerves (8). In the present study we investigated in a large group of GBS patients whether CMV infections are associated with anti-GM2 antibodies in the acute phase of the disease.

PATIENTS AND METHODS

Patients

Pretreatment serum samples, obtained within two weeks of onset of weakness, were available for serological testing in 130 of the 147 GBS patients who participated in the Dutch GBS trial (9). The 17 excluded cases did not differ from the other patients with regard to their clinical manifestations and course of disease. Serum samples were also tested from patients with other neurological diseases (OND) (50), controls infected with CMV (CMVC) (50) and controls infected with *C. jejuni* (CJC) (50) both without neurological involvement, and normal controls (NC) (50). The group of OND comprised patients with inflammatory polyneuropathy (PNP) (24), non-inflammatory PNP (12), and neurological diseases other than PNP (14).

Detection of antibodies against glycolipids

IgM and IgG antibodies against GM2 were tested by enzyme linked immunosorbent assay (ELISA) and thin-layer chromatography (TLC) overlay, as described previously (3). Serum samples, tested in a dilution of 1:100 in ELISA, with an optical density (OD) of more than 3 standard deviations above the mean value of 50 NC sera were tested in TLC overlay. Positive samples were tested again in ELISA, using serial dilutions starting at 1:100. The reciprocal of the highest dilution that resulted in an OD higher than the cut-off value was then taken to be the titre. The GBS patients with anti-GM2 antibodies were also tested for IgM and IgG antibodies against GM1, GM3, GD1b, GD2, asialo-GM1 (GA1) and asialo-GM2 (GA2).

All patients were serologically tested for CMV and *C. jejuni* infection. IgM antibodies against CMV indicated a recent CMV infection (6), and a recent *C. jejuni* infection was indicated by IgA, IgM, or high titres of IgG antibodies against *C. jejuni* (3).

Statistical analysis

Differences in proportions were tested with the Fisher's exact test. A p-value<0.05 was considered to be significant.

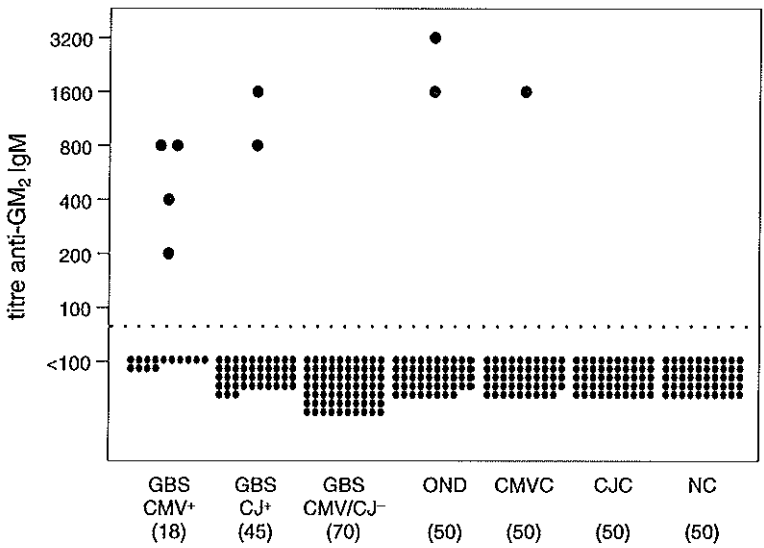

Figure. Titre of anti-GM2 IgM antibodies in patients with Guillain-Barré syndrome (GBS), other neurological diseases (OND), cytomegalovirus (CMV) infected controls (CMVC), and *Campylobacter jejuni* infected controls (CJC) both without neurological involvement, and normal controls (NC). The GBS patients were subdivided into patients with CMV infection (CMV^+), *C. jejuni* infection (CJ^+) and those without CMV and *C. jejuni* infection (CMV/CJ^-). Three GBS patients had a dual infection with CMV and *C. jejuni* and were negative for anti-GM2 antibodies.

RESULTS

Anti-GM2 IgM antibodies were detected in six (5%) of 130 GBS patients, two (4%) of 50 OND patients, one (2%) of 50 CMVC patients and in none of the 50 CJC patients or 50 NC (Figure). These antibodies were found in four (22%) of 18 GBS patients with a recent CMV infection. This frequency of anti-GM2 IgM antibodies was higher than in GBS patients without CMV infection (2%) ($p=0.003$), and patients with OND ($p=0.04$), CMVC ($p=0.02$), CJC ($p=0.004$) and NC ($p=0.004$). Anti-GM2 IgM antibodies were found in two (4%) of 45 GBS patients with a recent *C. jejuni* infection, but this frequency was lower than in GBS patients with a CMV infection ($p=0.04$). Three GBS patients had a dual infection with CMV and *C. jejuni*, but did not have anti-GM2 antibodies. In all GBS patients the titre of the anti-GM2 IgM antibodies decreased with clinical improvement. Anti-GM2 IgG antibodies could not be demonstrated in pretreatment sera. Isotype switch to anti-GM2 IgG or IgA antibodies during follow-up of the anti-GM2 positive GBS patients did not occur. Anti-GM2 IgM antibodies were found in two patients with OND. One patient had a chronic pure motor and demyelinating PNP with paraproteinaemia. The other patient had paresthesias and otherwise a chronic motor and demyelinating PNP without paraproteinaemia. In these patients no evidence for CMV infections was found.

The four CMV infected GBS patients with anti-GM2 antibodies had additional IgM antibodies against GA2, and in one case against GD2, but not against GM1, GM3,

GD1b and GA1. In these patients IgG antibodies against glycolipids were not found. Interestingly, the two *C. jejuni* infected GBS patients with anti-GM2 antibodies also had IgM antibodies against GM1, GD1b and GA1, but not against GA2 and GM3. In these two patients IgG antibodies against GM1 and GA1 were also found.

The clinical characteristics of the GBS patients with anti-GM2 antibodies were found to be related to the antecedent infections. The four CMV positive patients were relatively young females who had an antecedent upper respiratory infection. Thereafter, they had globally distributed moderate to severe weakness with facial palsy, sensory loss and paresthesias, and in three patients, respiratory insufficiency. The two *C. jejuni* infected patients with anti-GM2 IgM antibodies had antecedent diarrhoea, followed by a severe and predominantly distal weakness without involvement of cranial and sensory nerves, and without respiratory insufficiency. These two patients slowly recovered to independent locomotion (125 days and more than 181 days). The clinical symptoms of the GBS patients with anti-GM2 antibodies did not differ significantly from those of other GBS patients with CMV or *C. jejuni* infections.

DISCUSSION

The association between CMV infections and anti-GM2 antibodies described in this study, further supports the concept that antecedent infections are related to anti-glycolipid antibodies and clinical subgroups in GBS (1). It parallels the previously reported associations between *C. jejuni* infections and anti-GM1 antibodies (2,3), and between *Mycoplasma pneumoniae* infections and anti-galactocerebroside antibodies (10).

Our results are partly in accordance with those published by Irie et al (7). They found anti-GM2 antibodies in all three CMV infected GBS patients and in none of the GBS patients without CMV infection. In a large group of GBS patients we showed the presence of anti-GM2 antibodies in a lesser percentage (22%) of CMV infected patients. However, serum samples in the study of Irie et al. were obtained at rather a long time after neurological onset (mean 24, range 2 to 180 days) compared to our study (mean 6, range 1 to 14 days) which may lead to an underestimation of CMV infections in their patients. In addition, we also found anti-GM2 antibodies in some GBS patients with *C. jejuni* infections, but the frequency was significantly less than in CMV infected GBS patients.

Anti-ganglioside antibodies in GBS patients are predominantly IgG, although IgM and IgA antibodies are also found. Remarkably, the anti-GM2 antibodies in our study were all IgM. This is probably not related to specific antecedent infections, since the anti-GM2 antibodies in the *C. jejuni* infected patients were also IgM, and others found anti-GM2 IgG antibodies in CMV infected patients (7). As sera with these antibodies show no activity against similar gangliosides like GM3, the anti-GM2 IgM antibodies demonstrated in our study are not multireactive.

Instead, two specific patterns of antibody activity against GM2 may be present in patients with GBS. Firstly, some patients have additional antibodies that bind to GA2 or GD2 but not to GM1, which may suggest that the antibodies recognize the shared GalNAc(β1-4)Gal terminal in GM2, GA2 and GD2 (see Appendix 2) (7). The three

patients in the study of Irie et al., and the four patients in our study with this pattern of antibody activity all had a CMV infection. Secondly, other patients show additional antibody activity against GM1 but not against GA2 or GD2, indicating that these antibodies bind with the GalNAc(β1-4)[NeuAcα2-3]Gal(β1-4)Glc moiety which GM2 and GM1 have in common (see Appendix 2) (12). We found this pattern in two patients with antecedent *C. jejuni* infection (11). These antibodies may be produced initially during the antecedent infection, as LPS from several *C. jejuni* strains are recognized by monoclonal antibodies against GM1 and GM2 (12), and have a terminal tetrasaccharide identical to that of GM2 (13). However, the number of anti-GM2 positive patients is too small to conclude that these two antibody patterns are associated with specific antecedent infection.

The clinical characteristics of the GBS patients with anti-GM2 antibodies were related to the antecedent infection and did not differ from those of other GBS patients with CMV or *C. jejuni* infections. In previous studies we found that CMV infections are associated with severe sensory loss, cranial nerve involvement and respiratory insufficiency (6), and *C. jejuni* infections with severe and predominantly distal weakness without sensory or cranial nerve involvement (3). In the six anti-GM2 positive GBS patients, these clinical differences may be related to the fine specificity of the anti-GM2 antibodies.

At present, the role of CMV in the pathogenesis of GBS is unknown. Direct infection of peripheral nerves is unlikely, as the CMV genome was not detected in sural nerve biopsies of CMV infected GBS patients (14). Serum anti-GM2 antibodies have been demonstrated to bind with CMV infected cells (7), indicating that these antibodies in GBS may be induced by antecedent CMV infection. Only one patient with a CMV infection without neurological involvement had anti-GM2 antibodies. This suggests that CMV infections are not the only factor determining the production of anti-GM2 antibodies and the development of immune mediated polyneuropathy. Further research is needed to investigate whether anti-GM2 antibodies are induced by CMV infections, and whether they are involved in the pathogenesis of CMV related GBS.

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CHAPTER 3.5

CLINICAL SIGNIFICANCE OF THE SPECTRUM OF ANTI-GANGLIOSIDE ANTIBODIES IN GUILLAIN-BARRÉ SYNDROME

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ABSTRACT

The relation between clinical heterogeneity and antibodies against gangliosides in Guillain-Barré syndrome (GBS) has thus far been studied only in small groups of patients or with few gangliosides. Therefore, we studied the association between antibodies against a panel of gangliosides and the clinical symptoms in 154 well-documented GBS patients. Pretreatment serum samples from these patients, and from 63 patients with other neurological diseases (OND) and 50 normal controls (NC) were tested for IgM, IgG and IgA antibodies against LM1, GM1, GM3, GD1a, GD1b, GT1b and GQ1b by enzyme-linked immunosorbent assay and thin-layer chromatography overlay. Anti-ganglioside antibodies were detected in 44 (29%) GBS patients, 8 (13%) OND patients and 1 (2%) NC. In 28 (64%) of these 44 GBS patients antibodies were found against multiple gangliosides. Anti-LM1 antibodies were demonstrated in only 9 (6%) GBS patients, and their presence was not related to a specific clinical presentation. Anti-GM1 antibodies were associated with a rapidly progressive and more severe pure motor GBS, irrespective of the presence of anti-GD1b antibodies. Anti-GD1a antibodies were also associated with more severe weakness. Anti-GQ1b antibodies were related to respiratory insufficiency and involvement of oculomotor, trochlear, and abducens nerves. Antecedent *C. jejuni* infections were associated with antibodies against GM1 and GD1b. This study demonstrates a wide spectrum of anti-ganglioside antibody specificities in GBS, which may modulate the clinical features and thereby contribute to the heterogeneity.

INTRODUCTION

The Guillain-Barré syndrome (GBS) is a monophasic polyneuropathy (PNP), which presumably results from an infection induced transient immune response to peripheral nerves. GBS patients show a wide variation in clinical and pathological features. This heterogeneity may result from differences in severity and specificity of the immune responses between individual GBS patients (1).

Several lines of evidence suggest that antibodies against peripheral nerve gangliosides play a role in the pathogenesis and heterogeneity of GBS. Firstly, antibodies against various gangliosides have been demonstrated in subgroups of GBS patients (2-17). Gangliosides form a large and heterogeneous family of sialylated glycolipids which have a specific function, distribution and accessibility to antibodies (18). The predominant gangliosides in human peripheral nerve myelin are LM1, GM3, GD1b and GD3 (3,19), and in axons GM1, GD1a, GD1b and GT1b (see Appendix 2) (20). Some gangliosides are related to specific nerve fibres, like GM1 and GD1a in myelin of motor nerves (19), and GQ1b in oculomotor nerves (9). Secondly, some anti-ganglioside antibodies are associated with specific clinical symptoms, reflecting the tissue location of the targets of these antibodies. Motor involvement is related to antibodies against GM1 (6,15), and GD1a (8,13) and ophthalmoplegia in GBS and Miller Fisher syndrome (MFS) to antibodies against GQ1b (9). Thirdly, *in vitro* studies indicate that anti-ganglioside antibodies may have a direct pathogenic effect on peripheral nerves. Serum with anti-GM1 antibodies causes conduction block (21), and affects voltage gated Na^+ channel function (22). Serum with anti-GQ1b antibodies interferes with neuromuscular transmission, probably by blocking acetylcholine release (23). Antecedent infections may trigger the transient production of these anti-ganglioside antibodies in GBS patients (1).

So far, most studies investigating the clinical relevance of anti-ganglioside antibodies in GBS were conducted in selected and small groups of patients which were tested for antibodies against one or a limited number of gangliosides. Detailed studies on the clinical associations with antibodies against several major peripheral nerve gangliosides, like LM1 and GD1b, are presently not available. Furthermore, some GBS patients have antibodies against several gangliosides. The relation between clinical symptoms and combinations of anti-ganglioside antibody activity has not been investigated either. Therefore, we investigated the clinical relevance of serum antibodies against seven major peripheral nerve gangliosides in 154 well-documented GBS patients.

PATIENTS AND METHODS

Patients

Serum samples were obtained from GBS patients who participated in the Dutch GBS trial comparing the therapeutic effect of plasma exchange (PE) and intravenous immunoglobulins (IVIg) (24), or in the pilot study evaluating the effect of methyl-prednisolone and IVIg (MP-IVIg) (25). All patients fulfilled the criteria for GBS (26), were unable to walk 10 m independently and were admitted within 2 weeks of onset of weakness. The functional score and the Medical Research Council (MRC) sum score of 6 bilateral muscle groups, ranging from 60 (normal) to

0 (tetraparalytic), were determined at study entry and at 16 time points during a follow-up period of six months. Severity of sensory loss was evaluated and classified as described previously (27). Pretreatment serum samples from 154 of the 172 GBS patients were available for serological studies. The 18 excluded cases did not differ from the other patients with regard to their clinical manifestations and course of disease. Serum samples from 63 patients with other neurological diseases (OND) and from 50 normal controls (NC) were also tested. The group of OND included patients with chronic inflammatory demyelinating polyneuropathy (CIDP) (16), other immune-mediated PNP (8), non-immune-mediated PNP (7), multiple sclerosis (17), and other neurological disorders (15). All samples were taken from patients in the active phase of the disease before treatment was started, and were tested without knowledge of the clinical data.

Detection of antibodies against gangliosides

IgM, IgG and IgA antibodies against LM1 (sialosyl paragloboside), GM1, GM3, GD1a, GD1b, GT1b and GQ1b (see Appendix 2) were tested by enzyme linked immunosorbent assay (ELISA) and thin-layer chromatography (TLC) overlay, as reported previously (15). LM1 was isolated from human granulocytes according to methods previously described (28). Briefly, their lipid extract was chromatographied by a DEAE-Sepharose CL-6B column (Pharmacia Fine Chemicals, Uppsala, Sweden) followed by silica column and high-pressure liquid chromatography. On TLC the LM1 was demonstrated to be pure. Serum samples from all patients were tested in a dilution of 1:100 in ELISA. An isotype specific positive control sample was tested in each assay. To correct for inter-assay variations all extinctions were normalized against the positive control serum. Serum samples with an optical density (OD) of more than 3 standard deviations above the mean value of 50 NC sera were tested in a TLC overlay to exclude antibody binding to contaminants. Positive serum samples were tested again in ELISA, using serial dilutions starting at 1:100. The reciprocal of the highest dilution resulting in an OD higher than the cut-off value was then taken to be the titre.

In previous studies we described the frequency and clinical association of anti-GM1 antibodies and *Campylobacter jejuni* infections in the same GBS patients and controls (15).

Statistical analysis

Differences in proportions were tested with the Chi-square test without continuity correction or the two-tailed Fisher's exact test if appropriate. Differences in medians were tested with the Wilcoxon-Mann-Whitney *U* test. A p-value<0.05 was considered to be significant.

RESULTS

Anti-ganglioside antibodies were found in 44 (29%) of 154 GBS patients and less often in patients with OND (13%) (p=0.01) and NC (2%) (p<0.001) (Table 1). Anti-ganglioside antibodies of the IgG and IgA isotype were almost exclusively found in GBS patients (Table 1). Although in some GBS patients the anti-ganglioside antibodies were still present after six months, the titre declined in all of them (data not shown). No isotype switch of anti-ganglioside antibodies was found during follow-up.

Twenty-eight (64%) of the 44 GBS patients with anti-ganglioside antibodies had antibody activity against multiple gangliosides (Table 2). Significant associations were found between the presence of anti-GM1 andanti-GD1b antibodies, and between the presence of anti-GD1a, anti-GT1b and anti-GQ1b antibodies (Table 2). The overlap in anti-ganglioside activity was not associated with the isotype and titre of the antibodies.

TABLE 1. Frequency of anti-ganglioside antibodies in patients with GBS, OND and NC [a].

Patients	Isotype	Anti-ganglioside antibodies							
		LM1	GM1	GM3	GD1a	GD1b	GT1b	GQ1b	≥ 1 ganglioside
GBS	IgM	1 (1%) [b]	16 (10%) [b,c]	0	2 (1%)	11 (7%) [c]	0	3 (2%)	21 (14%) [c]
(n=154)	IgG	7 (5%)	22 (14%) [b,c]	2 (1%)	3 (2%)	16 (10%) [b,c]	2 (1%)	6 (4%)	34 (22%) [b,c]
	IgA	2 (1%)	11 (7%) [c]	0	1 (1%)	6 (4%)	0	1 (1%)	13 (8%) [c]
	≥ 1 isotype	9 (6%)	31 (20%) [b,c]	2 (1%)	5 (3%)	27 (18%) [b,c]	2 (1%)	6 (4%)	44 (29%) [b,c]
OND	IgM	3 (5%)	4 (6%)	0	0	2 (3%)	0	0	7 (11%)
(n=63)	IgG	0	0	0	0	0	0	0	0
	IgA	0	1 (2%)	0	0	0	0	0	1 (2%)
	≥ 1 isotype	3 (5%)	5 (8%)	0	0	2 (3%)	0	0	8 (13%)
NC	IgM	0	0	0	0	0	0	0	0
(n=50)	IgG	1 (2%)	0	0	0	0	0	0	1 (2%)
	IgA	0	0	0	0	0	0	0	0
	≥ 1 isotype	1 (2%)	0	0	0	0	0	0	1 (2%)

a. Abbreviations: GBS, Guillain-Barré syndrome; OND, other neurological diseases; NC, normal controls.
b. Significantly different from patients with OND ($p<0.05$).
c. Significantly different from NC ($p<0.05$).

TABLE 2. Additional anti-ganglioside activity in GBS patients with anti-ganglioside antibodies.

	N	None	Additional anti-ganglioside antibody activity [a]	
			Significant association ($p<0.05$)	No significant association
LM1	9	5 (56%)	GM3 (2)	GM1 (2), GD1a (1), GD1b (3), GT1b (1), GQ1b (1)
GM1	31	6 (19%)	GD1b (25)	GD1a (2), GT1b (1), GQ1b (1), LM1 (2)
GM3	2	0	GT1b (1), LM1 (2)	GD1a (1), GD1b (1), GQ1b (1)
GD1a	5	1 (20%)	GD1b (3), GT1b (2), GQ1b (3)	GM3 (1), GM1 (2), LM1 (1)
GD1b	27	1 (4%)	GM1 (25), GD1a (3), GT1b (2)	GM3 (1), GQ1b (2), LM1 (3)
GT1b	2	0	GM3 (1), GD1a (2), GQ1b (2)	GM1 (1), GD1b (2), LM1 (1)
GQ1b	6	3 (50%)	GD1a (3), GT1b (2)	GM1 (1), GM3 (1), GD1b (2), LM1 (1)
All	44	16 (36%) [b]		

a. Numbers of patients with antibodies against combinations of gangliosides are in parentheses.
b. Implicating that 28 (64%) of 44 GBS patients had antibodies against more than one ganglioside.

The OND patients with anti-ganglioside antibodies had CIDP or other immune-mediated PNP. One NC had anti-ganglioside antibodies, which were IgG against LM1.

Antecedent *C. jejuni* infections in GBS patients were associated with the presence of antibodies against GM1 ($p<0.001$), as published before (15), and GD1b ($p=0.006$), but not with antibodies against other gangliosides (Table 3). However, IgA antibodies against GD1a, GD1b, GQ1b and LM1 were only found in GBS patients with *C. jejuni* infections. In OND patients no association was found between *C. jejuni* infections and anti-ganglioside antibodies (data not shown).

Most GBS patients with anti-LM1 antibodies suffered from severe muscle weakness, indicated by the low MRC sum scores at entry and nadir, and from impairment of sensory and cranial nerves (Table 3). However, these clinical manifestations did not differ significantly from those in patients without anti-LM1 antibodies.

Antibodies against GM1 and GD1b were both associated with a rapidly progressive, more severe and predominantly distal weakness without paresthesias or sensory loss (Table 3), as was previously reported for anti-GM1 antibodies alone (15). We found no significant differences in the clinical features between patients with anti-GM1 and anti-GD1b antibodies compared to patients with only anti-GM1 antibodies (data not shown). Two patients had anti-GD1b antibodies without anti-GM1 antibodies. One of them also had antibodies against GD1a, GT1b and GQ1b.

Patients with anti-GD1a antibodies were older and suffered from a more severe weakness at entry and nadir compared to patients without these antibodies (Table 3). Mild sensory loss was found in one patient with antibodies against GD1a and GQ1b, and in one with antibodies against GD1a only. Ophthalmoplegia was present in two patients with anti-GD1a antibodies, who also had anti-GQ1b antibodies.

Anti-GQ1b antibodies were related to respiratory insufficiency and ophthalmoplegia (Table 3). Ophthalmoplegia was present in five (83%) of the six patients with anti-GQ1b antibodies compared to three (2%) of the 148 patients without these antibodies ($p<0.001$). Ophthalmoplegia was already present at entry and before nadir in four of

TABLE 3. Clinical and laboratory characteristics of GBS patients in association with anti-ganglioside antibodies [a].

	Anti-ganglioside antibodies					
	LM1 (n=9)	GM1 (n=31)	GD1a (n=5)	GD1b (n=27)	GQ1b (n=6)	None [b] (n=110)
Age	51 (16-75)	47 (9-77)	67 (35-79)*	47 (9-77)	66 (35-78)	46 (10-80)
Sex	2M/7F	18M/13F	2M/3F	13M/14F	2M/4F	58M/52F
MRC sum score at entry [c]	36 (6-53)	32 (0-50)*	27 (17-30)*	30 (1-48)*	26 (22-32)*	42 (11-56)
Days to nadir	8 (4-15)	6 (1-18)*	7 (5-12)	6 (1-18)*	9 (7-12)	9 (3-21)
Lowest MRC sum score [c]	21 (0-49)	20 (0-50)*	17 (9-26)*	21 (0-48)*	19 (0-30)*	36 (0-55)
Days to recovery [d]	69 (20-181)	55 (4-181)	69 (11-181)	55 (4-181)	83 (20-181)	55 (6-181)
Predom. distal weakness	2 (22%)	19 (61%)*	1 (20%)	16 (62%)*	0	35 (33%)
Respiratory insufficiency	5 (56%)	10 (32%)	4 (80%)	9 (33%)	5 (83%)*	44 (40%)
Cranial nerve impairment	7 (78%)	12 (39%)*	3 (60%)	12 (44%)	5 (83%)	68 (62%)
Ophthalmoplegia	1 (11%)	1 (3%)	2 (40%)*	2 (7%)	5 (83%)*	2 (2%)
Sensory loss	6 (67%)	9 (39%)*	2 (50%)	9 (47%)	4 (80%)	88 (91%)
Paresthesias	8 (89%)	17 (55%)*	3 (60%)	17 (63%)*	6 (100%)	96 (87%)
Diarrhoea	2 (22%)	12 (40%)*	2 (40%)	9 (35%)*	1 (17%)	11 (10%)
C. jejuni infection	3 (33%)	20 (65%)*	2 (40%)	14 (54%)*	2 (33%)	26 (23%)

a. Abbreviation: MRC, Medical Research Council. Data are expressed as medians (2.5-97.5% percentile) or numbers of patients (percentage).
b. No antibodies against the 7 gangliosides used in this study.
c. MRC sum score ranges from 60 (normal) to 0 (tetraparalytic).
d. Number of days to reach independent locomotion.
*. Significantly different from the subgroup of GBS patients without these specific anti-ganglioside antibodies ($p<0.05$).

the patients with anti-GQ1b antibodies. In one patient the presence of anti-GQ1b antibodies preceded the ophthalmoplegia. Serum from the three ophthalmoplegic patients without anti-GQ1b antibodies was also tested in 1:10 dilution to detect low titres of anti-GQ1b antibodies, but no additional patients were found to be positive. Severe weakness at entry and nadir was also found in anti-GQ1b positive patients, although this may be related to the additional activity against GM1 and GD1a in the most affected patients.

Detailed information on specific cranial nerve loss was available in 149 patients. The presence of anti-GQ1b antibodies was strongly associated with involvement of the oculomotor nerves ($p<0.001$) (Table 4). One anti-GQ1b positive patient without oculomotor weakness also had antibodies against GM1, GD1a, GD1b and GT1b. In patients with anti-GM1 antibodies facial palsy was significantly less often found than in patients without these antibodies ($p<0.001$).

Antibodies against GM3 and GT1b were not found to be associated with specific clinical manifestations (data not shown), but the number of patients with these antibodies was very small.

TABLE 4. Association between anti-ganglioside antibodies and specific cranial nerve involvement in 149 patients with GBS.

		Anti-ganglioside antibodies					
Cranial nerve	N [a]	LM1 (n=9)	GM1 (n=31)	GD1a (n=5)	GD1b (n=27)	GQ1b (n=6)	None [b] (n=105)
N.III	12	1 (11%)	2 (6%)	2 (40%)	3 (11%)	5 (83%)*	5 (5%)
N.IV	12	1 (11%)	2 (6%)	2 (40%)	3 (11%)	5 (83%)*	5 (5%)
N.V	9	0	1 (3%)	0	1 (4%)	1 (17%)	7 (7%)
N.VI	22	1 (11%)	1 (3%)	2 (40%)	2 (7%)	5 (83%)*	16 (15%)
N.VII	81	7 (78%)	8 (26%)*	3 (60%)	9 (33%)	5 (83%)	61 (58%)
N.IX/X	20	1 (11%)	4 (13%)	1 (20%)	3 (11%)	1 (17%)	13 (12%)
N.XI	8	0	4 (13%)	0	4 (15%)	0	4 (4%)
N.XII	10	1 (11%)	2 (6%)	1 (20%)	3 (11%)	2 (33%)	6 (6%)

a. Number of GBS patients with involvement of indicated cranial nerve.
b. No antibodies against the 7 gangliosides used in this study.
*. Significantly different from the subgroup of GBS patients without these specific anti-ganglioside antibodies ($p<0.05$).

DISCUSSION

This study demonstrates that the spectrum of anti-ganglioside antibodies in GBS patients is partly related to the clinical manifestations. This spectrum includes antibodies with a remarkable variety of fine specificities. In serum from 29% of 154 GBS patients we found antibodies against one or more of the major peripheral nerve gangliosides LM1, GM1, GM3, GD1a, GD1b, GT1b and GQ1b. Antibodies against these gangliosides (2-9,11-13,15,17), but also against minor peripheral nerve gangliosides like *N*-acetyl-galactosaminyl-GD1a (10), and GM1b (see Appendix 2) (14), were reported before, and the list of gangliosides will probably expand in the future.

Anti-ganglioside antibodies also differ with respect to their target carbohydrate epitopes and additional activity against other gangliosides. A considerable overlap was found between the presence of anti-GM1 and anti-GD1b antibodies, and between antibodies against GD1a, GT1b and GQ1b. Overlap may result from antibodies cross-reacting with shared oligosaccharide moieties in homologous gangliosides, like Gal(β1-3) GalNAc in GM1 and GD1b, NeuAc(α2-3)Gal(β1-3)GalNAc in GD1a and GT1b, and NeuAc(α2-8)NeuAc(α2-3)Gal in GT1b and GQ1b (see Appendix 2) (7). Alternatively or in addition, some patients may have several subpopulations of antibodies each recognizing different gangliosides (7).

Moreover, antibodies binding to these same ganglioside in laboratory assays may have different capacities to react with neural tissues, as was demonstrated for antibodies to GM1 (29). Antibody binding to gangliosides highly depends on the temperature and the presence of accessory lipids (30), which differ considerably between laboratory assays and neural tissue in the patients. The presumed pathogenetic effect and site of action of anti-ganglioside antibodies will depend on their ability to bind membrane-bound gangliosides in specific nerve fibres. Therefore, patients with the same serum anti-ganglioside activity in laboratory assays may vary in clinical symptoms.

LM1 is the predominant ganglioside in human peripheral nerve myelin. In previous studies anti-LM1 antibodies were demonstrated in 43 to 58% of GBS patients by TLC overlay (3,4), suggesting that LM1 is indeed a frequent target antigen in GBS. The percentage of anti-LM1 positive GBS patients was much lower in our and other studies using ELISA in addition to TLC overlay (7,11,17). Our report is the first to include a detailed description of the clinical manifestations in patients with anti-LM1 antibodies. Motor, sensory and cranial nerves were affected in most patients with anti-LM1 antibodies, which is in accordance with the ubiquitous distribution of LM1 in human peripheral nerves (3,19). However, the clinical symptoms in these patients did not differ significantly from those in patients without these antibodies. Additionally, the presence of a high titre of IgG antibodies against LM1 in a NC argues against the pathogenicity of these antibodies.

Anti-GM1 antibodies were found to be associated with a rapidly progressive, pure motor variant of GBS, independent of the presence of additional anti-GD1b activity. This may indicate that antibodies against GM1, whether directed against the Gal-(β1-3)GalNac-epitope or not, are associated with selective damage of motor nerves. This suggestion is in accordance with the preponderance of GM1, but not of GD1b, in the myelin of motor nerves compared to sensory nerves (19). The clinical relevance of

antibodies against GD1b alone was difficult to determine due to their low frequency in our patients.

Anti-GD1a antibodies in GBS patients were found to be associated with a pure motor variant with severe axonal degeneration in some studies (8,13), but not in others (11). Human peripheral nerve axons, and myelin of motor fibres have the highest concentrations of GD1a, while lower concentrations are found in the myelin of sensory nerves (19). In the present study, GBS patients with anti-GD1a antibodies suffered from severe muscle weakness, and some of them from mild sensory loss. This sensory nerve damage may result from antibodies against GD1a or GD1a-like glycoconjugates in these fibres.

We confirm the previously described high association between anti-GQ1b antibodies and ophthalmoplegia in GBS (9), although this association was not absolute. In one patient with anti-GQ1b IgG antibodies oculomotor weakness did not occur, but subclinical involvement of the oculomotor nerves could not be excluded. Alternatively, the fine-specificity of the anti-GQ1b antibodies in this patient may differ from those of the others with ophthalmoplegia, since additional antibody activity against GM1, GD1a, GD1b and GT1b was also demonstrated in this patient. Three (38%) of eight patients with ophthalmoplegia were negative for anti-GQ1b antibodies and may have antibodies against other oculomotor antigens. Anti-GQ1b antibodies were associated with oculomotor, trochlear and abducens palsy, which is in accordance with the presence of GQ1b in these nerves (9). In two patients with anti-GQ1b antibodies and ophthalmoplegia, the hypoglossal nerves were also affected. This is in accordance with the frequent hypoglossal deficits in patients with MFS. Also in patients with acute oropharyngeal palsy without ophthalmoplegia anti-GQ1b antibodies have been demonstrated (31). The concentration of GQ1b in the hypoglossal nerves is unknown.

Anti-ganglioside antibodies are also present in normal controls. These antibodies are mostly IgM and of low titres. In GBS patients IgG and IgA antibodies of relatively high titres predominate, which decrease with neurological improvement. This isotype switch and transient elevation of the titre may be induced by antecedent infections. In the present study, antibodies against GM1 and GD1b, and IgA antibodies against the other gangliosides were associated with *C. jejuni* infections. Binding studies with monoclonal anti-ganglioside antibodies indicate that *C. jejuni* lipopolysaccharides (LPS) contain a variety of ganglioside-like epitopes (32). The spectrum of anti-ganglioside specificities in GBS may result from variations in molecular mimicry between *C. jejuni* LPS and peripheral nerve gangliosides. Other infectious agents may also induce cross-reactive antibodies as is suggested by the reported association between anti-GM2 antibodies and CMV infections (16,33). and between anti-galactocerebroside antibodies and *Mycoplasma pneumoniae* infections in GBS (34).

In conclusion, we found a wide spectrum of antibody specificities against the major peripheral nerve gangliosides in a large group of GBS patients. Our results further suggest that the specificity of these antibodies may modulate the clinical features and thereby contribute to the clinical heterogeneity in GBS.

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CHAPTER 3.6

ELECTRODIAGNOSTIC FINDINGS RELATED TO ANTI-GM1 AND ANTI-GQ1B ANTIBODIES IN GUILLAIN-BARRÉ SYNDROME

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ABSTRACT

Antibodies against the gangliosides GM1 and GQ1b may induce conduction failure in mice. To investigate their possible site of action in the Guillain-Barré syndrome (GBS), we studied the relation between serum anti-GM1 and anti-GQ1b antibodies and electromyography in 124 GBS patients. Anti-GM1 antibodies were found in 22 (18%) and anti-GQ1b antibodies in 5 (4%) patients. Anti-GM1 antibodies were associated with low distal compound muscle action potential amplitudes and relatively high compound sensory nerve action potential (CSNAP) amplitudes. In none of the patients with anti-GQ1b antibodies, CSNAPs could be detected. Patients with anti-GM1 and anti-GQ1b antibodies were heterogenous with respect to electrodiagnostic features exclusive for demyelination or axonal degeneration, although the anti-GM1 positive patients tended to have more axonal degeneration. In conclusion, the electromyographic findings indicate selective and more severe damage of motor nerves in patients with anti-GM1 antibodies, while patients with anti-GQ1b antibodies have severe damage of sensory nerves. These antibodies may interfere with the electrophysiologic properties of different nerve fibres and thereby contribute to the clinical heterogeneity in GBS.

INTRODUCTION

The Guillain-Barré syndrome (GBS) is characterized by nerve conduction failure resulting in muscle weakness and sensory deficits. Different patterns of conduction failure, including conduction block, have been described in GBS patients, possibly reflecting different pathophysiological mechanisms (1). The variability of pathology found in obducted GBS patients further supports the presence of different pathophysiological subgroups (2).

Recently, it was suggested that antibodies against the gangliosides GM1 and GQ1b may have a direct pathogenic effect on nerve conduction. Serum from patients with these antibodies induces nerve conduction failure in a phrenic nerve/diaphragm preparation after passive transfer to mice (3-5). Anti-GM1 antibodies have been found in approximately 20-25% of large groups of GBS patients (6,7). Anti-GQ1b antibodies have been demonstrated in 5% of GBS patients (8). The association of anti-GM1 antibodies with a severe, pure motor variant (6,7,9) and of anti-GQ1b antibodies with ophthalmoplegia (8), suggests that these antibodies contribute to specific clinical manifestations in GBS.

Although these antibodies may play a role in the pathogenesis, the site of action in GBS patients has not been identified. The highest concentrations of GM1 are found in human peripheral nerve axons and in the myelin fraction of motor fibres (10,11). GQ1b is predominantly present in human oculomotor nerves but is also found in lower concentrations in dorsal and ventral roots (8). However, the possible site of action of anti-GM1 and anti-GQ1b antibodies will not only depend on the tissue distribution of the gangliosides, but also on their being shielded by other structures, their function and their accessibility to antibodies from the blood. If these antibodies contribute to the pathogenesis, the electrodiagnostic findings in GBS patients with anti-GM1 and anti-GQ1b antibodies would presumably reflect the localization of the antibodies on the nerves.

In this study we performed detailed serial electrodiagnostic studies and determined the presence of serum antibodies against GM1 and GQ1b in 124 GBS patients to determine the possible site of action of these antibodies in GBS.

MATERIALS AND METHODS

Patient population

The patients tested in this study were included in the Dutch GBS trial comparing the therapeutic effect of plasma exchange and intravenous immunoglobulins (12). All patients fulfilled the criteria for GBS (13), were unable to walk 10 m independently, and were admitted within 2 weeks of onset of weakness. A complete electrodiagnostic examination (EMG) from at least one timepoint and a pretreatment serum sample were available from 124 of the 147 GBS patients who participated in the trial. The excluded cases did not differ from the other patients regarding their clinical manifestations and course of disease.

Detection of antibodies against GM1 and GQ1b

IgM, IgG and IgA antibodies against GM1 and GQ1b were tested in an enzyme-linked immunosorbent assay (ELISA) as described previously (7). To correct for interassay variations

all extinctions were normalized against a positive control serum used in each assay. Serum samples with an optical density (OD) of more than 3 standard deviations above the mean value of 50 normal control sera were tested in a thin-layer chromatography (TLC) overlay, as described previously (7), to exclude antibody binding to contaminants in the GM1 and GQ1b preparations. Positive serum samples were tested again in ELISA, using serial dilutions starting at 1:100. The reciprocal of the highest dilution resulting in an OD higher than the cut-off value was then taken to be the titre. The samples were all tested without knowledge of the clinical and electrodiagnostic data. The clinical manifestations in relation to the presence of anti-GM1 antibodies in these GBS patients have been reported previously (7).

Electrodiagnostic examination

EMGs were performed on three occasions according to the trial protocol: within 2 days of randomization, 1 week later, and 1 month later. The EMG findings were not taken into account for inclusion in the trial. The methods of electrophysiological testing have been described in detail previously (14). In short, ulnar, median and peroneal nerves were used to determine the motor and sensory nerve conduction velocities (CV). Amplitudes and duration of the evoked motor and sensory responses were measured with surface electrodes. The abductor pollicis brevis, abductor digiti quinti and anterior tibial muscles were tested with concentric needle electrodes for the presence of denervation (fibrillation potentials or positive sharp waves) and the recruitment pattern on maximal voluntary effort.

The parameters included in EMG were: motor nerve CV, distal motor latencies (DML), F-response latency, compound muscle action potential (CMAP) amplitude at distal (wrist or ankle) and proximal (elbow or knee) stimulation, sensory nerve CV, compound sensory nerve action potential (CSNAP) amplitude at distal and proximal stimulation, recruitment pattern, and denervation potentials. Each electrodiagnostic variable was defined as abnormal if it fell outside the normal values in our laboratory (14). A severly decreased or absent recruitment pattern obtained on maximal voluntary effort was considered to be abnormal.

Conduction block (CB) was defined as an abnormal CMAP amplitude decay of at least 16% in ulnar nerve and 11% in median nerve if the CMAP amplitude at distal stimulation was $\geq$ 5 mV (14). In patients with a CMAP amplitude at distal stimulation < 5 mV, CB was defined as a decrease of at least 1 mV (14).

The patients were divided into five categories according to their EMG findings. We used the second EMG to study features indicating demyelination, since the clinical differences between the patients were most evident after 1 week. To study the axonal changes we used the third EMG, since most denervation potentials were found after 4 weeks. In the first group of patients, EMG showed features of demyelination with prolonged DML but without CB or decreased motor nerve CV, indicating distal demyelination. In the second group, features of demyelination were found with prolonged DML, CB and decreased motor nerve CV, indicating more randomly distributed demyelination. The EMG from the third group had features of both demyelination and axonal degeneration. The fourth group had denervation potentials without features of demyelination and reached independent locomotion within 55 days after randomization (the median time in the group of 124 patients), indicating that the axonal degeneration was presumably distally localized. The fifth group had denervation potentials without features of demyelination and reached independent locomotion 55 or more days after randomization, indicating that the axonal degeneration started more proximally. Residual patients were subgrouped as unclassifiable. Twenty-nine patients were excluded from this analysis because the data from the second and third EMG were incomplete.

Statistical analysis

Differences in proportions were tested with the Chi-square test without continuity correction or the Fisher's exact test, and differences in medians with the Wilcoxon-Mann-Whitney *U* test. A p-value<0.05 was considered to be significant.

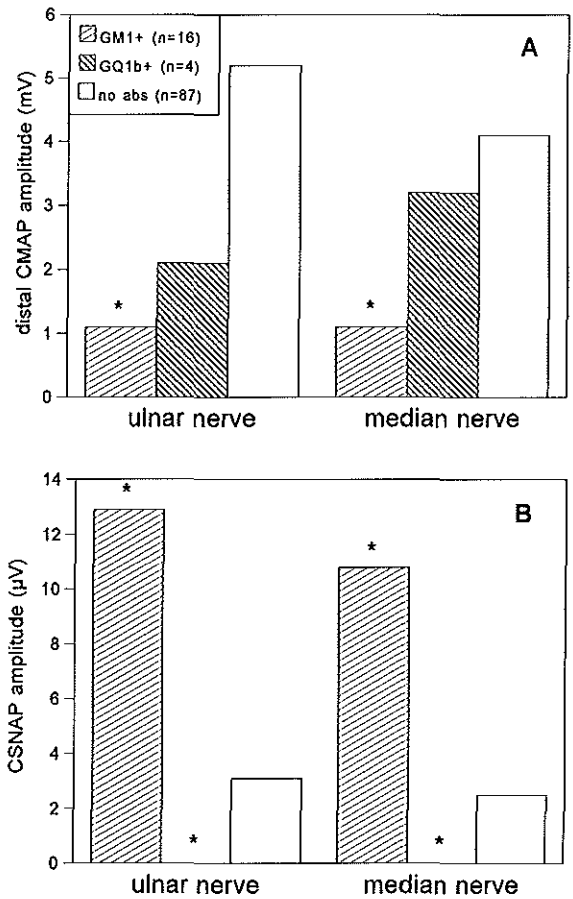

Figure 1. Median values of distal CMAP amplitudes (**A**) and CSNAP amplitudes (**B**) in the median and ulnar nerve 1 week after randomization in GBS patients with anti-GM1 antibodies ($GM1^+$), with anti-GQ1b antibodies ($GQ1b^+$) and without anti-GM1/GQ1b antibodies (no abs). Significantly different from patients without anti-GM1/GQ1b antibodies ($p<0.05$) (*).

RESULTS

Antibodies

Elevated titres of serum anti-GM1 antibodies were detected in 22 (18%) of the 124 GBS patients. IgM anti-GM1 antibodies were found in 13 (10%), IgG in 15 (12%), and IgA in 9 (7%) GBS patients. Elevated titres of anti-GQ1b antibodies were found in 5 (4%) patients; all had IgG antibodies, 2 had IgM, and 1 had IgA antibodies against GQ1b. None of the patients had both anti-GM1 and anti-GQ1b antibodies.

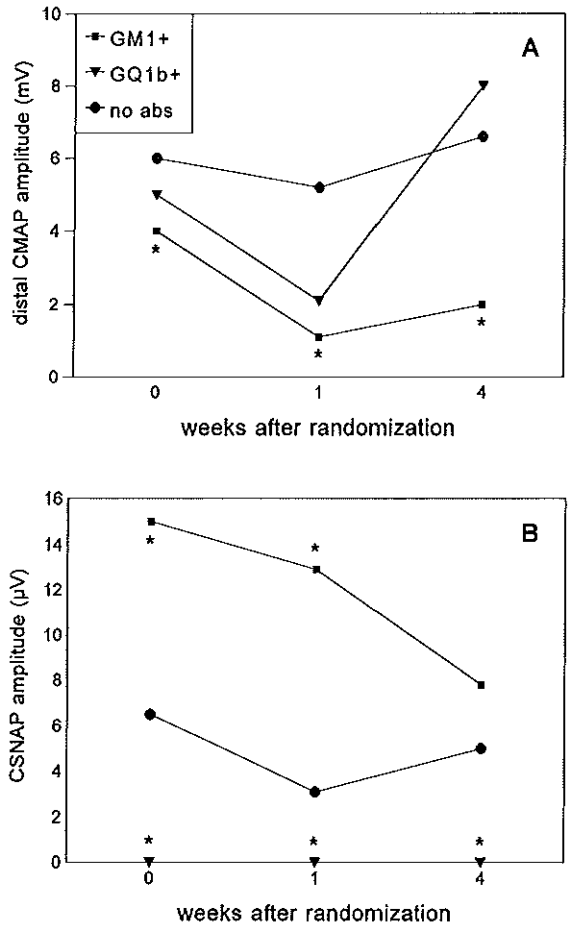

Figure 2. Follow-up of median values of distal CMAP amlitudes (**A**) and CSNAP amplitudes (**B**) in the ulnar nerve in GBS patients with anti-GM1 antibodies ($GM1^+$), with anti-GQ1b antibodies ($GQ1b^+$) and without anti-GM1/GQ1b antibodies (no abs). Significantly different from patients without anti-GM1/GQ1b antibodies ($p<0.05$) (*).

CMAP and CSNAP amplitudes

The patients with anti-GM1 antibodies had significantly lower distal CMAP amplitudes in the second EMG than the patients without anti-GM1 and anti-GQ1b antibodies (ulnar nerve, p=0.003; median nerve, p=0.006) (Fig. 1A). Anti-GM1 antibodies of the IgM, IgG and IgA isotype were all associated with lower CMAP amplitudes. The difference in distal CMAP amplitudes between patients with anti-GM1 antibodies and those without was already present in the first EMG of the ulnar nerve, shortly after randomization, but increased in the second EMG and persisted in the third EMG (Fig. 2A). The distal CMAP amplitudes in the patients with anti-GQ1b antibodies was lower than in the patients without antibodies, but this difference was not statistically significant.

The patients with anti-GM1 antibodies had significantly higher CSNAP amplitudes in the second EMG than the patients with anti-GQ1b antibodies (ulnar nerve, p=0.008; median nerve, p=0.005) or those without anti-GM1 and GQ1b antibodies (ulnar nerve, p=0.005; median nerve, p=0.003) (Fig. 1B). In 4 of 5 patients with anti-GQ1b antibodies no CSNAPs were detectable from either ulnar or median nerves in the first and second EMG. One patient with anti-GQ1b antibodies had a normal CSNAP amplitude of the median nerve in the first EMG. At that time the patient did not suffer from ophthalmoplegia. At the later time points the EMG showed an inexcitable sensory median nerve and the patient developed ophthalmoplegia. In this patient the presence of anti-GQ1b antibodies preceded both the sensory nerve inexcitability and ophtalmoplegia.

Nerve conduction

The changes in DML and motor nerve CV in the second EMG were not significantly associated with the presence of anti-GM1 or anti-GQ1b antibodies (Table 1). The sensory nerve CVs were higher in the patients with anti-GM1 antibodies compared to those without. In the patients with anti-GQ1b antibodies the sensory ulnar and median nerves were inexcitable in the second EMG despite supramaximal stimulation.

Needle EMG studies

The most obvious associations between anti-ganglioside antibodies and findings in needle EMG could be demonstrated in the third registration (4 weeks after randomiza-

TABLE 1. Nerve conduction characteristics in relation to antibodies against GM1 and GQ1b at 1 week after randomization [a].

Test	Anti-GM1 antibodies		Anti-GQ1b antibodies		No anti-GM1/GQ1b antibodies	
Motor ulnar nerve						
DML (ms)	3.3	(17)	2.6	(4)	3.6	(88)
CV (m/s)	55	(17)	54	(4)	57	(87)
Motor median nerve						
DML (ms)	3.8	(16)	3.7	(4)	5.4	(89)
CV (m/s)	52	(16)	60	(4)	52	(87)
Sensory ulnar nerve						
CV (m/s)	58	(17)*	0	(4)*	48	(43)
Sensory median nerve						
CV (m/s)	61	(17)	0	(4)*	54	(43)

a. Data are presented as medians with number of patients in parentheses. Abbreviations: DML, distal motor latency; CV, conduction velocity.

*. Significantly different from patients without anti-GM1/GQ1b antibodies ($p<0.05$).

tion) (Table 2). An abnormal recruitment pattern on maximal voluntary effort was more often found in the patients with anti-GM1 antibodies than in those without. This difference was already present in the first EMG registration.

Electrodiagnostic evidence of denervation (fibrillation potentials or positive sharp waves) was more often found in patients with anti-GM1 antibodies compared to those without, but the difference was only significant in the ulnar nerve (Table 2). In the patients with anti-GM1 or anti-GQ1b antibodies, as in most patients without antibodies, denervation potentials in ulnar and median nerves were only found in the third EMG and not in the first two. Severe axonal damage, indicated by the combination of denervation potentials, inexcitable motor nerves, and poor prognosis, was found in 5 (26%) of 17 patients with anti-GM1 antibodies and in none of the 5 patients with anti-GQ1b antibodies. The percentage of severe axonal damage was significantly higher in anti-GM1 positive patients than in the 77 patients without anti-GM1 or anti-GQ1b antibodies (3%) (p=0.002).

Demyelination

Electrodiagnostic criteria for demyelination in GBS patients were defined previously (14). According to these criteria, 9 (53%) of the 17 anti-GM1 positive patients and 2 (50%) of the 4 anti-GQ1b positive patients suffered from demyelination of motor nerves (Table 3). These proportions were not significantly different from the patients without anti-GM1 or anti-GQ1b antibodies. The frequency of conduction block did not differ between the three groups. The distribution of the demyelination, distal or at random, and the probable localization of the axonal degeneration were not associated with the presence of anti-ganglioside antibodies either (Table 4).

TABLE 2. Needle EMG characteristics in relation to antibodies against GM1 and GQ1b at 4 weeks after randomization [a].

Test	Anti-GM1 antibodies	Anti-GQ1b antibodies	No anti-GM1/GQ1b antibodies
Ulnar nerve			
Denervation potentials [b]	6/17 (35%)*	1/5 (20%)	9/67 (13%)
Abnormal recruitment pattern [c]	8/16 (50%)*	3/5 (60%)	13/68 (19%)
Median nerve			
Denervation potentials [b]	4/17 (24%)	1/5 (20%)	10/68 (15%)
Abnormal recruitment pattern [c]	7/15 (47%)	3/5 (60%)	17/72 (24%)

a. Data are presented as numbers of positive patients /numbers of tested patients with percentages in parentheses.
b. Fibrillation potentials or positive sharp waves.
c. Severely decreased or absent recruitment pattern.
*. Significantly different from patients without anti-GM1/GQ1b antibodies ($p<0.05$).

TABLE 3. EMG characteristics of demyelination in relation to antibodies against GM1 and GQ1b at 1 week after randomization [a].

EMG criteria for demyelination [b]	Anti-GM1 antibodies	Anti-GQ1b antibodies	No anti-GM1/GQ1b antibodies
1. DML > 150% of ULN	4/16 (25%)	1/4 (25%)	45/90 (50%)
2. Motor nerve CV < 70% of LLN	0/16 (0%)	0/4 (0%)	8/88 (9%)
3. F-wave latency > 150% of ULN	1/7 (14%)	0/2 (0%)	3/54 (6%)
4. CMAP amplitude decay > ULN	3/16 (19%)	0/4 (0%)	13/93 (14%)
5. Distal CMAP duration > 300% of ULN	0/13 (0%)	0/3 (0%)	2/66 (3%)
6. Distal to proximal CMAP duration ratio > 150% of ULN	2/13 (15%)	1/3 (33%)	3/64 (5%)
7. At least one of 1 to 6	9/17 (53%)	2/4 (50%)	56/93 (60%)

a. Data are presented as numbers of positive patients /numbers of tested patients with percentages in parentheses. Abbreviations: DML, distal motor latency; ULN, upper limit of normal; CV, conduction velocity; LLN, lower limit of normal; CMAP, compound muscle action potential.

b. Parameters 1 to 6 were tested in ulnar, median and peroneal nerve and considered to be abnormal when demonstrated in at least 2 nerves. The ULNs and LLNs were defined previously (14).

TABLE 4. EMG categories in relation to antibodies against GM1 and GQ1b [a].

EMG categories	Anti-GM1 antibodies	Anti-GQ1b antibodies	No anti-GM1/GQ1b antibodies
Distal demyelination	2 (14%)	1 (25%)	15 (20%)
More random demyelination	4 (29%)	1 (25%)	28 (36%)
Demyelination and axonal degeneration	3 (21%)	0 (0%)	12 (16%)
Distal axonal degeneration	0 (0%)	0 (0%)	1 (1%)
More proximal axonal degeneration	2 (14%)	1 (25%)	4 (5%)
Unclassifiable	3 (21%)	1 (25%)	17 (22%)
Total	14	4	77

a. Data are presented as numbers of patients with percentages in parentheses.

DISCUSSION

The present study demonstrates that anti-GM1 and anti-GQ1b antibodies in GBS patients are associated with two distinct electrodiagnostic patterns, suggesting that these antibodies have pathophysiological effects on specific subsets of nerve fibres.

Anti-GM1 antibodies may preferentially be involved in the conduction failure of motor nerves. Patients with anti-GM1 antibodies more often have a low distal CMAP amplitude and abnormal recruitment pattern than patients without these antibodies. In accordance with this finding, patients with anti-GM1 antibodies have a more severe and pure motor variant of GBS (6,7,9). However, mild sensory deficits and paresthesias also occur in some of these patients. The normal CSNAP amplitudes and conduction velocities in the majority of these patients indicate relative sparing of the sensory fibres. Abnormally low CSNAP amplitudes in patients with anti-GM1 antibodies occurred more often in the third EMG when the CMAP amplitudes were already very low. This suggests that in these patients the primary attack is directed against motor nerves, which may cause bystander damage of sensory nerves in severe cases.

The anti-GM1 positive subgroup is heterogeneous with respect to the electrodiagnostic patterns associated with demyelination and axonal degeneration, as was reported by others (15). Low distal CMAP amplitudes are compatible with axonal degeneration and with distal demyelination. The combination of low CMAP amplitudes and relatively spared DML in most patients with anti-GM1 antibodies suggests axonal changes. Denervation potentials and inexcitable motor nerves were also more often demonstrated in patients with anti-GM1 antibodies. These signs of axonal degeneration were rarely found in the first EMGs. In a subgroup of these patients the primary attack may be against the myelin. The finding that some anti-GM1 positive patients with electrodiagnostic signs of demyelination did not develop axonal degeneration supports this hypothesis. The extent of the axonal degeneration may be related to the heterogeneity of the anti-GM1 antibodies with respect to isotype, titre and fine-specificity.

The electrodiagnostic findings in GBS patients with anti-GM1 antibodies are partly in accordance with the distribution of GM1 on peripheral nerves. The highest concentra-

tions of GM1 have been demonstrated in the axons of both motor and sensory nerves (10). However, gangliosides are frequently shielded and not available for antibody binding on the cell surface (16). The preferential involvement of motor fibres may be related to the higher concentration of GM1 in the myelin of human motor compared to sensory nerves (11). Immunohistochemical studies have shown that human anti-GM1 antibodies bind with motor neurons and not with dorsal root ganglion cells (17). Another cause for the predilection of motor fibres may be their higher susceptibility to the effect of anti-GM1 antibodies, e.g., by a better access at the nerve terminals, where the blood-nerve barrier is deficient.

The present study suggests that anti-GQ1b antibodies may be involved in the conduction failure of sensory fibres of peripheral nerves. All patients with anti-GQ1b antibodies had inexcitable sensory nerves during the course of their disease, although clinical sensory deficits were generally absent or mild. In patients with the Miller Fisher syndrome (MFS), which is highly associated with the presence of anti-GQ1b antibodies (8,18,19), absent sensory responses have been reported also (15,20). Areflexia combined with lack of muscle weakness in MFS patients further suggests that sensory fibres in peripheral nerves are selectively damaged.

GQ1b is present in high concentrations in the oculomotor nerves (8), which parallels the ophthalmoplegia found in all GBS patients with anti-GQ1b antibodies in this study. Low concentrations of GQ1b have been found in sensory nerves, and a mouse monoclonal antibody against GQ1b weakly stains the dorsal root ganglia (8). Antibodies against GQ1b may bind with other structures on sensory nerves like GD1b, since additional anti-GD1b activity was demonstrated in some patients with anti-GQ1b antibodies (8,18,19). We found anti-GD1b antibodies in only 1 of the anti-GQ1b positive patients and in the majority of the anti-GM1 positive patients (data not shown). These findings indicate that antibodies against GD1b are probably not responsible for sensory nerve damage in the anti-GQ1b positive GBS patients.

Anti-ganglioside antibodies may cause conduction failure by demyelination through complement activation (21). In addition, antibodies against GM1 and GQ1b may have a direct effect on nerve conduction. Passive transfer and direct application of serum from patients with anti-GM1 or anti-GQ1b antibodies induces conduction block in a mouse phrenic nerve/diaphragm preparation (3-5). Rabbit monoclonal anti-GM1 antibodies can interfere with both voltage sensitive potassium and sodium currents in rat nerve fibres (22). The direct pathophysiological effects of these antibodies suggest that demyelination is not the only mechanism resposible for conduction failure in GBS patients.

In conclusion, the electrophysiological data in the present study give further support to the hypothesis that anti-GM1 and anti-GQ1b antibodies interfere with nerve conduction in distinct subgroups of nerve fibres in GBS patients.

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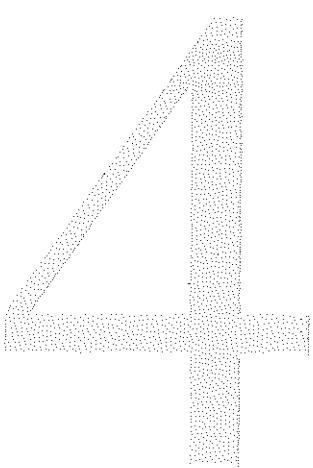
MOLECULAR MIMICRY BETWEEN *CAMPYLOBACTER JEJUNI* AND GANGLIOSIDES



MOLECULAR MIMICRY BETWEEN *CAMPYLOBACTER JEJUNI* AND GANGLIOSIDES

CHAPTER 4.1

INTRODUCTION

Antecedent infections may induce the immune response to peripheral nerves in the Guillain-Barré syndrome (GBS). In Chapter 2 we described that 68% of the GBS patients reported clinical symptoms of a preceding infection. Based on serology studies we identified a recent infection in 56% of the patients (Chapter 2). *Campylobacter jejuni* was found to be the predominant cause of antecedent infections in GBS.

The mechanism by which *C. jejuni* and other infectious agents related to GBS may trigger the immune response to peripheral nerves is presently not elucidated. Possibly they induce an immune response to peripheral nerve gangliosides, because GBS patients with a *C. jejuni* infection significantly more frequently have serum antibodies against GM1 and GD1b (Chapters 3.2 and 3.5). Antibodies to GM2, GD1a, GQ1b and LM1 were also found in patients with a *C. jejuni* infection. IgA anti-ganglioside antibodies were almost exclusively produced in patients with a *C. jejuni* infection, which further strengthens the relation with enteric infections. Recently, others reported that antibodies against *N*-acetyl-galactosamine-GM1b and *N*-acetyl-galactosamine-GD1a were also highly associated with *C. jejuni* infections (1).

C. jejuni infections precede the neurological symptoms also in patients with the Miller Fisher syndrome (MFS) (2-4). MFS is considered to be a variant of GBS, and is characterized by ophthalmoplegia, ataxia and areflexia (5). MFS is highly associated with the presence of serum antibodies against the ganglioside GQ1b (6-8). This antibody specificity reflects the clinical manifestations in MFS, since GQ1b is predominantly found in oculomotor nerves (8). *C. jejuni* may therefore also be involved in the production of anti-GQ1b antibodies in MFS patients.

Molecular mimicry between microbial and host antigens may be one of the mechanisms by which infections can lead to transient high titres of autoantibodies (9). Interestingly, biochemical analysis has demonstrated ganglioside-like structures in the lipopolysaccharides (LPS) from *C. jejuni* isolates from patients with GBS and MFS (10-12). Infections with *C. jejuni* expressing these strucures may lead to the formation of antibodies which cross-react with particular gangliosides in peripheral nerves and induce nerve dysfunction.

Antecedent *C. jejuni* infections may induce cross-reactive anti-ganglioside antibodies in patients with GBS and MFS. The studies in Chapter 4 tested this hypothesis, by investigating if serum anti-ganglioside antibodies from patients with GBS and MFS bind with whole bacteria and LPS fractions of *C. jejuni*. The titre course, isotype, and fine-specificity of these cross-reactive antibodies were also determined to further characterize the humoral immune response against *C. jejuni* LPS in patients with GBS and MFS. Since the specificity of anti-ganglioside antibodies and the ganglioside-like structures in LPS are extremely heterogeneous, we focused on anti-ganglioside antibodies and *C. jejuni* isolates from GBS and MFS patients themselves. The cross-reactivity of the serum anti-ganglioside antibodies from GBS and MFS patients with *C. jejuni* bac-

teria and LPS was investigated with inhibition enzyme-linked immunosorbent assay (ELISA).

To further characterize the ganglioside-like structures in *C. jejuni* LPS, we used purified monoclonal anti-ganglioside antibodies from patients with chronic ataxic neuropathy (13,14). These monoclonal antibodies enabled us to demonstrate direct binding of anti-ganglioside antibodies with LPS in ELISA and thin-layer chromatography overlay. Control experiments with sialidases and cholera toxin were conducted to exclude aspecific binding of anti-ganglioside antibodies with LPS. In addition to the isolates from the MFS and GBS patients, we used *C. jejuni* isolates from enteritis patients without neuropathy.

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CHAPTER 4.2

ANTI-GM1 IGG ANTIBODIES AND *CAMPYLOBACTER* BACTERIA IN GUILLAIN-BARRÉ SYNDROME: EVIDENCE OF MOLECULAR MIMICRY

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ABSTRACT

In Guillain-Barré syndrome (GBS) antibodies to GM1 and the presence of an antecedent *Campylobacter jejuni* infection are both correlated with a more severe course of the disease. From a group of 137 consecutive GBS patients, 11 sera had elevated titres of anti-GM1 IgG antibodies during the acute stage of disease. Each serum sample was preincubated with three different Penner serotypes of whole *C. jejuni* (PEN O:41, PEN O:4,59) and *Campylobacter coli* (PEN O:22) bacteria. The PEN O:4,59 serotype, isolated from the stools of a GBS patient, inhibited 63 to 93% of the anti-GM1 activity in 6 of 11 patients. The PEN O:41 inhibited 63 to 100% of the anti-GM1 antibody activity in 9 of 11 patients. The PEN O:22 inhibited anti-GM1 antibody activity in only 2 of 11 patients (80 and 86%). Two GBS 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-GM1 antibodies. The results of this study indicate that anti-GM1 IgG antibodies in GBS sera recognize surface epitopes on whole *Campylobacter* bacteria and that this recognition is strain-specific. This provides evidence for the involvement of molecular mimicry in the pathogenesis of GBS.

INTRODUCTION

Elevated antibody titres 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-GM1 IgM and IgG antibody titres in 41 (30%) of 137 GBS patients (4,5). Elevated anti-GM1 antibody activity was significantly correlated with a more severe disease course (5). Serological evidence of an antecedent *Campylobacter jejuni* correlated significantly with the presence of anti-GM1 antibodies (5), in accordance with other reports (6,7). This association led to the hypothesis that molecular mimicry between antigens on *Campylobacter* bacteria and GM1 or other glycoconjugates on peripheral nerve tissue could be an important autoimmune mechanism in the pathogenesis of a subgroup of GBS patients. We tested this hypothesis in all 11 GBS patients with high IgG antibody titres against GM1 by measuring the inhibition of anti-GM1 antibody activity in absorption experiments with different strains of *C. jejuni* and *C. coli* bacteria.

PATIENTS AND METHODS

Patients and healthy controls

A group of 147 consecutive GBS patients involved in the Dutch Guillain-Barré trial (5) was routinely screened for serum anti-GM1 IgG and IgM antibodies. All 11 GBS patients with high anti-GM1 IgG antibody titres 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-GM1 antibodies.

Campylobacter serology and serotyping

Serum IgM, IgG and IgA antibodies against *C. jejuni* were determined by an enzyme-linked immunosorbent assay (ELISA) (8). *C. jejuni* isolates from the stools of 25 patients without neurological involvement and from one 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 (LPS).

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 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 solution (PBS) with 1% formaldehyde, centrifuged at 10,000*g*, washed three times in PBS, and suspended in PBS. After the dry-weight concentration was determined, suspensions were aliquotted 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 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, Campylobacter* 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-GM1 IgG activity was measured in an ELISA and compared with the antibody activity in unabsorbed serum. The 96-well plates were coated with 100 µl of ethanol containing 2 µg bovine GM1-ganglioside (Sigma, St. Louis, MO) per well. After overnight ethanol evaporation, the plates were washed five times in 0.01 M PBS. 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 containing 0.05% Tween 20. As second antibody, peroxidase-labelled goat 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*-phenyl diamine (Sigma) and 0.012% hydrogen peroxide in 0.1 M citrate buffer. After 5 minutes the reaction was stopped with 4 M sulphuric acid and the optical densities (OD) were read at 492 nm with a Titretek Multiscan MCC (Labsystems, Finland). Antibody activity against the glycolipids asialo-GM1 (GA1), GD1a, GD1b and GT1b (Sigma), was also tested in ELISA.

Other control experiments

The possibility of aspecific binding of IgG to the bacteria led to absorption studies being performed using sera from systemic lupus erythematosus (SLE) patients with high levels of anticardiolipin IgG antibodies. Anticardiolipin antibodies 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 GM1 on the ELISA plate and inhibit anti-GM1 IgG binding. This would give a false-positive absorption of anti-GM1 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 bacteria solutions. After washing 5 times in PBS-1% BSA, patient serum was added as a second step followed by the procedure as mentioned.

Calculation of the antibody titre 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. Logit Y was calculated as:

$$\ln [(OD/ODmax)/(1-OD/ODmax)].$$

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-GM1 antibody titre. The anti-GM1 IgG antibody

activity following preincubation of patient serum with the different bacteria was compared with the antibody activity of PBS-incubated serum. A decrease of anti-GM1 IgG antibody activity after preincubation with one of the serotypes of *Campylobacter* was considered to be caused by specific antigen-antibody recognition on the bacteria surface only if the preincubation with *E. coli* did not show such a decrease in titre. The inhibition percentage of anti-GM1 IgG antibody activity was used as a measure of specific antibody binding to bacterial surface antigens and was calculated as:

$$\frac{\text{antibody titre (serum without bacteria) - antibody titre (serum preincubated with bacteria)}}{\text{antibody titre (serum without bacteria)}} \times 100\%.$$

RESULTS

Disease severity and antecedent infections

Clinical data of the 11 anti-GM1 IgG positive GBS patients used in this study 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 2 or 4 weeks after disease onset that was suggestive of axonal degeneration.

IgG antibodies to GM1, GD1a, GD1b, GT1b and GA1

The anti-GM1 IgG titres of the GBS sera ranged from 1:210 to 1:4,410, compared to titres from 0 to 1:50 in 50 healthy age- and sex-matched control subjects. No high serum anti-GM1 antibody titres 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-GM1 antibodies 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 nontypable.

The presence of antibody activity against GD1a, GD1b, GT1b and GA1 in the 11 anti-GM1 IgG positive GBS sera is given in Table 2. In 5 of 11 patients, additional IgG antibody binding to GD1b and GA1 was found. In 2 of the 11 patients, additional binding was found to GA1 and in 1 patient to GD1a.

TABLE 1. Clinical parameters of the 11 GBS patients with anti-GM1 IgG antibodies [a].

	Age (yrs)	Sex	Treatment	Anti-GM1 antibodies		*C. jejuni* infection [b]	Antecedent disease [c]	Sensory deficit	Recovery time [d]	Artificial respiration	Denervation activity
				IgG	IgM						
Patient 1	61	M	IVIg	+	–	+	–	–	69	–	+
Patient 2	74	M	PE	+	–	+	Diarrhoea	+	>181	+	+
Patient 3	57	F	IVIg	+	–	+	Diarrhoea	–	Dead	–	nt
Patient 4	9	M	IVIg	+	+	+	URTI	–	4	–	nt
Patient 5	47	M	IVIg	+	+	+	Diarrhoea	+	>181	+	+
Patient 6	15	M	IVIg	+	+	+	–	–	20	–	+
Patient 7	47	M	IVIg	+	–	–	URTI	+	20	–	–
Patient 8	77	F	IVIg	+	–	+	Diarrhoea	+	>181	–	+
Patient 9	67	F	PE	+	+	+	Diarrhoea	–	>181	–	+
Patient 10	73	F	PE	+	–	–	URTI	–	55	+	nt
Patient 11	19	M	PE	+	+	+	Diarrhoea	–	>181	–	nt

a. Abbreviations: M, male; F, female; IVIg, intravenous immunoglobulin; PE, plasma exchange; *C. jejuni*, *Campylobacter jejuni*; URTI, upper respiratory tract infection; nt, not tested; +, present; –, absent.

b. As indicated by positive *C. jejuni* serology according to criteria previously described (8).

c. As indicated by clinical symptoms in the 4 weeks preceding the onset of weakness.

d. Number of days to reach independent walking for > 10 m (F=2, according to Hughes score (13)). The patients were evaluated during a follow-up of 181 days.

TABLE 2. Presence of serum IgG antibodies to GM1, GD1a, GD1b, GT1b and GA1 in 11 GBS patients [a].

	GM1	GD1a	GD1b	GT1b	GA1
Patient 1	+	–	+	–	+
Patient 2	+	–	-	–	+
Patient 3	+	–	–	–	–
Patient 4	+	–	–	–	+
Patient 5	+	–	+	–	+
Patient 6	+	–	+	–	+
Patient 7	+	–	–	–	–
Patient 8	+	–	+	–	+
Patient 9	+	+	–	–	–
Patient 10	+	–	–	–	–
Patient 11	+	–	+	–	+

a.Abbreviations: GA1, asialo-GM1; +, present; –, absent (titre <100).

Antibody absorption experiments

An example of the ELISA results after preincubation of patient serum containing anti-GM1 antibodies 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-GM1 activity was found, whereas preincubation with *E. coli* and *C. coli* PEN O:22 did not give any inhibition of the signal when compared with the results of serum incubated with PBS. The percentages of inhibition of anti-GM1 IgG activity in the sera from 11 GBS patients by the four bacterial strains are given in Table 3. The inhibition percentagesshowed a bimodal distribution that defined a group with low (0 to 50%) and a group with high (60 to 100%) inhibition. Since all the *E. coli* results fell in the first group, we interpreted this low inhibition as caused by aspecific antibody absorption, and the high inhibition values as a result of specific antigen-antibody binding. This cut-off 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-GM1 IgG activity in 9 of 11patients; the PEN 100%) inhibition. Since all the *E. coli* results fell in the first group, we interpreted this low inhibition as caused by aspecific antibody absorption, and the high inhibition values as a result of specific antigen-antibody binding. This cut-off 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-GM1 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-GM1 IgG absorption with PEN O:4,59 were also inhibited by PEN O:41. Of these, 2 demonstratedabsorption with the PEN O:22 serotype as well. In 3 patient sera anti-GM1 IgG was inhibited exclusively by the PEN O:41 serotype. Only in Patient 3 and 7 no absorption with any of the three *Campylobacter* serotypes was found.

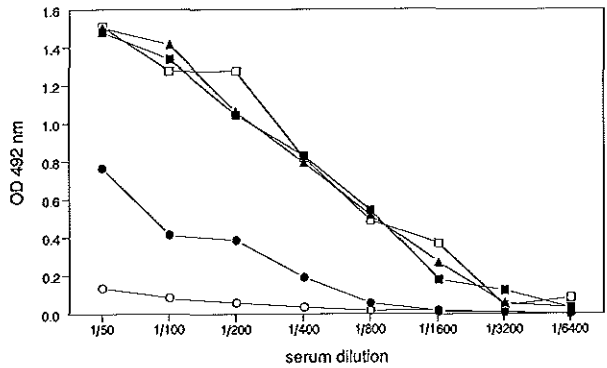

Figure. Example of the results in inhibition-ELISA. Anti-GM1 IgG activity in Patient 6 was measured at different serum dilutions following serum preincubation in PBS without bacteria (*open squares*), and with *E. coli* (*closed squares*), and three *Campylobacter* serotypes: PEN O:4,59 (*closed circles*), O:41 (*open circles*) and O:22 (*triangles*). Preincubation with O:4,59 and O:41 inhibits 93% and 100% of the serum anti-GM1 IgG activity, respectively, whereas preincubation with *E. coli* and O:22 does not result in any decrease in antibody titre.

TABLE 3. Inhibition of anti-GM1 IgG antibody activity in serum from 11 GBS patients by preincubation with *E. coli* and different *Campylobacter* strains.

		Percentage of inhibition by preincubation with [a]:			
	Anti-GM1 IgG titre (x10^2)	*E. coli*	*C. jejuni* O:4,59	*C. jejuni* O:41	*C. jejuni* O:22
Patient 1	3.1	47	76 (+)	63 (+)	80 (+)
Patient 2	9.9	0	0	82 (+)	37
Patient 3	3.1	9	0	38	2
Patient 4	5.1	2	63 (+)	100 (+)	16
Patient 5	2.1	0	71 (+)	75 (+)	0
Patient 6	12.0	7	93 (+)	100 (+)	20
Patient 7	3.2	0	33	23	37
Patient 8	31.0	8	80 (+)	92 (+)	0
Patient 9	44.1	0	31	83 (+)	0
Patient 10	2.1	29	74 (+)	89 (+)	86 (+)
Patient 11	21.9	0	1	86 (+)	0

a. Data represent the percentage of inhibition of antibody activity by preincubation with bacteria, as defined in the materials and methods. Percentages of inhibition higher than the mean + 2 standard deviations of the inhibitions found with *E. coli*, were considered to be specific and were indicated by (+).

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 antibodies. Preincubation of these sera with the *C. jejuni* and *E.coli* strains did not decrease the anticardiolipin titres measured by ELISA (data not shown).

Adhesins

Interference due to the presence of free adhesins was ruled out since no inhibition of anti-GM1 antibody activity was found using GM1-coated plates incubated with supernatant from the bacterial solutions (data not shown).

DISCUSSION

This study demonstrated that anti-GM1 IgG antibody 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:41, PEN O:22 and PEN O:4,59). This indicates the presence of surface epitopes on *Campylobacter* that cross-react with GM1. Others identified oligosaccharide structures in purified LPS fractions from *C. jejuni* serotypes PEN O:4 and PEN O:19 (16-19) and have shown that monoclonal anti-GM1 IgM antibody 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 antibodies to GM1 under the conditions of our absorption assay. This could be due to the lower titre of serum IgM antibodies compared to IgG in our GBS patients, or to the lesser affinity of these polyclonal IgM antibodies, compared to the monoclonal anti-GM1 antibodies used by Wirguin et al. (20). However, other as yet unknown factors related to the assay method may be involved.

The specific pattern of anti-GM1 IgG antibody absorption in the 11 GBS sera by the two *C. jejuni* and one *C. coli* serotypes, and the absence of clear antibody absorption by the *E. coli*, which also has a lipopolysaccharide capsule, illustrates the specificity the cross-reacting surface epitopes. In addition, the same bacteria did not absorb IgG antibodies to cardiolipin, which further indicates the lack of aspecific, false-positive binding.

Assessment of antibody reactivity to other gangliosides and GA1 demonstrated differences in binding patterns between the 11 patient sera. Five patient sera showed binding to GD1b and GA1, suggesting cross-reaction to the carbohydrate structure Gal(β1-3)GalNAc. Two sera reacted also with GA1, one with GD1a, and three others only to GM1 (Table 2). These binding patterns do not discriminate between cross-reactive antibodies and the presence of several different antibody specificities in the same sample. Therefore, we can not make definite conclusions as to the fine specificity of these antibodies. In agreement with the study of Wirguin et al. (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 antibody reactivity to GM1, GD1b and AGM1, GA1 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 antibody 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 GM1 could therefore be stimulated

aspecifically during a *C. jejuni* infection, giving high anti-GM1 antibody titres without pathogenic relevance. However, the serotype-specific antibody 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-GM1 antibodies 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-GM1 antibody titres in two patients with diarrhoea 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, the results of this study provided experimental evidence for the observed epidemiological correlation between the presence of anti-GM1 antibodies and *C. jejuni*. Anti-GM1 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-GQ1b IgG antibodies that recognized epitopes on *C. jejuni* in patients with Miller Fisher syndrome, using a similar assay method.

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CHAPTER 4.3

SERUM ANTI-GQ1B IGG ANTIBODIES RECOGNIZE SURFACE EPITOPES ON *CAMPYLOBACTER JEJUNI* FROM PATIENTS WITH MILLER FISHER SYNDROME

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ABSTRACT

Three patients who had diarrhoea prior to the development of Miller Fisher syndrome (MFS) are presented. *Campylobacter jejuni* was isolated from stool specimens from all patients. High titres of anti-GQ1b IgG antibodies were demonstrated in the serum of these patients by enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatography overlay. In ELISA inhibition studies the anti-GQ1b IgG antibodies bound specifically to whole bacteria of the MFS-associated *C. jejuni* strains. The presence of anti-GQ1b IgG binding epitopes on the surface of the *C. jejuni* from the MFS patients was not exclusively associated with a specific Penner serotype. It is suggested that anti-GQ1b antibodies are formed during the initial infection that elicits MFS. The cross-reactivity of anti-GQ1b IgG antibodies with surface epitopes on MFS-associated *C. jejuni* strains supports the hypothesis of molecular mimicry between bacteria and neural tissue.

INTRODUCTION

The syndrome described by Miller Fisher is characterized by the acute onset of ophthalmoplegia, ataxia, and areflexia and is considered to be a variant of the Guillain-Barré syndrome (GBS) (1). In the majority of patients, Miller Fisher syndrome (MFS) and GBS are preceded by an infection. In GBS patients, *Campylobacter jejuni* was identified as a frequent cause of antecedent infections (2-4). Also, patients with MFS or bilateral abducens paresis following *C. jejuni* infection have been described (5-8). A specific association was found between the presence of serum IgG antibodies to the ganglioside GQ1b and both MFS (9-11) and GBS with ophthalmoplegia (12). Although the origin of anti-ganglioside antibodies in MFS and GBS is unknown, it can be speculated that they are formed initially against bacteria during an antecedent infection. In this study we describe 3 patients in whom MFS developed after they had diarrhoea. *C. jejuni* was isolated from stool specimens of all 3 patients. We tested serum from these patients for the presence of antibodies against GQ1b and other glycolipids. Furthermore, we investigated whether these antibodies could recognize *C. jejuni*, which could suggest molecular mimicry between GQ1b and surface epitopes on MFS-associated *C. jejuni* strains.

PATIENTS AND METHODS

The clinical features and laboratory findings of the 3 patients with MFS are given in Table 1. The patients received supportive care and improved spontaneously without treatment with intravenous immunoglobulins or plasma exchange.

Serum samples were obtained on admission, which was within 1 week (patient B and C) or 2 weeks (patient A) after the onset of neurological symptoms, and 3 weeks after admission and were stored at -80°C until use. Cerebrospinal fluid (CSF) samples obtained on these occasions were available for further studies for patient A only.

C. jejuni was isolated from stool specimens from all 3 patients and serotyped according to the Penner classification system (13). This method is based on the passive hemagglutination technique which detects soluble heat-stable antigens that have been identified as lipopolysaccharides (LPS). Serum and CSF IgM, IgG, and IgA antibodies against *C. jejuni* were determined by an enzyme-linked immunosorbent assay (ELISA) (14).

Serum samples from 50 healthy blood donors were used as controls for anti-ganglioside antibody detection by an ELISA.

Enzyme-Linked Immunosorbent Assay

IgM, IgG and IgA antibodies against the glycolipids asialo-GM1 (GA1), GM1, GM2, GM3, GD1a, GD1b, GD2, GD3, GT1b, GQ1b, LM1, LK1 and globoside were tested in an ELISA. All antigens except GQ1b, LM1, and LK1 were purchased from Sigma (St.Louis, MO). GQ1b was purchased from Biocarb (Lund, Sweden). We isolated LM1 from human buffy coats and LK1 from human cauda equina. The purity of LM1 and LK1 was confirmed by thin-layer chromatography (TLC). ELISA was performed by coating 96-well polystyrene microtitre trays (Immuno Maxisorb, Nunc, Roskilde, Denmark) with 150 pmol of antigen in 100 µl of ethanol per well and evaporating to dryness. Unspecific binding sites were blocked with 200 µl of phosphate-buffered saline solution (PBS) (pH 7.4) containing 1% bovine serum albumin (BSA) (Sigma) for one hour at room temperature and subsequently for 30 minutes at 4°C. After blocking, the plates were incubated with 100 µl of serum diluted 1:100 in PBS-1% BSA per well for 4 hours at 4°C.

After washing six times with PBS, the plates were incubated for 90 minutes at room temperature with peroxidase-conjugated goat-antihuman IgM (μ-chain specific), peroxidase-conjugated goat-antihuman IgG (γ-chain specific), or peroxidase-conjugated goat-antihuman IgA (α-chain specific) (Sigma) diluted 1:2,500 in PBS-1% BSA. After six washings with PBS, the plates were developed with 0.05% *O*-phenyl diamine (Sigma) and 0.012% hydrogen peroxide in 0.1 M citrate buffer (pH 5.0). The reaction was stopped after 5 minutes with 2 M hydrochloric acid and the optical densities (OD) were read at 492 nm with a Titretek Multiscan MCC (Labsystems, Finland). All samples were assayed in duplicate. The serum was tested in both antigen-coated and blank wells, allowing the OD values and titres to be calculated after subtracting the blank well OD from the coated-well OD.

A serum sample was considered positive for anti-GQ1b antibodies if it had OD of more than 3 standard deviations above the mean value for 50 normal control sera. To confirm positivity, the same sample was tested again in a subsequent ELISA, using serial dilutions starting at 1:100. The reciprocal of the dilution that still resulted in OD larger than the cut-off value was then taken to be the titre.

Thin-Layer Chromatography Overlay

TLC was performed on silica gel 60 high-performance TLC plates (Merck, Darmstadt, Germany) that were coated with 300 pmol of each antigen and developed in chloroform/methanol/0.2% calcium chloride (50:40:10, by volume). After chromatography, the plates were dried under vacuum and dipped in a solution of 0.1% polyisobutylmethacrylate in *n*-hexane. After air-drying, the plates were blocked with PBS-1% BSA for 1 hour at 37°C and overlaid with serum diluted 1:50 in PBS-1% BSA and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20, the plates were incubated with alkaline phosphatase-conjugated goat-antihuman IgM (μ-chain specific) or with alkaline phosphatase-conjugated goat-antihuman IgG (γ-chain specific) (Sigma) diluted 1:1,000 in PBS-1% BSA for 90 minutes at room temperature. After washing with PBS-0.05% Tween 20, the plates were developed with 0.5 mg/ml 5-bromo-4-chloro-3-indolylphosphate in 0.1 M glycine-sodium hydroxide buffer (pH 10.4) for 1 hour at 30°C.

Binding of anti-ganglioside IgG antibodies to *C. jejuni*

The binding of serum anti-ganglioside antibodies to *C. jejuni* was investigated by measuring the inhibition of antibody activity after preincubation of each serum sample with various concentrations of whole bacteria of each *C. jejuni* strain. In the binding assays we used the highest serum dilution that still gave maximal OD (of at least 1.0 in all patients) in the ELISA. The *C. jejuni* strains were grown in brain-heart infusion broth in a microaerobic atmosphere. After 48 hours the bacteria were harvested, inactivated in 1% formaldehyde, and washed in PBS. In the experiments the concentrations of each bacteria strain were 12.5, 25, 50, 100, 200, and 400 μg/ml. Each serum sample was preincubated with *C. jejuni* from the 3 MFS patients and 3 control *C.jejuni* strains. The control strains (1, 2, and 3) were isolated from patients with diarrhoea but without neurological involvement and were serotyped according to the Penner classification system as PEN O:22, PEN O:23 and PEN O:41, respectively. Control strain 3 was known to be able to bind anti-GM1 IgG antibodies (15). In control studies, serum of a GBS patient with an antecedent *C. jejuni* infection and a high titre of anti-GM1 IgG antibodies was incubated in the same way as the serum samples from the MFS patients. After preincubation with *C. jejuni* for 2 hours at 4°C, the samples were centrifuged at 10,000 *g* for 10 minutes. The supernatant was tested in the ELISA for residual anti-GQ1b IgG antibody activity in the MFS sera and for residual anti-GM1 IgG antibody activity in the GBS serum. The percentage of inhibition was calculated as:

$$\frac{\text{OD (serum without bacteria) - OD (serum preincubated with bacteria)}}{\text{OD (serum without bacteria)}} \times 100\%.$$

RESULTS

The *C. jejuni* strains isolated from stool specimens were classified according to the Penner serotyping system as PEN O:4,50 in Patient A, PEN non-typable in Patient B, and PEN O:23 in Patient C (Table 1). Elevated anti-*C. jejuni* antibody titres in two or more immunoglobulin classes were found in serum samples from all 3 patients, demonstrating an immune response against a recent infection with *C. jejuni* bacteria. In the CSF of Patient A no antibodies against *C. jejuni* could be detected.

Using the ELISA, high titres of anti-GQ1b IgG antibodies were found in the serum from the 3 MFS patients (Table 2). Anti-GQ1b IgM antibodies were only detected in the serum from Patient B and anti-GQ1b IgA antibodies were not found at all. A low anti-GQ1b IgG antibody titre was found in the CSF samples from Patient A. The anti-GQ1b IgG and IgM titres decreased with spontaneous clinical improvement in all patients (Table 2).

Low titres of anti-GD1b and anti-GD3 IgG antibodies were detected in the serum from Patients A and C. No IgM, IgG, or IgA antibodies against GA1, GM1, GM2, GM3, GD1a, GD2, GT1b, LM1, LK1, or globoside could be demonstrated. The ELISA results were confirmed using the TLC overlay technique.

The anti-GQ1b IgG antibody activity in the serum from all 3 MFS patients was dose dependently inhibited by preincubation with *C. jejuni* from Patients B and C (see Figure). The *C. jejuni* strain isolated from Patient A was found to bind with the anti-GQ1b IgG antibodies present in the autologous serum. The binding of this strain with the anti-GQ1b IgG antibodies from the serum of Patient C was weak and the antibodies from Patient B were not bound at all. The strongest inhibition was not always achieved by the patient's autologous *C. jejuni* strain. The anti-GQ1b IgG antibody activity was not inhibited by preincubation with control *C. jejuni* strains 1, 2, and 3 (see Figure). Inhibition of anti-GM1 IgG antibody activity in the serum from a GBS patient could be demonstrated by preincubation with control strain 3 but not with the strains isolated from the MFS patients or with the other control strains (see Figure).

TABLE 1. Clinical and laboratory findings in the 3 patients with Miller Fisher syndrome [a].

Characteristics	Patient A	Patient B	Patient C
Sex/age (yrs)	M/41	M/29	M/22
Diarrhoea	+	+	+
Ataxia	+	+	+
Hyporeflexia or areflexia	+	+	+
Oculomotor weakness	+	+	+
Facial weakness	+	–	+
Oropharyngeal weakness	–	+	+
Sensory symptoms	+	+	+
Sensory signs	–	–	–
Weakness arms or legs	–	–	–
Electromyography	nt	N	N
Cerebrospinal fluid cells/protein	N/↑	N/N	N/N
Magnetic resonance imaging	N	N	nt
C. jejuni Penner serotype	O:4,50	non-typable	O:23

a. Abbreviations: M, male; +, present; –, absent; N, normal; ↑, increased; nt, not tested.

TABLE 2. Anti-GQ1b antibody titres on admission and 3 weeks after admission [a].

	Material	IgM	IgG	IgA
Patient A	serum	–/–	1600/800	–/–
	cerebrospinal fluid	–/–	16/4	–/–
Patient B	serum	200/100	400/100	–/–
Patient C	serum	–/–	800/400	–/–

a. Data represent the titres of the anti-GQ1b antibodies on admission/ 3 weeks after admission. Abbreviation: –, absence of antibody activity (titre < 100 in serum, or < 2 in cerebrospinal fluid).

DISCUSSION

In this study we describe 3 patients with MFS and serum anti-GQ1b antibodies following a *C. jejuni* infection. Whole bacteria of the *C. jejuni* strains isolated from the stools of these patients were able to bind with the serum anti-GQ1b IgG antibodies. The antibody binding was strain-specific since control *C. jejuni* strains were not able to inhibit the anti-GQ1b antibody activity. Furthermore, the antibody binding was not non-specific and probably not Fc-mediated since anti-GM1 IgG antibodies were not bound by the MFS-associated *C. jejuni* strains. These findings support the hypothesis that molecular mimicry exists between GQ1b and surface epitopes on *C. jejuni* strains from patients with MFS.

Serum anti-GQ1b IgG antibodies were found to be associated with ophthalmoplegia in MFS and GBS patients (12). Yuki et al. (10) argued that the presence of anti-GQ1b antibodies in MFS patients was associated with the severity of ataxia. Immunostaining of the paranodal regions of human oculomotor, trochlear, and abducens nerves with anti-GQ1b monoclonal antibodies supports a relationship between anti-GQ1b antibodies and ophthalmoplegia (12). However, GQ1b has also been demonstrated in low concentrations in other human neural tissues like motor and sensory roots (12).

Immune responses against *C. jejuni* may be involved in the pathogenesis of GBS and MFS by cross-reactivity with neural tissue. *C. jejuni* is a frequent cause of antecedent infections in GBS (2-4), especially in patients with serum anti-ganglioside antibodies (16,17). Anti-GM1 antibodies from GBS patients cross-react with some *C. jejuni* strains (15). The antigen initiating the anti-ganglioside antibodies may be the LPS which in some strains can be bound by monoclonal anti-ganglioside antibodies (18,19) and shows homology with gangliosides (20,21,22). On the other hand, LPS generally induces an IgG2 response and antibodies against GM1 and GQ1b are mainly restricted to the IgG1 and IgG3 subclasses (23).

It is suggested that most *C. jejuni* strains associated with GBS have the same Penner (24) or Lior serotype (25). The presence of ganglioside-like structures may depend on the serotype of the strain. The various serotypes of the MFS-associated *C. jejuni* strains in our study suggest that the presence of GQ1b cross-reactive epitopes is not restricted to a specific Penner serotype. Besides, of the two PEN O:23 *C. jejuni* strains tested, only the strain isolated from the MFS patient was able to bind anti-GQ1b antibodies. Therefore, we conclude that the presence of anti-GQ1b antibody binding epitopes on the surface of *C. jejuni* strains is not exclusively associated with a specific Penner serotype.

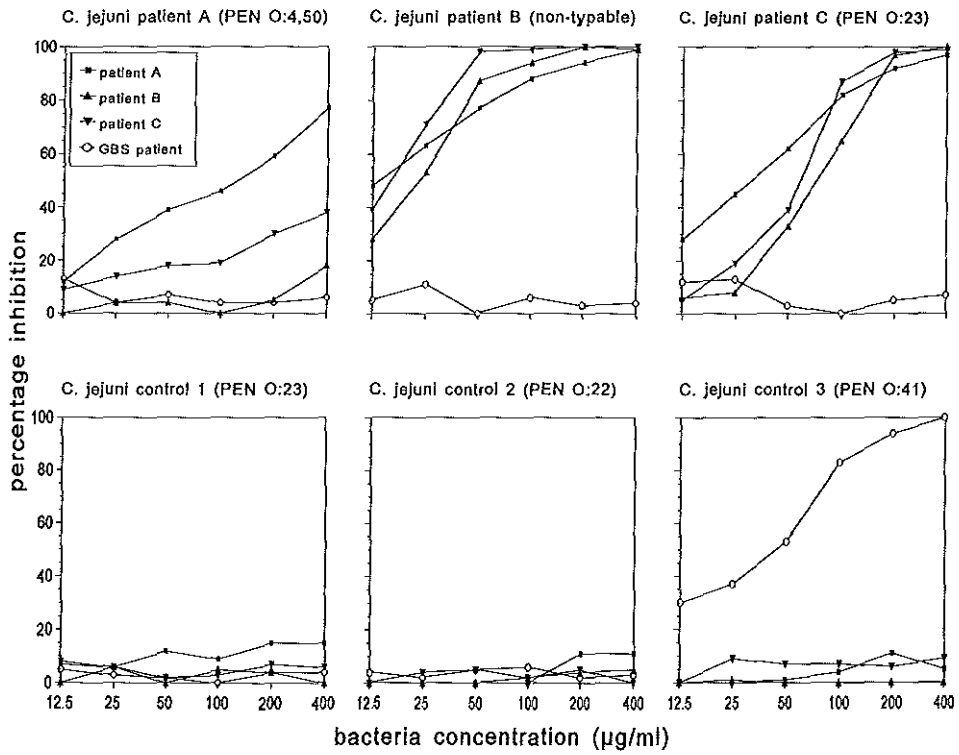

Figure. Inhibition of anti-GQ1b IgG and anti-GM1 IgG antibody activity by preincubation with various concentrations of *C. jejuni* from Patients A, B and C and control *C. jejuni* strains 1, 2 and 3. Percentages of inhibition are represented by closed symbols for anti-GQ1b IgG antibodies in serum from Patients A, B and C, and by open symboles for anti-GM1 IgG antibodies in serum from the patient with GBS.

Whether anti-GQ1b antibodies are pathogenic in MFS is unknown. Roberts and coworkers (26) demonstrated that sera from MFS patients with anti-GQ1b antibodies could induce unresponsiveness in a mouse phrenic nerve/diaphragm preparation, suggesting a failure of acetylcholine release from motor nerve terminals. However, they did not show that the anti-GQ1b antibodies were responsible for this effect.

We conclude that *C. jejuni* isolated from patients with MFS bear anti-GQ1b antibody binding epitopes, and that the presence of these epitopes is not exclusively associated with a specific Penner serotype. It remains to be shown that these cross-reactive epitopes are also immunogenic and furthermore that anti-GQ1b antibodies are involved in the pathogenesis of MFS.

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CHAPTER 4.4

CROSS-REACTIVE ANTIBODIES AGAINST GANGLIOSIDES AND *CAMPYLOBACTER JEJUN I* LIPOPOLYSACCHARIDES IN PATIENTS WITH GUILLAIN-BARRÉ OR MILLER FISHER SYNDROME

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ABSTRACT

Campylobacter jejuni was isolated from stool specimens of 3 patients with Miller Fisher syndrome (MFS) and 2 patients with Guillain-Barré syndrome (GBS). Anti-GQ1b antibodies in serum from all MFS patients cross-reacted with sialidase-sensitive epitopes in the lipopolysaccharide (LPS) fraction of *C. jejuni* from these 3 MFS patients. One GBS patient had anti-GM1 antibodies that bound with LPS of *C. jejuni* from a control patient and from the other GBS patient without anti-GM1 antibodies. This binding was inhibited by cholera toxin but not by pretreatment with sialidase. The *C. jejuni* isolate from the GBS patient with serum anti-GM1 antibodies did not contain anti-GM1 antibody-binding epitopes. Our results strongly support the hypothesis that anti-GQ1b antibodies in MFS patients are induced during the antecedent *C. jejuni* infection. In GBS patients, mechanisms other than molecular mimicry may also be involved in the production of anti-GM1 antibodies.

INTRODUCTION

The Guillain-Barré syndrome (GBS), including its variant the Miller Fisher syndrome (MFS), is an acute inflammatory polyneuropathy that is often preceded by an infection. The clinical manifestations of the disease are related to the presence of serum anti-ganglioside antibodies. Anti-GM1 antibodies are associated with a rapidly progressive and pure motor form of GBS (1). Anti-GQ1b antibodies are associated with both MFS and GBS with ophthalmoplegia (2). The high concentrations of GM1 in the myelin of motor peripheral nerves (3) and of GQ1b in oculomotor nerves (2), correspond with the clinical manifestations of GBS and suggest that anti-ganglioside antibodies are involved in its pathogenesis.

Although the origin of anti-ganglioside antibodies is unknown, it can be speculated that they are initially formed against micro-organisms during the antecedent infection. *Campylobacter jejuni* was identified as a frequent cause of such infections in GBS patients (4) and has been described in MFS patients as well (5). Moreover, anti-GM1 antibodies are associated with *C. jejuni* infections in GBS (1). In previous studies, we demonstrated that anti-GM1 antibodies and anti-GQ1b antibodies from GBS and MFS patients bind with whole cells of different *C. jejuni* isolates (5,6). Biochemical analysis showed that the lipopolysaccharide (LPS) fraction of a *C. jejuni* isolate from a GBS patient with anti-GM1 antibodies contained an oligosaccharide with similarities to GM1 (7). The *C. jejuni* LPS may induce the production of cross-reactive anti-ganglioside antibodies with the specificity found in GBS and MFS patients. However, whether serum anti-GM1 antibodies in GBS patients and anti-GQ1b antibodies in MFS patients actually recognize these sialylated epitopes in *C. jejuni* LPS needs to be clarified.

In this study we investigated if anti-ganglioside antibodies from the serum of GBS and MFS patients specifically cross-react with sialic acid-containing epitopes in the LPS of *C. jejuni* isolates from the same and other patients.

PATIENTS AND METHODS

Patients

This study included *C. jejuni* isolates and pretreatment serum samples from 3 patients with MFS (A, B and C) and 2 patients with GBS (D and E). All patients had diarrhoea and positive *C. jejuni* serology. Patients A, B and C had ophthalmoplegia, ataxia and areflexia without limb weakness, and patients D and E fulfilled the diagnostic criteria for GBS (8).

C. jejuni and lipopolysaccharides

The *C. jejuni* isolates were serotyped according to the Penner classification system, based on the bacterial LPS, as PEN O:4,50 (A), non-typable (B), 0:23 (C), O:1 (D) and O:24 (E). We used *C. jejuni* isolates from 3 enteritis patients (1,2 and 3) without neurologic involvement for control studies, that were serotyped as O:23, O:22 and O:41, respectively. The *C. jejuni* bacteria were grown on blood agar plates in a microaerobic atmosphere. After 48 hours the bacteria were harvested, inactivated in 1% formaldehyde, and washed in phosphate-buffered saline solution (PBS) (pH 7.4). The LPS fraction from the *C. jejuni* bacteria was isolated by phenol-water extraction (9) and demonstrated to contained less than 1% proteins.

Detection of antibodies against gangliosides

Anti-ganglioside antibodies were tested by using enzyme linked immunosorbent assay (ELISA) and thin layer-chromatography (TLC) overlay as described previously (1). In ELISA, a 1:100 dilution of serum samples was tested for IgM, IgG and IgA antibodies against the gangliosides GM1, GM3, GD1a, GD1b, GD2, GD3, GT1b (all from Sigma, St. Louis, MO, USA) and GQ1b (Biocarb, Lund, Sweden). Serum samples with an optical density (OD) of more than 3 standard deviations above the mean value of 50 normal control sera were tested in TLC overlay and ELISA, using serial dilutions, to determine the titre.

Binding of anti-ganglioside antibodies to *C. jejuni*

Each serum sample was preincubated with *C. jejuni* isolates from the patients and enteritis controls, according to methods previously described (5). The concentrations of each isolate were 0.8, 3.1, 12.5, 50, 200 and 800 µg/ml and of each LPS were 0.2, 0.8, 3.1, 12.5, 50 and 200 µg/ml. After incubation for 3 hours at 4°C, the samples were centrifuged, and the supernatant was tested in ELISA for residual anti-ganglioside IgG antibody activity. The percentage of inhibition was calculated as:

$$\frac{\text{OD (serum without bacteria) - OD (serum preincubated with bacteria)}}{\text{OD (serum without bacteria)}} \times 100\%$$

Sialidase treatment

Whole cells and LPS of *C. jejuni* were incubated with 0.05 U/ml sialidase from *Clostridium perfringens* (Sigma) or *Arthrobacter ureafaciens* (Sigma) in 50 mM of sodium acetate buffer (pH 5.5) overnight at 37°C. After incubation, the whole cells were washed in PBS, and the LPS were incubated at 80°C for 30 minutes to inactivate the sialidases.

Treatment with cholera toxin

Whole cells of *C. jejuni* were incubated with 100 µg/ml of B subunit of cholera toxin (CT) (Sigma), which recognizes GM1 (10), in PBS-1% bovine serum albumine (BSA) for 3 hours at 37°C. After incubation, the cells were washed with PBS.

RESULTS

Serum anti-ganglioside antibodies

The titres of the anti-ganglioside antibodies in serum from the MFS and GBS patients are given in the Table. High titres of anti-GQ1b antibodies were found in the 3 MFS patients, as reported previously (5). Anti-GM1 and anti-GD1b antibodies were demonstrated in serum from GBS patient D but not in serum from GBS patient E. The anti-ganglioside titres decreased with clinical improvement in all patients (data not shown).

Binding of serum anti-ganglioside antibodies with *C. jejuni*

The anti-GQ1b IgG antibody activity in serum from MFS patient C was dose-dependently inhibited by preincubation with whole cells and LPS of *C. jejuni* from the 3 MFS patients but not with *C. jejuni* from the 2 GBS patients or the 3 controls (Figure 1). The same percentage of inhibition was achieved with a much lower concentration of LPS than with whole cells of *C. jejuni* from all 3 MFS patients. The same results were found with the anti-GQ1b antibodies in sera from MFS patient A and B (data not shown).

TABLE. Titre of anti-ganglioside antibodies in serum from patients with Guillain-Barré syndrome or Miller Fisher syndrome [a].

	Miller Fisher patients			Guillain-Barré patients	
Antibodies to:	A	B	C	D	E
GM1	–	–	–	800 (IgG) 1600 (IgM) 200 (IgA)	–
GM3	–	–	–	–	–
GD1a	–	–	–	–	–
GD1b	100 (IgG)	–	200 (IgG) 200 (IgM)	100 (IgG) –	–
GD2	–	–	–	–	–
GD3	200 (IgG)	–	200 (IgG)	–	–
GT1b	–	–	–	–	–
GQ1b	1600 (IgG)	400 (IgG) 200 (IgM)	800 (IgG)	–	–

a. Data represent titres of anti-ganglioside antibodies with the isotype of the antibodies in parentheses; –, absence of antibody activity (titre < 100).

The anti-GM1 IgG antibody activity in serum from GBS patient D was dose-dependently inhibited by preincubation with whole cells and LPS of *C. jejuni* from GBS patient E and control 3 (Figure 1). However, anti-GM1 antibodies from GBS patient D were not bound by the autologous isolate nor by *C. jejuni* from the 3 MFS patients or the control patients 1 and 2. Again, LPS was more effective than whole cells of *C. jejuni* from GBS patient E and control patient 3 in binding anti-GM1 antibodies.

Control studies with sialidases and CT

After preincubation of the whole cells with sialidase, the *C. jejuni* isolates from the 3 MFS patients could not inhibit anti-GQ1b antibody activity (figure 2A). LPS fractions from the MFS isolates also lost their capacity to bind anti-GQ1b antibodies after treatment with sialidase (data not shown). However, the capacity of the *C. jejuni* from GBS patient E and control patient 3 to bind anti-GM1 antibodies was not decreased after treatment of the bacteria with sialidase (Figure 2B). The binding of anti-GM1 antibodies to these isolates was completely blocked after treatment of these bacteria with CT (Figure 2B). The capacity of the MFS-associated *C. jejuni* to bind anti-GQ1b antibodies was not reduced after preincubation with CT (Figure 2A). We also investigated whether peroxidase-conjugated B subunit of CT could bind to *C. jejuni* by inhibition ELISA. After preincubation with whole cells of *C. jejuni* from GBS patient E and control 3, the residual activity of peroxidase-conjugated CT against GM1 in supernatant was lost completely (data not shown). The capacity to bind CT was not influenced by treatment of these isolates with the sialidases (data not shown).

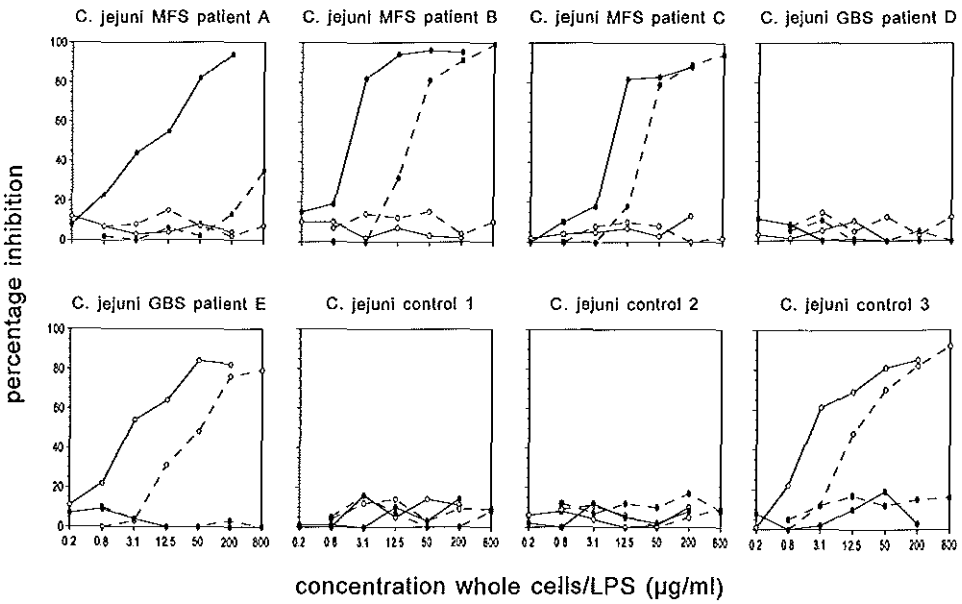

Figure 1. Inhibition of anti-GQ1b IgG antibody activity in serum from MFS patient C (•) and anti-GM1 IgG in serum from GBS patient D (o) by preincubation with various concentrations of *C. jejuni* whole cells (dotted lines) and LPS (solid lines) from MFS patient A, B and C, GBS patients D and E and enteritis controls without neurologic involvement (controls 1, 2 and 3).

DISCUSSION

This study demonstrates the cross-reactivity of serum anti-ganglioside antibodies with LPS of *C. jejuni* isolates from patients with MFS and GBS. Anti-GQ1b IgG antibodies from the MFS patients bound with whole cells and LPS fractions of *C. jejuni* isolates from 3 MFS patients. Treatment of the *C. jejuni* whole cells and LPS with sialidase prevented the binding, implicating that sialic acids on the surface of the bacteria are apparently essential for the anti-GQ1b antibodies to cross-react. These findings strongly support the hypothesis that anti-GQ1b antibodies in MFS patients with an antecedent *C. jejuni* infection are initially formed against sialic acid containing, GQ1b-like epitopes in *C. jejuni* LPS.

Our results are in accordance with the study of Yuki et al. who demonstrated that mouse monoclonal antibodies against GQ1b can bind with LPS from MFS-associated *C. jejuni* isolates (11). However, the fine specificity of anti-GQ1b antibodies is very heterogenous and may differ between mice and MFS patients. The mouse monoclonal antibodies have been demonstrated to bind with a variety of *C. jejuni*, including isolates from GBS patients (12) and enteritis patients without neurological involvement (11). The authors suggest that the presence of the GQ1b-like epitopes in the LPS is associated with the PEN O:2 LIO:4 serotype (11). However, in the present study, anti-GQ1b antibodies from the sera from MFS patients themselves could be absorbed only

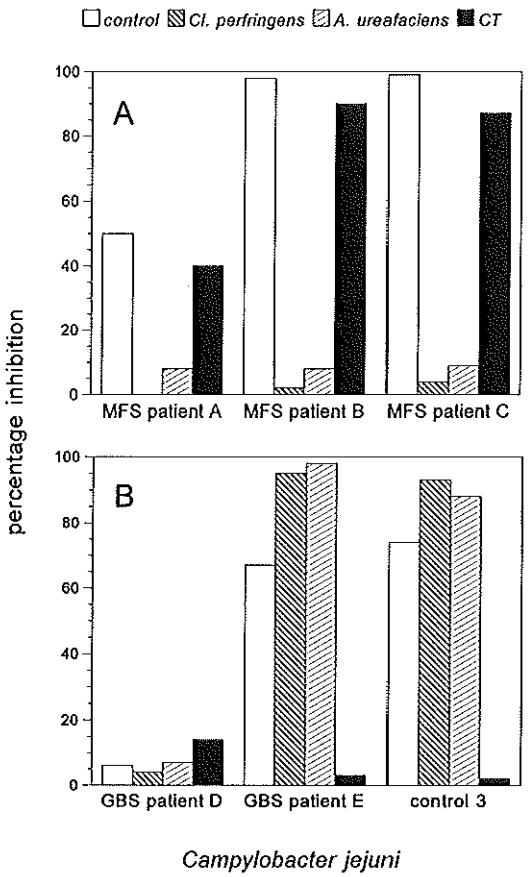

Figure 2. Whole cells of *C. jejuni* were preincubated with sialidase from *Clostridium perfringens* or *Arthrobacter ureafaciens* in 50 mM sodium acetate buffer (pH 5.5), with cholera toxin (CT) in PBS-1%BSA (pH 7.4), or as a control with 50 mM sodium acetate buffer only. Results with PBS-1%BSA only, as a control for preincubation with CT in PBS-1%BSA, are not shown since they did not differ from control treatment with sodium acetate buffer. (**A**) Inhibition of anti-GQ1b IgG antibody activity in serum from MFS patient C by preincubation with *C. jejuni* from MFS patients A, B and C. (**B**) Inhibition of serum anti-GM1 IgG antibody activity in serum from GBS patient D by preincubation with *C. jejuni* from GBS patients D and E and enteritis control patient 3.

by bacteria from MFS patients. Also the PEN O:23 *C. jejuni* isolate from control 1 did not bind anti-GQ1b antibodies although it had the same Penner serotype as the isolate from MFS patient C. This suggests that the capacity to bind polyclonal anti-GQ1b antibodies from the serum of MFS patients is restricted to *C. jejuni* isolates from MFS patients and is not associated with a particular Penner serotype (5). Further investigations are needed to determine the incidence and geographical distribution of anti-GQ1b antibody binding epitopes on *C. jejuni* isolates from patients and controls.

Anti-GM1 antibodies from the serum of GBS patient D did not bind with the autologous *C. jejuni*. This isolate may have expressed the GM1 epitope during infection in

the patient but lost it due to the culture circumstances *in vitro*. The sialylation of the LPS higly depends on the presence of nucleotide sugars for sialic acids (13) and sialidases in the host. Other mechanisms than molecular mimicry with surface epitopes may also be involved in the production of anti-GM1 antibodies after infection. The enterotoxin produced by some *C. jejuni* preferentially binds with GM1 and may serve as a carrier molecule in the production of antibodies against the hapten GM1 (reviewed by Willison and Kennedy (14)).

Moreover, anti-GM1 antibodies were bound by the LPS of *C. jejuni* from GBS patient E and control enteritis patient 3 without neurologic involvement. Therefore, the presence of GM1-like epitopes may be a common finding in *C. jejuni* that is not restricted to isolates from GBS patients with anti-GM1 antibodies. The induction of anti-GM1 antibodies probably not only depends on the presence of GM1-like epitopes on bacteria but also on host factors.

The binding of anti-GM1 antibodies could not be inhibited by treatment of *C. jejuni* with sialidases. These enzymes may not be able to desialate the GM1-like structures in the LPS, or the binding of the anti-GM1 antibodies in GBS patient D may not depend on the presence of sialic acid. In accordance with the last option is the finding of additional low antibody activity in serum from GBS patient D against asialo-GM1 (data not shown) and GD1b, which have the Gal(β1-3)GalNAc epitope in common with GM1. The binding of anti-GM1 antibodies could be blocked by CT, indicating that they recognize the same epitope. CT preferentially binds with GM1 but also binds with GD1b and asialo-GM1 (10), as was also demonstrated in our ELISA and TLC overlay (data not shown). These results are in accordance with those of others who demonstrated that the binding of monoclonal anti-GM1 IgM antibodies to *C. jejuni* LPS in patients with chronic motor neuropathy could be inhibited by treatment with CT (15).

Molecular mimicry between epitopes on micro-organisms and peripheral nerves may be involved in the pathogenesis of inflammatory polyneuropathies. This study gives strong support to the hypothesis that MFS-associated *C. jejuni* contain sialylated epitopes that induce serum anti-GQ1b antibodies with the specificity found in MFS patients. However, our findings on anti-GM1 antibodies suggest that the production of anti-ganglioside antibodies may also depend on other mechanisms than molecular mimicy. Other bacterium and host factors, in addition to the variety of ganglioside-like epitopes on *C. jejuni*, may determine the specificity of the anti-ganglioside antibodies and thereby contribute to the clinical manifestations in GBS and MFS.

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CHAPTER 4.5

HUMAN IGM PARAPROTEINS DEMONSTRATE SHARED REACTIVITY BETWEEN *CAMPYLOBACTER JEJUNI* LIPOPOLYSACCHARIDES AND HUMAN NERVE DISIALYLATED GANGLIOSIDES

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ABSTRACT

IgM paraproteins from patients with chronic ataxic neuropathy of the CANOMAD phenotype react with disialylated epitopes on a wide range of gangliosides including GQ1b, GT1a, GD1b and GD3. The tissue distribution of reactive antigens in human peripheral nerve has not been addressed in detail. In addition, the origin of these antibodies is unknown. Here we report that purified anti-disialosyl paraproteins from two affected patients bind a wide array of human peripheral nerve structures including dorsal root ganglia, dorsal and ventral root axons, femoral and oculomotor nerves. We also show that these paraproteins bind lipopolysaccharides of *Campylobacter jejuni* isolates from all 3 cases of Miller Fisher syndrome, and to a less frequent extent, from cases of Guillain-Barré syndrome and enteritis controls. In conjunction with our previous studies, these data provide a possible causal link between the origin and pathogenic effects of anti-disialosyl antibodies in human paraproteinaemic neuropathy.

INTRODUCTION

Phenotypic variants of autoimmune neuropathy are characterized by specific patterns of serum anti-ganglioside antibodies. One form of peripheral neuropathy associated with such a unique pattern of anti-ganglioside antibodies is an IgM paraproteinaemic neuropathy with the core clinical features of chronic sensory ataxia and areflexia and with variably present ophthalmoplegia and/or red blood cell cold agglutinating (cold agglutinin) activity (1-8). This has recently been reported under the acronym CANOMAD: Chronic Ataxic Neuropathy with Ophthalmoplegia, M-protein, Agglutination and Disialosyl antibodies (9). The IgM paraproteins found in patients with CANOMAD react with NeuAc(α2-8)NeuAc(α2-3)Gal disialosyl epitopes present on a wide range of neural gangliosides including GQ1b, GT1a, GT1b, GD1b, GD3 and GD2. This or a structurally similar epitope is also present on red blood cell glycophorins, thereby accounting for the concurrent cold agglutinin disease due to red cell cold agglutinating activity of anti-Pr specificity in some CANOMAD cases (10).

Some CANOMAD patients have fixed or intermittent episodes of ophthalmoplegia and/or bulbar dysfunction (1,7,8) reminiscent of Miller Fisher syndrome (MFS), a variant of Guillain Barré syndrome (GBS) characterised by acute ataxia, areflexia and ophthalmoplegia (11). Over 90% of acute phase MFS sera also contain anti-GQ1b and anti-GT1a IgG antibodies (12-14) which react with GD1b and GD3 in around half the cases (15). Thus, in addition to sharing similar clinical features, CANOMAD and MFS also share a similar pattern of serum anti-ganglioside antibodies. In CANOMAD the antibodies are of IgM class and chronically elevated, whereas in MFS, which is an acute and self limiting disease, the antibodies are IgG, subclass restricted to IgG1 and IgG3, and transiently present (16).

The distribution of immunoreactive epitopes identified with CANOMAD sera in the human peripheral nervous system has not been thoroughly examined. In the mouse, we have shown that an IgM antibody cloned from an affected patient binds to many peripheral structures including dorsal root ganglion (DRG neurons), sensory and motor peripheral nerve trunks, motor nerve terminals and muscle spindles (9). One previous study using whole serum from a CANOMAD case has shown IgM binding to human DRG neurons but no staining was seen in other sites (17). In view of our studies in the mouse this seems surprising. Other studies have performed immunocytochemical localisation studies using murine monoclonal antibodies raised by ganglioside immunization which have been selected for their high specificity for individual gangliosides including GQ1b (18) and GD1b (19). Although of interest in their own right, these antibodies are neither representative of the CANOMAD specificity, nor directly comparable with human autoantibodies. Here we report our findings that highly purified IgM paraproteins from CANOMAD cases have broader tissue immunoreactivity in man than previously recognized.

Campylobacter jejuni enteritis is a commonly recognized infection known to precipitate MFS and we and others have isolated strains of *C. jejuni* from MFS subjects (20-22). Evidence indicates that anti-GQ1b ganglioside antibody responses in MFS are triggered as a result of molecular mimicry with structurally similar epitopes on lipopolysaccharides (LPS) from MFS-associated strains of *C. jejuni.* Serological studies using anti-GQ1b monoclonal antibodies and MFS sera have demonstrated GQ1b-

like epitopes on whole organisms and LPS from strains of *C. jejuni* isolated from patients with MFS (20-23). Structural characterization of an MFS associated LPS core oligosaccharide has shown it to contain a terminal trisaccharide comprising NeuAc(α2-8) NeuAc(α2-3)Gal which is common to GQ1b, GT1a and GD3 gangliosides (22). We therefore considered the possibility that the IgM paraproteins found in CANOMAD sera might also arise through antigenic stimulation by disialylated or polysialylated epitopes on core oligosaccharides of *C. jejuni* derived LPS. Here, we tested the reactivities of highly purified monoclonal IgM fractions containing anti-disialosyl antibodies from two CANOMAD patients with LPS from a panel of *C. jejuni* isolates from patients with MFS, GBS and controls.

MATERIALS AND METHODS

Clinical subjects

Clinical reports on patients VP (7) and CH (6,9) have been previously published. Both had features of a chronic, predominantly sensory neuropathy comprising peripheral sensory loss, profound limb ataxia, areflexia and mild or absent limb weakness. Interspersed on the chronic background in both patients (recognized subsequent to the 1993 report of patient CH) was episodic deterioration with craniobulbar dysfunction, including ophthalmoplegia. Both patients had IgM lambda paraproteins with anti-Pr cold agglutinating activity and very high titre ($>10^5$) of anti-disialylated ganglioside antibodies, thereby comprising the CANOMAD phenotype. Neither subjects had detectable IgG or IgM antibodies to *C. jejuni* proteins, suggesting no recent infection.

Anti-ganglioside antibody assays and antibody purification

Serum anti-ganglioside antibodies in CANOMAD patients VP, CH and in neuropathy subjects (A-E) from whom *C. jejuni* strains were isolated were determined by enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatography overlay (TLC overlay) using a panel of commercially available gangliosides as previously described (6). IgM cold agglutinating antibodies from VP and CH were affinity purified from plasma by temperature-dependent elution on human group O red blood cells (RBC), as previously described (6). These fractions were monitored by quantitative immunoassay for IgM and isoelectric focusing (IEF) with Western blotting as previously described for CH and are referred to as rbc-M (9).

Campylobacter jejuni isolates and lipopolysaccharide extraction

The characteristics of the *C. jejuni* strains and subjects from whom they were derived are shown in the Table. Organisms were isolated from stool cultures of 3 patients with MFS (A, B and C), 3 patients with GBS (D, E and F) and 3 enteritis patients without neurological involvement (1, 2 and 3), as described previously (23). Patients A, B and C had clinical features of ophthalmoplegia, ataxia and areflexia without limb weakness, and patients D, E and F fulfilled the diagnostic criteria for GBS. *C. jejuni* bacteria were grown on blood agar plates in a microaerobic atmosphere. After 48 hours the bacteria were harvested, inactivated in 1% formaldehyde and washed in phosphate-buffered saline solution (PBS) (pH 7.4). The LPS fractions from the *C. jejuni* bacteria were isolated by phenol-water extraction (24), and were demonstrated to contain less than 1% proteins. For control studies the LPS fractions were incubated with 0.05 U/ml of sialidase from *Clostridium perfringens* or *Arthrobacter ureafaciens* (Sigma, St. Louis, MO) 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 minutes to inactivate the sialidases.

TABLE. Serotypes of *C. jejuni* isolates and antibody profiles of neuropathy and enteritis control subjects from whom they were derived [a].

	Diagnosis	Anti-ganglioside antibody profile [b]	*C. jejuni* Penner serotype
Patient A	MFS	GQ1b, GD1b, GD3	O:4,50
Patient B	MFS	GQ1b	non-typable
Patient C	MFS	GQ1b, GD1b, GD3	O:23
Patient D	GBS	GM1, GD1b	O:1
Patient E	GBS	not detected	O:24
Patient F	GBS	nt	non-typable
Patient 1	enteritis [c]	nt	O:23
Patient 2	enteritis [c]	nt	O:22
Patient 3	enteritis [c]	nt	O:41

a. Abbreviations: MFS, Miller Fisher syndrome; GBS, Guillain-Barré syndrome; nt, not tested.
b. Serum IgM, IgG, and IgA antibodies against the panel of gangliosides described in Patients and Methods. The gangliosides against which antibodies were demonstrated are indicated.
c. Enteritis patients without neuropathy.

Detection of antibodies reactive with lipopolysaccharides

IgM antibodies against LPS were tested by ELISA and TLC-overlay. ELISA was performed by coating 96-well polystyrene microtitre trays (Immuno Maxisorb, Nunc, Roskilde, Denmark) with 1mg of LPS in 50 ml PBS per well overnight. After the coating all steps were performed at 4°C. Non-specific binding sites were blocked with PBS containing 1% bovine serum albumin (BSA) for 4 hours. After blocking, the plates were incubated with 0.1 to 10 mg/ml rbc-M from VP or CH in PBS-0.1% BSA overnight. After washing with PBS, the plates were incubated with peroxidase-conjugated goat antihuman IgM (μ-chain specific) (Sigma) diluted 1:2,500 in PBS-0.1% BSA for 4 hours. After washing with PBS, the plates were developed with *O*-phenyl diamine (Sigma) in citrate buffer (pH 5.0) and the optical densities were read at 490 nm. TLC was performed on aluminium backed Kieselgel 60 $WF_{254}S$ high-performance TLC plates (Merck, Darmstadt, Germany) coated with 7 mg of LPS, and developed in *n*-propanol/water/25% NH_4OH (24:12:8, by volume). After chromatography, the plates were air-dried and dipped in a solution of 0.1% polyisobutylmethacrylate in *n*-hexane. The overlays and washings were performed at 4°C. The plates were air-dried and blocked with PBS-1% BSA for 1 hour and incubated with 0.1 to 10 mg/ml of rbc-M in PBS-0.1% BSA for 4 hours. After washing with PBS, the plates were incubated for 2 hours with peroxidase-conjugated goat-antihuman IgM (μ-chain specific) (Sigma) in PBS-0.1% BSA and washed with PBS. The plates were developed for 10 to 150 seconds using enhanced chemiluminescence (ECL, Amersham, UK).

Immunofluorescence studies

Human spinal cord, DRG, dorsal and ventral roots, femoral nerve and oculomotor nerve were obtained from a middle aged male without neurological disease at post mortem subject to local ethical approval and guidelines. Tissues embedded in Tissue-Tek O.C.T. mounting medium (Miles Inc. Diagnostics Div. Elkhart, IN) were frozen in a slurry of ethanol and dry-ice and stored at -70°C. Cryostat sections (5-15 m) were cut onto 3-aminopropyltriethoxysilane coated slides, and allowed to air-dry before immediate use or storage at -20°C. Mounted tissue sections were incubated with the rbc-M fractions from VP and CH at 40 mg/ml, diluted in PBS containing 10% goat serum and 0.1% Triton X100 for 3 hrs at 4°C. Mouse anti-neurofilament antibody (clone 1217, Affiniti Research Products, Exeter, UK) was used at 1:1,000 dilution. After primary antibody incubations, sections were drained, rinsed in four washes of cold PBS

and incubated with rhodamine-labelled goat-antihuman IgM and fluorescein-labelled goat-antimouse IgG secondary antibodies (Southern Biotechnology Associates, Birmingham, AL) at 1:300 dilutions in staining medium for 1 h at 4°C. The slides were again rinsed 4 times in cold PBS before being mounted in Citifluor antifade (Citifluor Products, Canterbury, UK), ringed with nail-varnish to minimise drying and stored at 4°C in the dark prior to viewing. Consistent fluorescence signals were totally dependent on strict maintenance of all solutions at 4°C at all times throughout the staining procedure.

Sections were also stained with the anti-neurofilament antibody plus an irrelevant human IgM monoclonal antibody and/or secondary antihuman IgM antibody to control for non-specific binding and fluorescence bleed-through between detection channels: the threshold of the image acquisition equipment was calibrated such that the control level of staining was close to zero. Images were obtained by a Sony colour CCD camera mounted onto a Zeiss Axioplan fluorescent microscope and linked to a PC driven image archiving system. Images were printed directly using a photographic-quality colour printer (ColourEase, Eastman Kodak Co., NY).

RESULTS

Serological studies on CANOMAD subjects

Patients VP and CH had anti-ganglioside IgM antibody titres previously reported as follows. For VP: GM1, <100; GM2, 30,000; GM3, <100; GD1a, 30,000; GD1b, 120,000; GT1b, 100,000; GQ1b, 170,000; GD3 150,000. For CH: GM1, <100; GM2, <100; GM3, 660; GD1a, 570; GD1b, 850,000; GT1b, 300,000; GQ1b, 450,000; GD3 260,000. The rbc-M fractions in both patients had an identical pattern of anti-ganglioside antibody reactivity as the sera from which they were derived (data not shown). IEF western blotting of serum and rbc-M (Figure 1) showed resolution of the rbc-M as a pure monoclonal ladder corresponding to the serum monoclonal IgM and free of polyclonal IgM when compared with the IEF profile of whole serum.

Reactivity of rbc-M fractions from CH and VP with *C. jejuni* lipopolysaccharides

The rbc-M fractions from VP and CH bound with LPS from the *C. jejuni* isolates from all 3 MFS patients (patients A, B and C), and from one GBS patient (patient E) and one enteritis control (patient 3) as shown in Figure 2. These patterns of binding were confirmed in TLC-overlay (Figure 3). Binding of rbc-M from VP and CH to LPS was found to be higher at 4°C than at 37°C as was also previously seen for ganglioside binding (data not shown). The capacity of the LPS to bind rbc-M from both VP and CH decreased after treatment of the LPS with sialidases from *Clostridium perfringens* (Figure 2) and *Arthrobacter ureafaciens* (data not shown). No activity was demonstrated against LPS from the *C. jejuni* isolates of GBS patients D and F and control patients 1 and 2. There was no obligate association between the presence of the paraprotein binding epitopes on LPS and the Penner serotype of the bacteria: enteritis control 3 is PEN O:23 and does not bind the paraproteins, although it has the same O:23 serotype as the isolates from MFS patient C, which does bind the paraproteins.

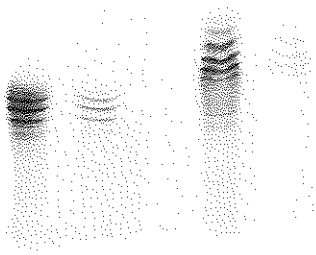

Figure 1. Western blots of IEF gels of whole serum (lanes 1 and 3) and rbc-M (lanes 2 and 4) from VP (lanes 1 and 2) and CH (lanes 3 and 4) stained with antihuman IgM showing the monoclonal IgM on a background of polyclonal IgM in serum compared with the pure paraprotein of identical isoelectric mobility in rbc-M. Monoclonal immunoglobulins appear as ladders in IEF gels due to charge microheterogeneity.

Immunofluorescence studies on human peripheral nerve

The binding patterns of rbc-M from VP and CH to a wide range of human peripheral nerve structures were identical. In spinal cord, the rbc-M bound amorphously to grey matter including weak cytoplasmic labeling of motorneuron cell bodies. There was very faint binding to white matter tracts. In the DRG (Figure 4) the majority of neuronal cell bodies displayed granular staining of an area including, and extending slightly beyond, the neurofilament-positive region (Figure 4, panels a and b), although a small population were unstained. In some areas of the DRG there was also weaker staining of extracellular regions surrounding the cell bodies. In fibre bundles within the DRG, there was strong staining associated with the axons of large fibres (Figure 4, panels c and d), little or no staining of the myelin sheath, and surrounding haloes of staining in the extracellular space (Figure 4, panels e and f). Strong labelling of blood vessel walls was seen throughout the nervous system, here shown in the DRG (Figure 4, panel d, arrow). In the spinal roots (Figure 5, panels a and b), the dorsal and ventral root large fibre axons and surrounding extracellular regions were similarly stained, with little labelling evident in their compact myelin sheaths. A similar pattern was observed in the oculomotor nerve (Figure 5, panels c and d). Large fibres in the femoral nerve were also stained in this pattern. Longitudinal sections confirmed this pattern and did not show significant compact or paranodal myelin staining. Small diameter fibre bundles in the femoral nerve, identifiable by clusters of neurofilament positive profiles were strongly immunoreactive (Figure 5, panels e and f) although it was not possible to identify specific structures within the bundles at this resolution. These small fibre bundles were also readily identifiable within the dorsal root but were absent (as expected from the fibre size distribution) in the ventral root.

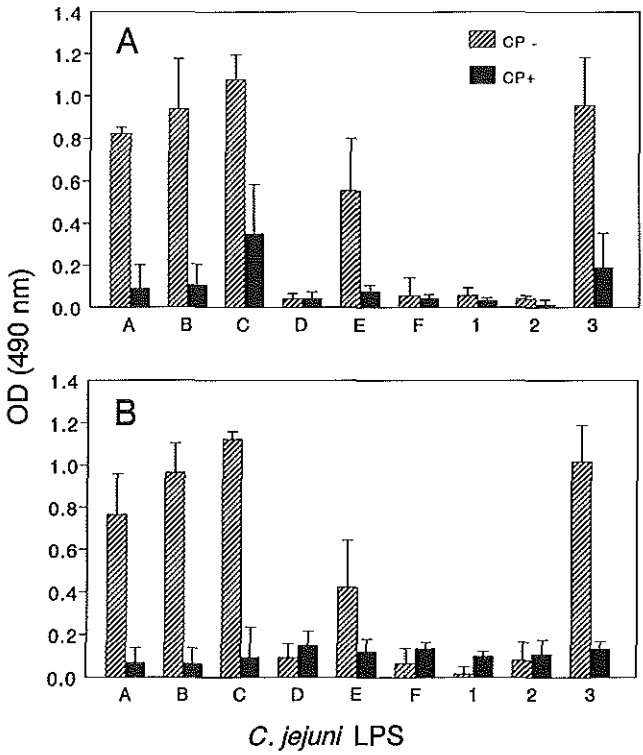

Figure 2. ELISA analysis of the reactivity of rbc-M from patients VP (1 µg/ml)(**A**) and CH (10 µg/ml)(**B**) with LPS from *C. jejuni* isolates without (hatched bars) and with (solid bars) treatment with *Clostridium perfringens* sialidase. The rbc-M from patients VP and CH bind with LPS from all 3 MFS-associated *C. jejuni* strains (lanes A, B and C), one of the GBS-associated *C. jejuni* strains (lanes D, E and F), and one of the enteritis control strain (lanes 1, 2 and 3). Means ± 2 standard deviations.

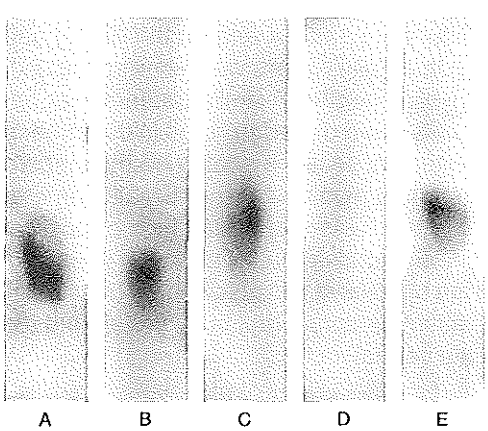

Figure 3. TLC-overlay analysis of LPS (7 µg/lane) from 3 MFS-associated *C. jejuni* strains (panels A, B and C) and 2 of the GBS-associated *C. jejuni* strains (panels D and E) immunostained with rbc-M from patient CH (10 µg/ml). Reactivity patterns correspond to those seen in ELISA.

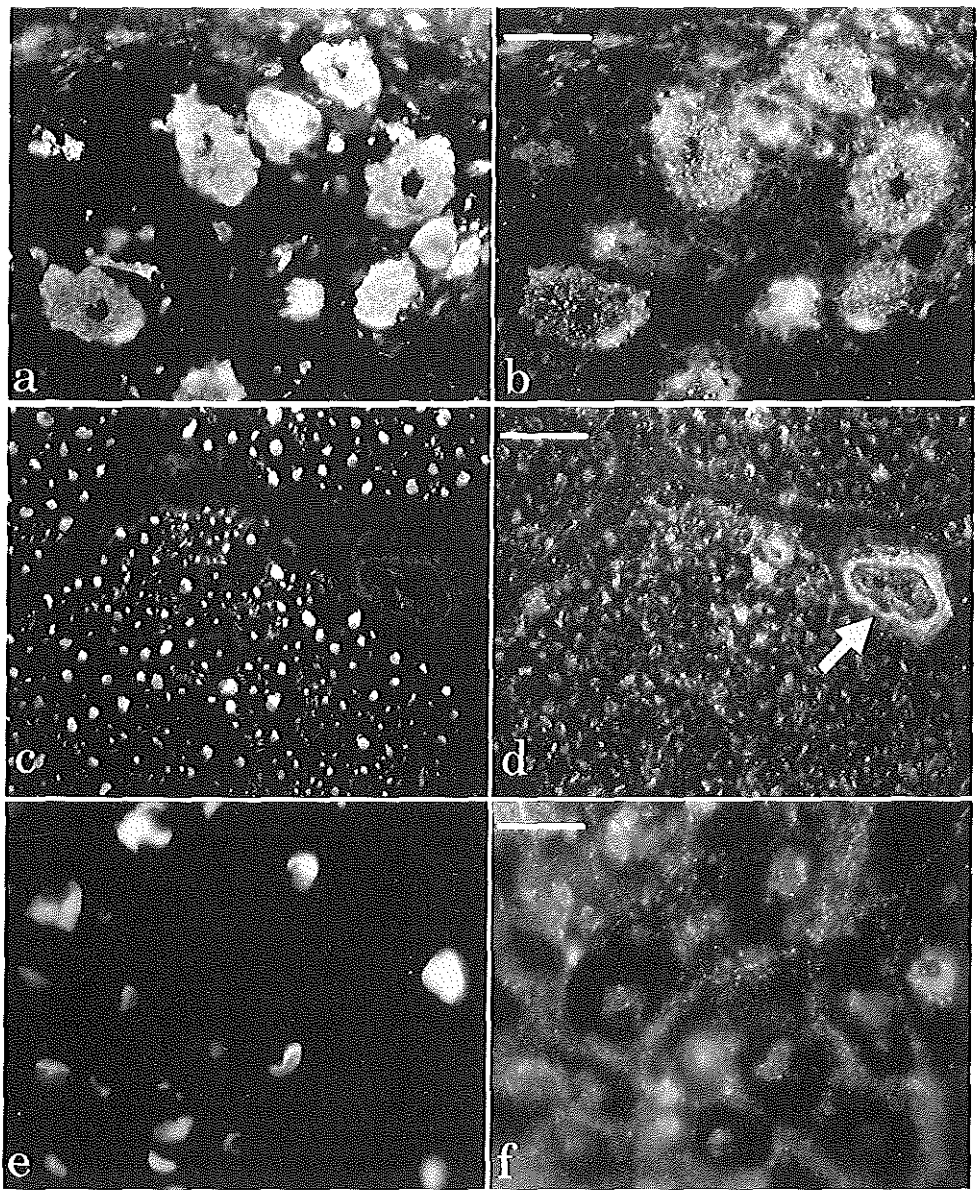

Figure 4. Double-labelled pairs of fluorescent micrographs showing the immunolocalization of rbc-M (panels b, d and f) and anti-neurofilament antibodies (panels a, c and e) in human dorsal root ganglion. The majority of cell bodies are labelled (panels a and b; Bar = 50 μm). In fibre bundles within the DRG, staining was localized to axonal profiles, connective tissue and blood vessels (arrow; panels c and d; Bar = 50 μm). At higher magnification, labelling of large axons and the extracellular regions surrounding their myelin sheaths is seen (panels e and f; Bar = 20 μm).

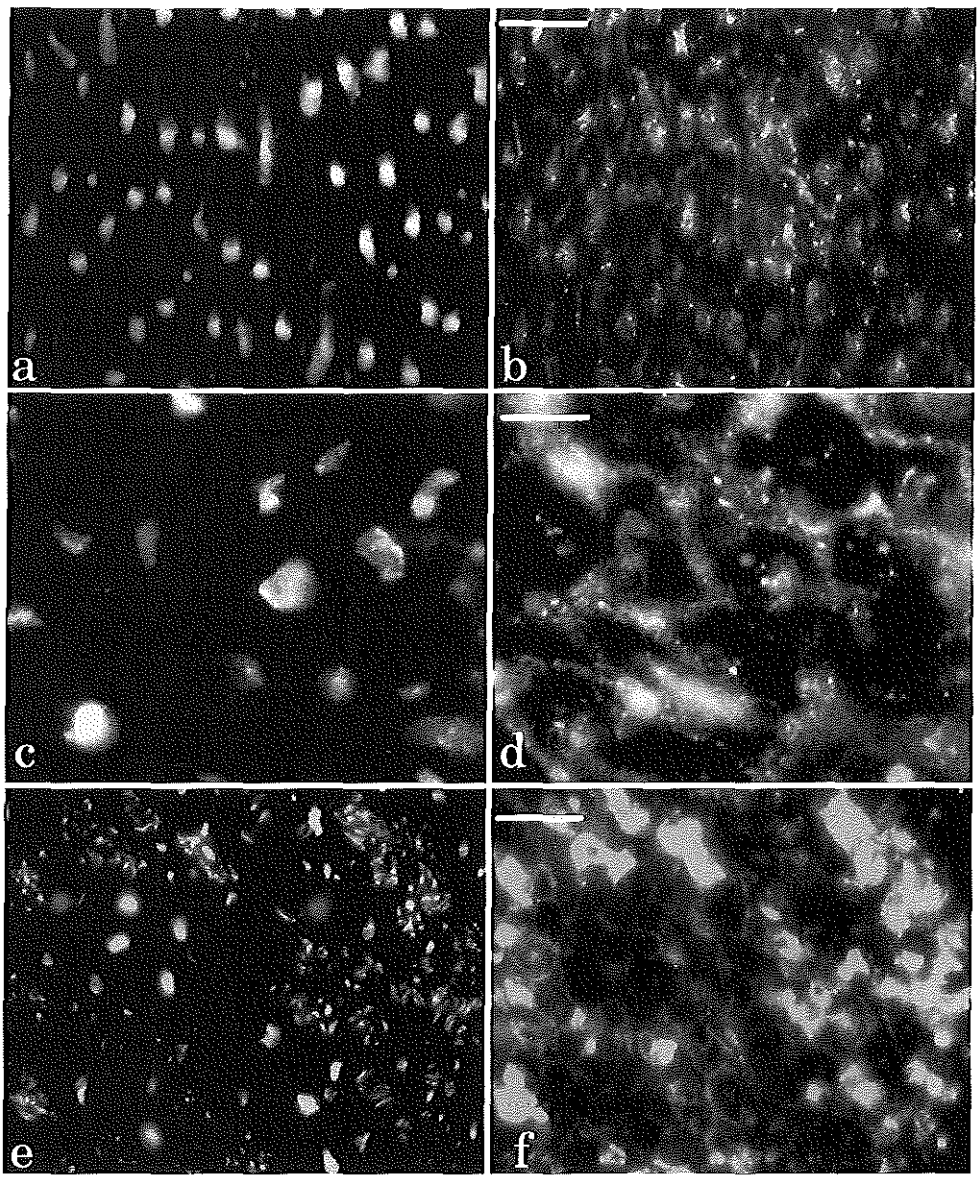

Figure 5. Double-labelled pairs of fluorescent micrographs showing the immunolocalization of rbc-M (panels b, d and f) and anti-neurofilament antibodies (panels a, c and e) in human peripheral nerve. Axonal profiles and extracellular regions are stained in the ventral root (panels a and b; Bar = 20 μm) and oculomotor nerve (panels c and d; Bar = 10 μm). In the femoral nerve staining is intense over small fibre bundles (panels e and f; Bar = 20 μm).

DISCUSSION

In this study we have shown that IgM paraproteins from patients with CANOMAD bind with human peripheral nerve antigens and *C. jejuni* LPS derived from MFS patients. These data provide a possible pathogenic link between the origin and the effect of anti-disialylated ganglioside IgM paraproteins in humans. They also add further support to the view that CANOMAD and MFS are related polyneuropathies, characterized by anti-ganglioside antibodies with overlapping but distinct specificity.

All MFS sera that bind GQ1b also bind GT1a and 50% of these MFS sera also bind other gangliosides bearing at least one NeuNAc(α2-8)NeuNAc-residue, such as GD1b and GD3 (16). The specificity of CANOMAD paraproteins for gangliosides is broader in that both paraproteins described here bind to all NeuNAc(α2-8)NeuNAc-bearing gangliosides we have studied. Furthermore, they may also bind very weakly to other gangliosides including GD1a and GM2 (6,7). Anti-GQ1b antibodies in sera from MFS patients and murine monoclonal anti-GQ1b antibodies react with *C. jejuni* LPS derived from MFS cases, indicating that these LPS molecules bear a GQ1b-like epitope (20,21). Interestingly, the LPS core oligosaccharide from the only MFS associated *C. jejuni* structurally characterized to date bears a GD3-like terminal trisaccharide rather than a GQ1b or GT1a motif (22). In addition, Aspinall et al. (25) have reported the structure of a GBS-associated LPS as containing a GT1a-like motif. This finding may indicate that there is not an exact relationship between the LPS oligosaccharide structure and the fine specificity of the corresponding anti-ganglioside antibody serology. Our data show that CANOMAD paraproteins not only react with LPS from all 3 MFS-associated *C. jejuni* strains but also bind 1 of 3 GBS strains and 1 of 3 control strains, which are not bound by MFS sera. This indicates that CANOMAD paraproteins have a broader specificity for sialidase sensitive LPS structures than MFS sera and thus may have arisen through an immune response to a wider range of disialylated LPS structures than MFS associated antibodies.

There is no direct evidence that the paraproteins in CANOMAD have arisen in response to *C. jejuni* LPS. Neither of the CANOMAD cases reported enteritis as an initiating event at the onset of neurological symptoms as is seen in MFS. Equally, neither sera contained antibodies to *C. jejuni* proteins used in a diagnostic assay for recent *C. jejuni* infections. These findings indicate that a putative *C. jejuni* infection is currently inactive and must have occurred in the distant past such that serum antibody levels have decayed to subdetectable levels. Our V-region sequencing studies on the paraprotein which we have cloned from patient CH have shown that the antibody is encoded by immunoglobulin variable region genes with extensive somatic mutation. This is indicative of an origin through antigen driven maturation rather than dysregulated proliferation of a naive B-cell with a random antigen specificity as the primary event (9). The appearance of paraproteins in CANOMAD would be consistent with an antigen specific response to bacterial LPS, possibly chronically sequestered in gut associated lymphoid tissue and possibly arising from *C. jejuni* or other infections with cross-reactive motifs. Assuming that MFS-associated anti-GQ1b antibodies and CANOMAD paraproteins arise in response to *C. jejuni* LPS, the major difference is that the former comprises an acute, self limiting primary immune response with class switching from IgM to higher affinity IgG antibodies whereas the latter comprises a chronic accumula-

tion of IgM produced by a slowly expanding dysregulated clone of B-cells.

We have already demonstrated in the MF1 mouse that monoclonal CANOMAD paraproteins can bind to a variety of murine peripheral nerve structures including dorsal root ganglia, motor nerve terminals, peripheral nerve axons and myelin and nodes of Ranvier. In addition, the paraprotein can immunolocalize to and induce electrophysiological abnormalities in motor nerve terminals in the *ex vivo* hemi-diaphragm model of nerve transmission, selected for study as one site out of many in the peripheral nerve unit that could be pathologically affected in the human disease (9). In our immunolocalization studies in the human peripheral nervous system described here we have shown widespread binding to sensory and motor nerves, including the oculomotor nerve, with prominent staining in groups of dorsal root ganglion neurons. In view of the sensory ataxia seen in CANOMAD cases, the dorsal root ganglion, a site with a limited blood nerve barrier into which antibody can easily penetrate, seems a likely target for immunopathological attack. The pattern of binding to human DRG neurons we have seen with CANOMAD monoclonal paraproteins is similar to that seen in previous reports using anti-GD1b antibodies. Using a murine anti-GD1b monoclonal antibody, Kusonoki et al. (19) have immunolocalized GD1b to dorsal root ganglion neuronal cytoplasm and spinal root axons. In studies by Oka et al. (17), using an unpurified CANOMAD serum of very similar specificity to those used here, a granular pattern of binding was seen in the DRG neuronal cell bodies, again very similar to that seen in this study. However, they observed no staining elsewhere in peripheral nerve in marked contrast to our CANOMAD IgM antibodies which stain human dorsal and ventral roots and peripheral nerve axons extensively. Thus, assuming that technical factors have not created the difference between our study and that of Oka et al., there must be critical differences in antibody fine specificity which dictate immunoreactivity to different peripheral nerve regions. These may be reflected by clinical differences between patients.

Combined data from this and our previous studies suggest the following pathophysiological sequence of events in the evolution of CANOMAD: a) individuals are infected with *C. jejuni* or other pathogens bearing disialylated ganglioside-like epitopes, b) a low level, low affinity IgM response is mounted with insufficient autopathogenic potential to cause an acute post-infectious neuropathy, c) transformation from a state of antigen dependent B-cell proliferation to autonomous B-cell proliferation occurs, with the formation of a monoclonal IgM gammopathy, d) serum IgM antibody levels rise to a level at which binding kinetics favour sufficient antibody "on" time to activate pro-inflammatory pathways in target membranes of the DRG and other antigen rich sites.

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CHAPTER 4.6

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 high titres 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 to baseline 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.

INTRODUCTION

The Guillain-Barré syndrome (GBS) is an acute monophasic polyneuropathy leading to a heterogeneous spectrum of motor and sensory deficits (1). 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 preceeds the neurological onset.

Campylobacter jejuni is a frequent cause of antecedent infections in GBS (2), and MFS (3,4). These infections are associated with the presence of antibodies against gangliosides in patients with GBS (5-7) and MFS (3,4). Anti-GM1 antibodies are associated with severe motor involvement in GBS (7), and anti-GQ1b antibodies with MFS and oculomotor involvement in GBS (8). The high concentration of GM1 in myelin of motor nerves (9) and of GQ1b in oculomotor nerves (8) 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 (10) and MFS (11). Furthermore, anti-GM1 IgG antibodies from GBS patients cross-react with GBS associated *C. jejuni* (12), and anti-GQ1b IgG antibodies with MFS associated isolates (3,7).

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 (4-6). 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 groups of GBS patients with anti-GM1 antibodies, but not in GBS patients without these antibodies.

Secondly, ganglioside-like epitopes have also been demonstrated in *C. jejuni* isolates from enteritis patients without GBS or MFS (3,4,13). 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 (14). Antibodies against gangliosides in GBS and MFS patients, however, are predominantly IgG1 and IgG3 (15-17). 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.

PATIENTS AND METHODS

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 to E and 1 to 3 and their *C. jejuni* isolates were described previously (4,18). 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 (19). Serum anti-ganglioside antibodies were found in MFS patients 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 to 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 (patient A), non-typable (patients B and F), O:23 (patients C and 1), O:1 (patient D), O:24 (patient E), O:22 (control 2) and O:41 (control 3). *C. jejuni* was grown for 48 hours on blood agar plates in a microaerobic atmosphere, inactivated in 1% formaldehyde and washed in phosphate-buffered saline solution (PBS) (pH 7.4). The *C. jejuni* LPS were isolated by phenol-water extraction (20) and were demonstrated to contain less than 1% protein.

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 titres of IgG antibodies against *C. jejuni* in ELISA, as previously described (7). The CJC all had diarrhoea 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 immunosorbent assay (ELISA) and thin-layer chromatography overlay, according to methods described previously (7).

Detection of antibodies against lipopolysaccharides

Serum antibodies against LPS were tested in ELISA, by coating 96-well polystyrene microtitre trays (Immuno Maxisorb, Nunc, Roskilde, Denmark) with 1 μg of LPS in 50 μl PBS per well overnight at 37°C. Unspecific binding sites were blocked for 4 hours with PBS containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO). After blocking, the plates were incubated overnight at 4°C with serum diluted 1:1,000 in PBS-1%BSA. After washing with PBS, the plates were incubated with peroxidase-conjugated goat antihuman IgG (γ-chain specific) (Sigma), diluted 1:2,500 in PBS-1%BSA, for 90 minutes at room temperature. After washing

TABLE 1. Presence of serum IgG anti-ganglioside antibodies and recent *C. jejuni* infections in 6 groups of patients tested for antibodies against *C. jejuni* LPS [a].

	Patients		IgG to:			
Group	Number	Diagnosis	GM1	GQ1b	*C. jejuni* infection [b]	Including patient:
1	12	GBS	12	0	9 (75%)	D
2	8	GBS/MFS	0	8	5 (63%)	A, B, 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	

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

b. Defined as positive *C. jejuni* serology according to criteria previously reported (7).

with PBS, the plates were developed with *O*-phenyl diamine (Sigma) in citrate buffer (pH 5.0) and the optical densities (OD) 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 by subtraction of the blank well OD from the coated-well OD. Serum samples with OD of more than 3 standard deviations above the mean value of 12 NC sera were considered to be of high titre, 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, The Netherlands) 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, The Netherlands), a mixture of capsular polysaccharides from 23 different serotypes of *Streptococcus pneumoniae*, by routine techniques.

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 minutes 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 in ELISA according to the same method as described for antibodies against LPS.

Cholera toxin treatment of lipopolysaccharides

The LPS fractions were coated according to the method described above, and incubated with 25 µg/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 under the same conditions without CT. Antibodies against the CT blocked LPS were further tested in ELISA according to the same method as described for antibodies against LPS.

RESULTS

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 (Figure 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* infection. The 3 patients without anti-LPS activity had no *C. jejuni* infection and 2 had lower anti-GM1 IgG titres than 1:1,000. 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 (Figure 1). IgG

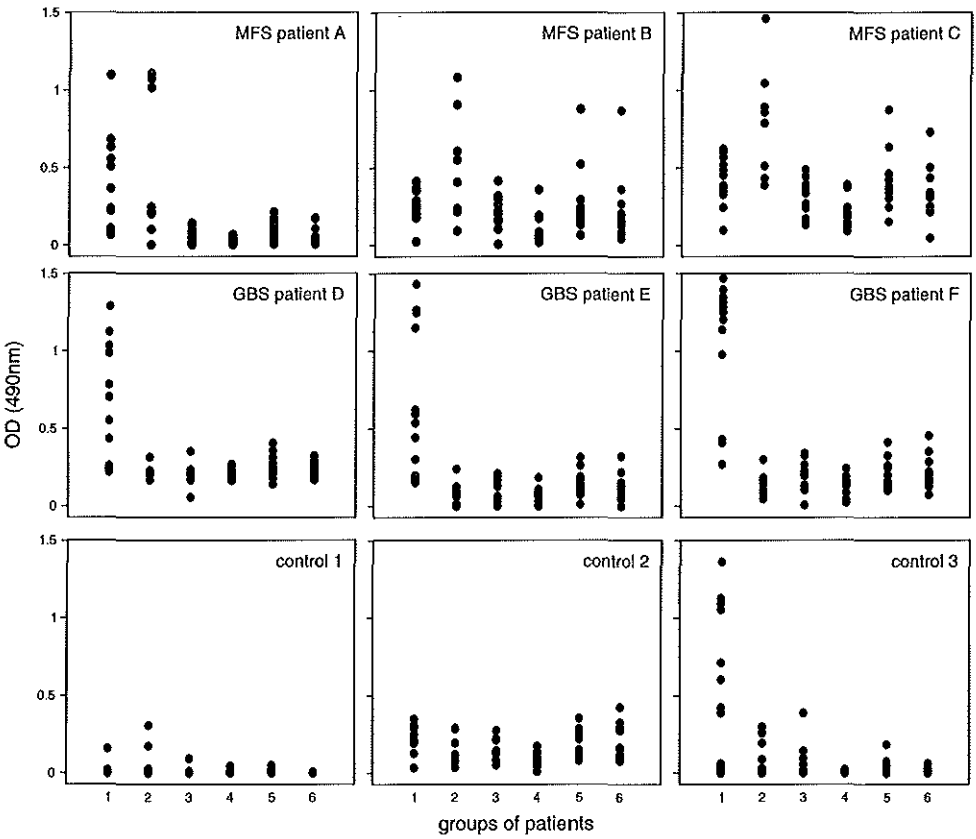

Figure 1. Serum IgG activity against LPS from *C. jejuni* isolates from MFS patients (A, B, C), GBS patients (D, E, F), and enteritis controls without neurological involvement (1, 2, 3). The patients tested for IgG antibodies against LPS were subdivided into six groups according to diagnosis, presence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

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 titre 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) (Figure 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 (Figure 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 (Figure 2).

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.

Subclasses of IgG antibodies against gangliosides and LPS

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

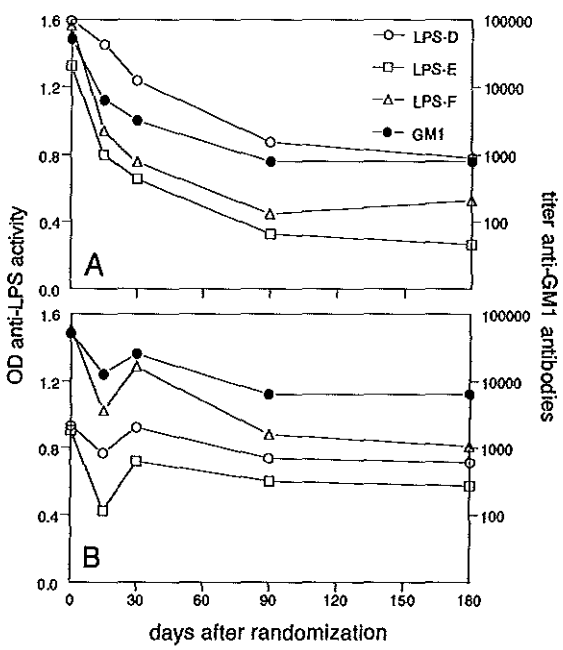

Figure 2. Follow-up of IgG antibody activity against LPS from 3 GBS associated *C. jejuni* isolates (D, E, F) (expressed as extinctions) and of IgG activity against GM1 (expressed as titres) in two GBS patients with *C. jejuni* infection, one treated with intravenous immunoglobulins (**A**), and one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

TABLE 2. Subclasses of IgG antibodies against GM1, GQ1b and *C. jejuni* LPS in serum from patients with GBS, MFS, CJC, and NC with high levels of anti-LPS antibodies [a].

				LPS [b]						
				MFS			GBS			CJC
Group	Patient	GM1	GQ1b	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	–	–	–	–

a. Abbreviations: LPS, lipopolysaccharides; GBS, Guillain-Barré syndrome; MFS, Miller Fisher syndrome; CJC, *C. jejuni* enteritis control without neurological deficits; NC, normal control; –, absence of high titre of IgG antibodies to LPS.

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

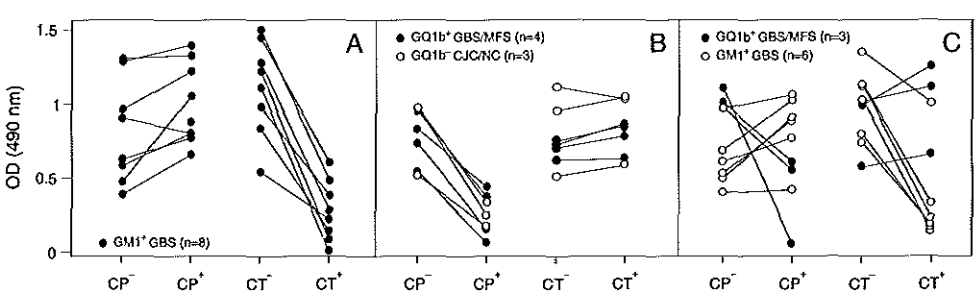

Figure 3. Serum IgG antibody activity against LPS preincubated with sialidase from *Clostridium perfringens* in 50 mM sodiumacetate buffer (CP^+), 50 mM sodiumacetate buffer only (CP^-), cholera toxin in PBS-1%BSA (CT^+), or PBS-1%BSA only (CT^-). These experiments were performed with LPS of *C. jejuni* from GBS patient E (**A**), MFS patient B (**B**), and MFS patient A (**C**). Serum was tested from all patients with high levels of IgG antibodies against these LPS (see Table 2).

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 (Figure 3A). Treatment of the LPS with sialidase CP had no or an increasing effect on the antibody binding (Figure 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 (Figure 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 (Figure 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.

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 titres of IgG against LPS from the MFS related strains. Secondly, the titre 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 (17). 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 antibodies that cross-react with gangliosides.

Molecular mimicry between gangliosides and LPS has been demonstrated with *C. jejuni* isolates from GBS and MFS patients (10,11). Previously we found that anti-GQ1b antibodies cross-react with LPS of *C. jejuni* from MFS patients A, B and C, and anti-GM1 antibodies with *C. jejuni* from GBS patient E and control 3 (18). 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 antibodies that cross-react with 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 (21,22).

In some patients with anti-ganglioside antibodies we did not find high IgG activity against LPS. This may result from relatively low titres 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 (18). 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(β1-3) 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(β1-3)GalNAc-containing glycolipids other than GM1 (23).

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 (13). 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 titres 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 the response against polysaccharides differs 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 responses are restricted to LPS from specific *C. jejuni* strains.

Long-lasting, high titres of IgG1 and IgG3 antibodies are characteristic for T-cell dependent immune responses (24). 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 (25,26). 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 (27). A candidate for this protein may be enterotoxins, which specifically bind with gangliosides and are produced by specific *C. jejuni* strains (28,29). 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 (30). Interestingly, the suppression of sodium current in sciatic nerve by rabbit anti-GM1 antibodies was found to be complement-dependent (31). In addition, IgG1 and IgG3 have a higher affinity for Fc-recep-

tors which play a role in opsonization and phagocytosis (32).

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 fine-specificity and isotypes of the anti-ganglioside antibodies.

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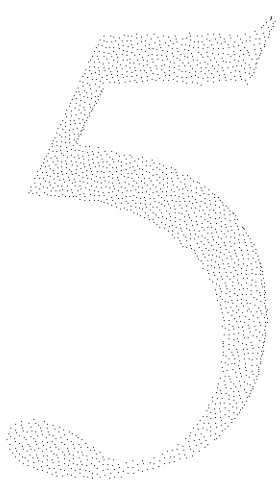
GENERAL DISCUSSION



GENERAL DISCUSSION

CHAPTER 5

GENERAL DISCUSSION

The Guillain-Barré syndrome (GBS) is an acute polyneuropathy with a marked patient to patient variation in pathological substrate, clinical presentation and prognosis (reviewed in Chapter 1.1). The Miller Fisher syndrome (MFS) is considered to be a variant of the GBS, characterized by ophthalmoplegia, ataxia, and reflexia. A variety of preceding infections has been reported in patients with GBS and MFS (see Chapter 1.2). These infections may trigger an immune response against peripheral nerves (see Chapter 1.3), possibly by molecular mimicry between microbic and nerve antigens (see Chapter 1.4). Peripheral nerve gangliosides are possible targets for the immune system because high titres of anti-ganglioside antibodies are often found in GBS and MFS patients, and ganglioside-like structures were identified in *Campylobacter jejuni*, the most frequent cause of antecedent infections in GBS. We hypothesize that the distinct patterns of peripheral nerve damage, which underlie the clinical heterogeneity in GBS, are partly determined by the fine-specificity of the anti-ganglioside antibodies, which are induced by specific micro-organisms during antecedent infections (Chapter 1.5). The objectives of the studies described in this thesis were (i) to identify the various antecedent infections and anti-ganglioside antibodies in GBS and to determine their relation with the clinical presentation (Chapters 2 and 3), and (ii) to investigate the role of *C. jejuni* in triggering the production of anti-ganglioside antibodies in GBS and MFS (Chapter 4).

5.1 RELATION BETWEEN ANTECEDENT INFECTIONS, ANTI-GANGLIOSIDE ANTIBODIES AND CLINICAL MANIFESTATIONS

Study design

Previous studies reported controversial results with respect to the frequency and clinical association of antecedent infections and serum anti-ganglioside antibodies in GBS (reviewed in Chapter 1.2 and 1.3). This controversy predominantly results from (i) the small number of patients and selection bias in most studies, (ii) the lack of detailed clinical and electrodiagnostic information, (iii) the use of serum samples obtained a long time after neurological onset, or after treatment, and (iv) differences in techniques used to detect anti-ganglioside antibodies or to determine the presence of recent infections.

In the studies presented in this thesis, we had the opportunity to investigate the clinical significance of antecedent infections and anti-ganglioside antibodies in a large group of GBS patients who were included in two therapeutic studies (1,2). All 172 patients fulfilled the diagnostic criteria for GBS, and were admitted within two weeks of onset of weakness. The patients were carefully evaluated with respect to a detailed array of clinical features according to a previously established protocol during

a follow-up of 6 months. Pretreatment serum samples, obtained within two weeks of neurological onset, were available in 154 (90%) of these patients. The 18 excluded patients did not differ significantly from the other patients regarding their antecedent illness, neurological manifestations, and course of the disease. To identify the spectrum of antecedent infections in these patients, the serum samples were tested for the presence of antibodies against 16 infectious agents. In addition, the presence of IgM, IgG and IgA antibodies to 8 major peripheral nerve gangliosides was determined. The anti-ganglioside antibodies were detected by enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatography (TLC) overlay. In our opinion, both techniques have to be used for a reliable detection of anti-ganglioside antibodies. ELISA is necessary to quantitate the antibody activity in titres, and TLC overlay is essential to confirm the positive results found in ELISA, since the ganglioside preparations provided by the manufacturers are usually contaminated with other glycolipids.

Antecedent infections in relation to clinical subgroups of GBS

The study described in Chapter 2 confirms the high frequency and diversity of antecedent infections in GBS. In the group of 154 patients, 68% reported some form of infectious illness, and 56% had a positive serology for a recent infection (Table 2, Chapter 2). *C. jejuni* was the most frequent cause of infection, followed by cytomegalovirus (CMV), Epstein-Barr virus (EBV), and *Mycoplasma pneumoniae.* These infections occurred significantly more often in GBS patients than in age-, sex- and season-matched patients with other neurological diseases. In addition, recent infections with *Haemophilus influenzae,* influenza A and B virus, herpes simplex virus, varicella zoster virus, adenovirus, and parainfluenza 1 virus, were also demonstrated in GBS patients, but their frequency was low and their relation to GBS remains unclear.

Two antecedent infections were clearly related to separate clinical subgroups. *C. jejuni* infections, identified in 32% of patients, were associated with a severe, pure motor variant of GBS (Chapters 2 and 3.2) (3). CMV infections, preceding GBS in 13% of patients, were also related to a severe form of GBS, but in addition these patients frequently had respiratory insufficiency, cranial nerve involvement, and severe sensory loss (Chapter 2) (4). These distinct clinical presentations indicate that *C. jejuni* and CMV infections trigger immune responses to different nerve antigens, leading to separate patterns of peripheral nerve damage. The clinical heterogeneity in GBS may thereby at least partly result from the variety of antecedent infections. On the other hand, infections with EBV and *M. pneumoniae* may induce more heterogeneous antibody responses since they were not related to specific clinical features in GBS.

Anti-ganglioside antibodies and their relation to antecedent infections

The study in Chapter 3.5 reports the presence of anti-ganglioside antibodies in 32% of GBS patients, which show a remarkable variety of fine-specificities (Table). Binding of antibodies to the major peripheral nerve gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b and LM1 was present in different subgroups of patients. In addition, antibodies to minor peripheral nerve gangliosides, like GM1b, *N*-acetyl-galactosamine-GM1b, *N*-acetyl-galactosamine-GD1a, and many others, were also reported in GBS (5-7). Furthermore, antibodies binding with a specific ganglioside may have different fine-specificities as indicated by the additional activity against other gangliosides. Some patients have antibodies to GM1 only, while others have additional antibody activity to GD1b or GM2. The anti-GM1 antibodies in patients with additional activity against GD1b may bind with the common Gal(β1-3)GalNAc moiety in GM1 and GD1b (see Appendix 2) (8). Anti-GM1 antibodies in patients with additional anti-GM2 activity, however, may recognize the shared GalNAc(β1-4) [NeuAc2-3]Gal(β1-4)Glc moiety (see Appendix 2). Alternatively or in addition, patients may have several subpopulations of antibodies, each recognizing different gangliosides. Together, these studies indicate that antibodies in GBS may bind to a variety of ganglioside epitopes in peripheral nerves.

Patients with *C. jejuni* infection significantly more often have antibodies to GM1 and GD1b than patients without these infections (Table) (Chapter 3.2 and 3.5). Antibodies to GM2, GD1a, GQ1b and LM1 were also found in patients with *C. jejuni* infection. Anti-ganglioside antibodies of the IgA class are almost exclusively produced in patients with *C. jejuni* infections, which further strengthens the relation with enteric infections. Recently, antibodies against *N*-acetyl-galactosamine-GM1b and *N*-acetyl-galactosamine-GD1a were also reported to be highly associated with *C. jejuni* infections (6). Patients with CMV infections frequently have IgM antibodies against GM2 and asialo-GM2, not cross-reacting with GM1 (Table) (Chapter 3.4) (9). In parallel, *Mycoplasma pneumoniae* infections in GBS patients were reported to be associated with antibodies binding with the glycolipid galactocerebroside (10). These serological studies indicate that the spectrum of antecedent infections partly determines the fine-specificity of the antibodies to gangliosides and other nerve antigens.

Anti-ganglioside antibodies in relation to clinical manifestations

Antibodies to GM1 (with or without GD1b), GD1a and GQ1b were associated with specific pathological subgroups in GBS (Table). The clinical and electrodiagnostic presentation of patients with anti-GM1 antibodies indicates that these patients have a severe and selective demyelination of motor nerves, and a variable degree of axonal degeneration. This association between anti-GM1 antibodies and pure motor GBS was irrespective of the presence of additional anti-GD1b antibodies. Anti-GD1a antibodies were also related to severe damage of motor nerves, although the number of patients with these antibodies was small. Severe involvement of oculomotor and sensory nerves was found in the subgroup with anti-GQ1b antibodies. The presence of these antibodies may reflect the dysfunction of specific nerve fibres, since GM1 and GD1a are preferentially present in axons (11), and in myelin of motor nerves (12), and GQ1b in myelin of oculomotor nerves (13). This is consistent with the clinical presentation in patients with other forms of neuropathy associated with these

TABLE. Frequency of anti-ganglioside antibodies in 154 GBS patients: association with antecedent infections, and clinical and electrodiagnostic features [a].

		Associated with:	
Antibodies to:	Frequency	Infection [b]	Clinical and electrodiagnostic features
GM1	20%	*C. jejuni*	(i) severe distal weakness and low CMAP amplitudes (ii) minor sensory loss and high CSNAP amplitudes (iii) minor cranial nerve deficiency
GM2	5% [c]	CMV [d]	severe weakness and sensory disturbances, cranial nerve deficiency, respiratory insufficiency
GM3	1%	none	none
GD1a	3%	none	severe weakness
GD1b	18%	*C. jejuni*	same features as associated with anti-GM1 antibodies [e]
GT1b	1%	none	none
GQ1b	4%	none	ophthalmoplegia, severe weakness with respiratory insufficiency, inexcitable sensory nerves
LM1	6%	none	none
All	32%		

a. Summarizing and extending the results from the studies in Chapters 2 and 3. Abbreviations: *C. jejuni, Campylobacter jejuni*; CMAP, compound muscle action potential; CSNAP, compound sensory nerve action potential; CMV, cytomegalovirus.
b. Infection with at least one of the 16 agents tested in Chapter 2.
c. Result of testing all 154 patients, including 130 patients described in Chapter 3.4.
d. In the absence of anti-GM1 antibodies.
e. Probably due to overlap with anti-GM1 antibodies.

anti-ganglioside antibodies. Anti-GM1 IgM antibodies are also present in patients with multifocal motor neuropathy (MMN), and other forms of chronic pure motor neuropathy (14,15). Anti-GD1a antibodies are also associated with other forms of neuropathy with severe weakness (16,17). Anti-GQ1b antibodies are highly associated with the MFS variant (13,18,19), and with acute ophthalmoparesis (13,20). In GBS patients the titre of the antibodies to GM1, GD1a and GQ1b usually decreases with clinical improvement. Together, the studies in patients with GBS, MFS, and other neuropathies strongly support the concept that the presence of these anti-ganglioside antibodies reflects dysfunction of specific subsets of nerve fibres, and that the fine-specificity of the anti-ganglioside antibodies is related to the clinical heterogeneity of GBS.

Direct evidence for the pathogenicity of these antibodies in GBS is presently not available. However, anti-GM1 antibodies from patients with MMN, serum immunoglobulins from patients with MFS, and monoclonal antibodies against disialylated gangliosides from patients with chronic ataxic neuropathy, ophthalmoplegia, M-protein, agglutination, and antibodies against disialylated gangliosides (CANOMAD), interfere

with peripheral nerve conduction or neuromuscular transmission in the mouse phrenic nerve/diaphragm preparation (21-23). Immunization of rabbits with GD1b induces anti-ganglioside antibodies and gives a polyneuropathy with clinical features similar to those found in patients with CANOMAD (24). The reported association with specific clinical and electrodiagnostic features in our studies in GBS patients, further suggests that some of the anti-ganglioside antibodies also have a pathogenetic effect on specific nerve fibres.

If these antibodies indeed are pathogenetic, their site of action will be determined by their fine-specificity. This may also explain why antibodies against some gangliosides are not associated with distinct clinical patterns. Firstly, the fine-specificity of antibodies against a ganglioside may vary between patients, thereby not leading to a homogeneous clinical presentation. This may be the case in the anti-GM2 positive patients: additional activity against GM1 was related to pure motor GBS, and additional activity against asialo-GM2 was related to cranial nerve involvement and sensory nerve impairment (Chapter 3.4). Secondly, antibodies against ubiquitously distributed gangliosides, as LM1, may not interfere with specific nerve fibres, and thereby not lead to discriminating clinical features. Thirdly, antibodies against various gangliosides may be associated with the same clinical pattern. Finally, some anti-ganglioside antibodies may be unrelated to GBS because some of these antibodies are also found in normal controls, although mostly in a lower titre. In our studies, high titres of anti-ganglioside antibodies were only found in two patients without neurological diseases: anti-LM1 IgG antibodies in a normal control (Chapter 3.5) and anti-GM2 IgM in a CMV infected control (Chapter 3.4). In addition, the number of patients positive for a specific anti-ganglioside antibody may be too small to demonstrate a statistically significant association.

One has to keep in mind, however, that the specificity of the anti-ganglioside antibodies is presently defined by laboratory assays, which may not reflect the targets and site of action of these antibodies in peripheral nerves. The binding characteristics of anti-ganglioside antibodies highly depend on the material used to adsorb the antigen and on the incubation temperature (25,26). Most assays, including ours, determine the reactivity of antibodies to gangliosides on polystyrene or silica at 4°C, which is significantly different from the *in vivo* circumstances. In addition, anti-ganglioside antibodies also bind with nerve glycoproteins (27,28). Therefore, antibodies with the same anti-ganglioside activity in ELISA and TLC-overlay may bind with different structures in peripheral nerves. Studying the binding of anti-ganglioside antibodies to tissues by immunohistochemistry, and determining their effect in physiological models like the mouse phrenic nerve/diaphragm preparation will give further information about the site of action and pathogenicity of these antibodies.

Prognostic relevance of antecedent infections and anti-ganglioside antibodies

GBS patients are also heterogeneous with regard to recovery after therapy. Antecedent *C. jejuni* infections and anti-GM1 antibodies seem to define a distinct subgroup of patients in which plasma exchange (PE) is less effective than intravenous immunoglobulins (IVIg) (Chapters 3.2 and 3.3). This parallels MMN, since these patients also frequently have high titres of anti-GM1 antibodies and do not respond to PE either (14), and are claimed to recover after IVIg (29,30). However, the number of tested

GBS patients is relatively small, and prospective studies are needed to investigate whether such patients indeed should be treated preferentially with IVIg.

It is difficult to speculate on the mechanisms underlying this difference in recovery, since both the pathogenetic factors in this subgroup of GBS patients, and the effects of PE and IVIg on these factors are poorly understood (31). Both forms of treatment may have a short-term effect on the level of anti-neural antibodies: PE removes antibodies from the blood and IVIg may neutralize antibodies by anti-idiotypic binding. Serum antibodies to a neuroblastoma cell line, which are found in approximately half of GBS patients, were found to be directly neutralized by IVIg (32). Moreover, anti-GM1 antibody activity was claimed to be inhibited by IVIg (33). In the long run, however, antibodies may show a rebound effect after PE (33), while IVIg inhibit the proliferation of B- and T-cells, thereby inducing a permanent decrease of antibody titres (35). These forms of treatment also effect other immune components than antibodies which may play a pathogenetic role in this subgroup of GBS patients. PE will also decrease the level of cytokines and other inflammatory mediators in the blood. IVIg may also contain neutralizing antibodies against cytokines, or inhibit activation of the complement cascade by blocking complement receptors or by scavenging of complement factors (31). In addition, IVIg may interfere with presumed Fc-dependent mechanisms, such as the phagocytosis of axons and myelin by macrophages. IVIg also contain antibodies against many infectious agents, thereby blocking antigens which stimulate a possible ongoing immune response. More research is needed to clarify which of these effects may account for the unfavorable response to PE compared to IVIg in GBS patients with *C. jejuni* infection and anti-GM1 antibodies. Insight in these mechanisms may lead to a more causal and individual treatment of GBS patients, based on laboratory tests for recent infections and anti-neural antibodies, which can be performed at an early stage in the disease.

5.2 CROSS-REACTIVITY OF ANTIBODIES TO *C. JEJUNI* LIPOPOLYSACCHARIDES AND GANGLIOSIDES

Several mechanisms may underlie the formation of antibodies to damaged tissue in postinfectious diseases. Firstly, infections may directly damage the tissue, leading to release or exposure of tissue antigens against which antibodies are produced. This mechanism is probably involved in mice developing myocarditis after infection with Coxsackievirus (36). In these mice antibodies against cardiac myosin are found which do not cross-react with Coxsackievirus (36). Immunization with cardiac myosin, however, does induce high titres of antibodies to myosin (37). These findings indicate that anti-myosin antibodies in the Coxsackievirus infected mice are not formed against the virus, but are secondarily produced, against myosin from damaged cardiac tissue. Secondly, infectious agents may mimic structures in the host, inducing antibodies during infection which cross-react with the corresponding structures in host tissue. This mechanism may apply to patients who develop rheumatic carditis after streptococcus infection (38,39). In these patients, antibodies against cardiac myosin are found which cross-react with streptococcal polysaccharides (38,39), indicating that they are primarily produced against the bacteria.

The studies described in Chapter 4 indicate that the second mechanism may also apply to the formation of anti-ganglioside antibodies in some patients with GBS and MFS. In accordance with this hypothesis, we demonstrated the binding of serum anti-GM1 IgG antibodies from GBS patients to *C. jejuni* isolates from GBS patients and enteritis controls by inhibition ELISA (Chapter 4.2). In addition, serum anti-GQ1b IgG antibodies from MFS patients specifically cross-react with *C. jejuni* isolates from MFS patients (Chapter 4.3). The structures which are recognized by the anti-ganglioside antibodies are located in the LPS fraction of these bacteria (Chapter 4.4). The binding of anti-GM1 or anti-GQ1b antibodies to LPS was inhibited by treatment of the LPS with cholera toxin and sialidases respectively, indicating that the responsible structures are quite similar to epitopes on gangliosides (Chapter 4.4). Together, these findings strongly support the hypothesis that anti-ganglioside antibodies in GBS and MFS patients with antecedent *C. jejuni* infection are initially formed against ganglioside-like structures in the *C. jejuni* LPS.

Molecular mimicry between LPS and gangliosides was further indicated by the binding of mouse monoclonal anti-ganglioside antibodies with LPS of *C. jejuni* isolates from GBS and MFS patients, and enteritis controls (40,41). Biochemical analysis of the LPS core oligosaccharides identified the presence of sialic acid containing ganglioside-like moieties which show a large variation between different *C. jejuni* strains (reviewed in Chapter 1.4). The GBS associated *C. jejuni* expresses core oligosaccharide with a tetrasaccharide identical to GM1 (42), but also oligosaccharides similar to GD1a, GD3 and GT1a (43). Interestingly, the LPS core oligosaccharide from an MFS associated *C. jejuni* bears a GD3-like terminal trisaccharide rather than a GQ1b moiety (44). It would thus appear that the ganglioside-like structures in LPS may not always correspond exactly with the specificity of the serum anti-ganglioside antibodies. However, our studies have clearly indicated that LPS from GBS and MFS associated *C. jejuni* are recognized by anti-ganglioside antibodies with the specificity found in GBS and MFS patients. The variety of ganglioside-like structures among *C. jejuni* strains may contribute to the heterogeneity of the anti-ganglioside antibody specificities in GBS.

Monoclonal IgM antibodies against disialylated gangliosides from patients with CANOMAD specifically recognize sialidase sensitive epitopes in *C. jejuni* LPS, as was demonstrated by direct staining in ELISA and TLC-overlay (Chapter 4.5). In immunofluorescence, these antibodies also bind to human dorsal root ganglion cells which are affected in patients with CANOMAD, indicating that the cross-reactivity has pathogenetic relevance. The antibodies bind to the *C. jejuni* isolates from the MFS patients, but also to the isolates from one GBS patient and one enteritis control. The broader reactivity of the monoclonal antibodies compared to anti-GQ1b antibodies from MFS patients is in accordance with their broader reactivity to gangliosides. Others reported that monoclonal IgM anti-GM1 antibodies from patients with chronic motor neuropathy also bind to *C. jejuni* LPS (45). Their report and ours may suggest that *C. jejuni* infections are also one of the mechanisms underlying the induction of monoclonal anti-ganglioside antibodies in paraproteinaemic neuropathies.

An important issue is whether the ganglioside-like structures in LPS are immunodominant epitopes in the antibody response against *C. jejuni* in GBS and MFS patients. We demonstrated a close association between the presence, titre course and isotype of antibodies to LPS and antibodies to gangliosides in patients with GBS and

MFS (Chapter 4.6). Furthermore, control experiments with cholera toxin and sialidases showed that most of the antibody activity against LPS is directed against the ganglioside-like structures (Chapter 4.6). Accordingly, immunization of rabbits with LPS from *C. jejuni* PEN O:1 and O:36 serostrains, which show molecular mimicry with GM2, induces high titres of IgM and IgG antibodies against GM2 (46). Together these studies give evidence for the immunogenicity of the ganglioside-like structures in *C. jejuni* LPS.

The antibodies to LPS and gangliosides in patients with GBS and MFS are predominantly IgG1 and IgG3, and have long-lasting high titres (Chapter 4.6), which is characteristic of a T-cell-dependent immune response. This is a remarkable finding because immune responses against polysaccharides are usually T-cell-independent, and predominantly induce IgM and IgG2 antibodies. The role of T-cells in the production of cross-reactive antibodies against LPS and gangliosides in GBS and MFS patients has not yet been elucidated. *In vitro* studies indicate that mitogen-activated T-cells stimulate the production of antibodies against both bacterial polysaccharides and GM1 (35,47). The reported weak association between *C. jejuni* infections and HLA haplotype in GBS patients further suggests that T-cells are involved in this subgroup of patients (48,49).

However, glycolipids can not be bound by MHC molecules, indicating that these structures can not directly activate T-cells in an MHC-restricted manner (50). For an MHC-restricted T-cell response to glycolipids, a carrier protein is needed which binds with the glycolipid and which can be presented in the MHC groove. Possible candidates for such carrier proteins are the enterotoxins which are produced by some *C. jejuni* strains and can bind specifically to gangliosides (51,52). If a *C. jejuni* expresses both this enterotoxin and the ganglioside-like LPS, the enterotoxin may bind to LPS, providing an epitope for T-cells. Alternatively, some microbial glycolipids may induce a specific MHC-unrestricted T-cell response, as was recently demonstrated for lipoglycans (53). These antigens are presented to T-cells in association with the CD1 molecule (53), which shows a close structural similarity to MHC (54). Presently, it is unknown whether these CD1-restricted, MHC-independent T-cell responses can also be induced against LPS and whether these T-cells can provide help to B-cells for antibody production.

After activation, T-cells may skew LPS-reactive B-cells to switch to IgG1 and IgG3 production. This subclass switch may be crucial for the pathogenicity of anti-ganglioside antibodies, since IgG1 and IgG3 antibodies are more effective than IgG2 in activating the classical complement pathway (55). Recently, we could clearly demonstrate that in the mouse phrenic nerve/diaphragm preparation the pathophysiological effects of anti-ganglioside antibodies from patients with MFS and CANOMAD only occur in the presence of complement (dr. J.J. Plomp, personal communication). Also, the effect of rabbit anti-GM1 antibodies on sodium currents was also found to be complement-dependent (56). Moreover, IgG1 and IgG3 have a higher affinity for Fc-receptors which is an important feature in opsonization and phagocytosis by macrophages (57). This is in accordance with the close association between macrophages and damaged myelin or axons in histopathology studies on nerves from GBS patients (58,59).

Recently, it was reported that chickens fed with a *C. jejuni* PEN O:19 isolate from a patient with the axonal variant of GBS, 12 days later develop paresis (60). The

sciatic nerves of these animals show nodal lengthening and paranodal demyelination, followed by Wallerian-like degeneration. Macrophages are identified in the periaxonal space of affected nerves, separating the inner myelin lamellae from the axon, but lymphocytic infiltrations are uncommon. These pathological changes in paralyzed chickens are remarkably similar to those found in the axonal variant of GBS patients. Most animals sacrificed on the first day of their weakness, however, have nearly normal sciatic nerves. This may be due to anti-neural antibodies which interfere with nerve conduction, but do not immediately lead to morphological changes. However, the presence of anti-ganglioside antibodies and the possibility of passive transfer of the disease to other animals were not determined.

The presence of the ganglioside-like structures in *C. jejuni* LPS is strain specific and not exclusively associated with the Penner serotype. Anti-GQ1b antibodies do not cross-react with isolates from enteritis controls, even with the same Penner serotype as the MFS isolates, although only a limited number of controls was tested so far (Chapters 4.3 and 4.4). Murine monoclonal antibodies against GQ1b, however, also bind with LPS of *C. jejuni* from GBS patients and enteritis controls (40,41). The specificity of mouse monoclonal anti-GQ1b antibodies probably differs from those in MFS patients, illustrating the importance of investigating the isolates with antibodies from the patients themselves. The cross-reactivity of anti-GM1 antibodies from GBS patients is not restricted to *C. jejuni* isolates from anti-GM1 positive GBS patients (Chapters 4.2 and 4.4). This broader binding pattern is probably related to the fine-specificity of anti-GM1 antibodies in GBS patients. Most GBS patients with anti-GM1 antibodies have additional activity against GD1b and asialo-GM1 (Chapter 3.5), indicating that these antibodies recognize the shared Gal(β1-3)GalNAc-epitope on these glycolipids (see Appendix 2). This structure lacks the restrictive sialic acids, and has been demonstrated in many glycoproteins, suggesting that it may also be wide-spread in *C. jejuni* strains.

Infection with *C. jejuni* expressing LPS with ganglioside-like epitopes may therefore not necessarily lead to the production of anti-ganglioside antibodies. In patients with *C. jejuni* enteritis without neuropathy we did not find high titres of anti-GM1 and anti-GQ1b antibodies and only low antibody activity against *C. jejuni* LPS (Chapter 4.6). In an outbreak of *C. jejuni* PEN O:19 enteritis, only one case of GBS was reported (61). Moreover, immunization of rabbits with GM2-like LPS from *C. jejuni* serostrain PEN O:23, which has an O-chain different from the PEN O:1 and O:36, did not induce these antibodies (46). The immunogenicity of the ganglioside-like structures may depend on other bacterial factors, like the LPS O-chain, which modulates the antibody response against LPS (62), and may explain the predominance of certain Penner serotypes in GBS. The expression of the hyaluronic acid-like polymer of the O-chain, which is the antigenic determinant of the Penner serotypes, is higher in *C. jejuni* isolates from GBS patients than in isolates from enteritis controls (63). The immunogenicity of the ganglioside-like structures may further depend on the presence of carrier molecules which bind to these structures and provide an epitope for T-cell help. In addition, host factors may determine whether anti-ganglioside antibodies will be produced in response to *C. jejuni* infection, since epidemics of *C. jejuni* associated GBS do not occur. Most likely, the immunogenetic background is important, as indicated by the signficant, albeit weak HLA association with *C. jejuni* infections in GBS, and the

occurrence of GBS in two sisters, and of MFS in a brother and sister after a *C. jejuni* infection (64,65).

Ganglioside-like structures may also be present in other infectious agents, as indicated by the association between CMV infections and specific antibodies against GM2 in GBS patients (Chapter 3.4) (9). Anti-GM2 antibodies from GBS patients were found to cross-react with CMV infected cells (9). This may indicate that at least some CMV particles carry GM2 or GM2-like structures, which may be incorporated in the viral envelope during budding from the host plasma membrane (66). Alternatively, viral infection of cells may induce the expression of these cross-reactive structures at the cellular surface. Antibodies against GM1 and GD1b were also reported in a GBS patient with *M. pneumoniae* infection (67), although we could not confirm this in our patients.

In conclusion, infectious agents which are related to GBS may express molecular mimicry with gangliosides or other peripheral nerve components. Cross-reactive immune responses may therefore frequently underlie the development of GBS. Further research is necessary to elucidate which factors, in addition to molecular mimicry, will determine if an infection is followed by an immune response to peripheral nerves.

5.3 MODEL FOR THE ROLE OF *C. JEJUNI* INFECTIONS AND ANTI-GANGLIOSIDE ANTIBODIES IN THE PATHOGENESIS OF GBS AND MFS

GBS does probably not result from one general pathogenetic mechanism. Instead, distinct pathogenetic factors may play a role in subgroups of patients, as indicated by the heterogeneous pathological, immunological, and clinical findings. The studies described in this thesis focused on GBS and MFS patients with antecedent *C. jejuni* infections and anti-ganglioside antibodies. Based on these studies we propose the following model for the pathogenesis and heterogeneity in these patients (Figure). Ganglioside-like structures are present in the LPS from *C. jejuni*, which show a large variation between different strains and may be further modulated by sialidases present in the gut. The immunogenicity of these structures may depend on other bacterial factors, like the O-chain of the LPS. T-cells probably give help to pre-existing B-cells against gangliosides, which may be normally present in healthy subjects. Whether these T-cells will be activated during infection may depend on the HLA haplotype of the host and on the availability of adequate T-cell epitopes. These epitopes may occur on bacterial enterotoxins which bind to ganglioside-like structures and may function as a carrier protein. B-cells which recognize the ganglioside-like epitopes by their surface membrane immunoglobulin will capture and endocytose the proposed hapten-carrier complex, and present the processed fragment of the carrier protein to T-cells. Other antigen-presenting cells may also be involved. We hypothesize that after activation these T-cells will provide help to B-cells, which results in affinity maturation and immunoglobulin switching, predominantly to the IgG1, IgG3 and IgA classes. The activated B-cells differentiate into clones of plasma cells secreting anti-LPS antibodies which cross-react with gangliosides or other glyco-conjugates in peripheral nerves. The antibodies with anti-ganglioside activity may directly interfere with nerve conduction or neuromuscular transmission, or activate pro-inflammatory pathways leading to complement

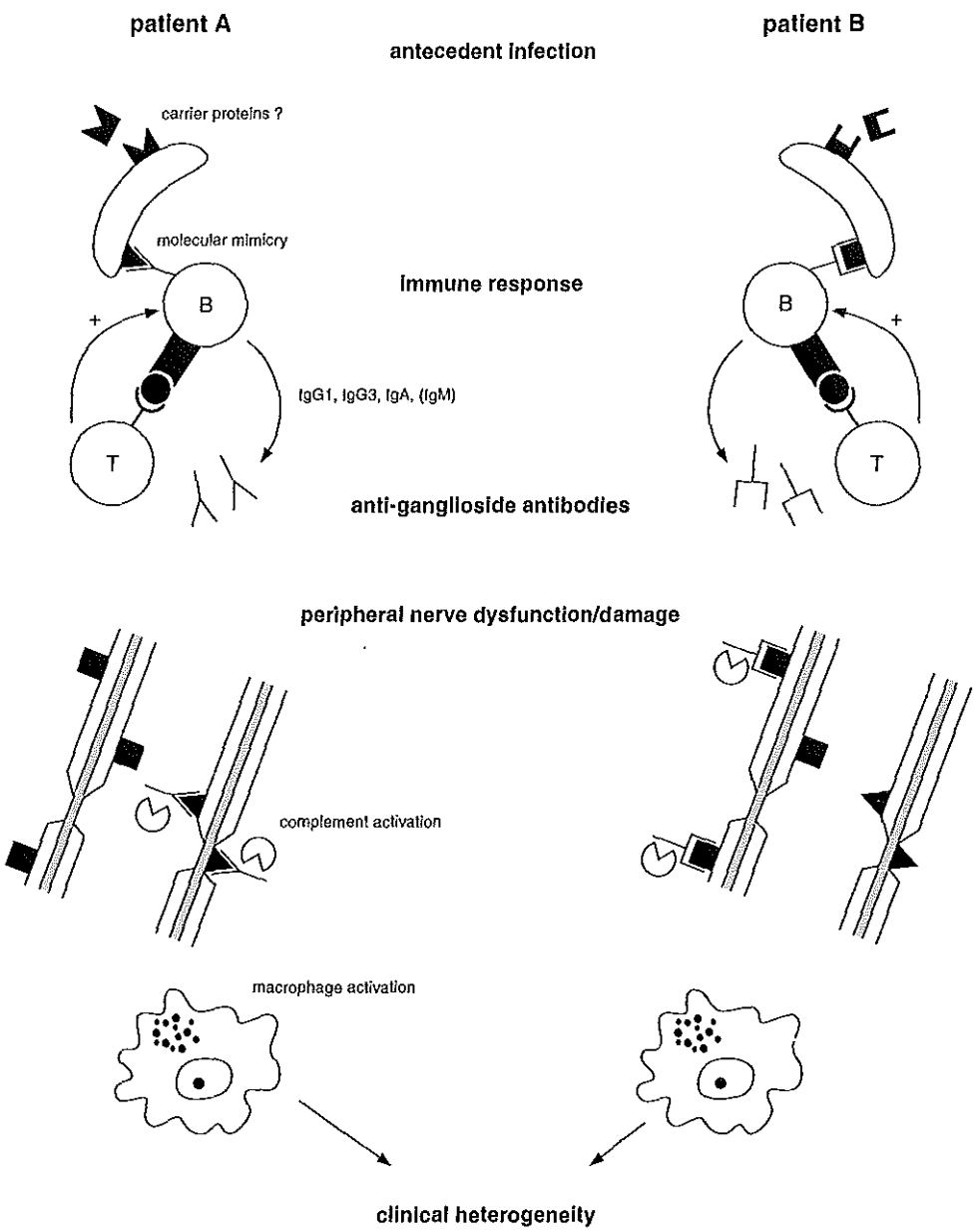


Figure. Schematic representation of the proposed model on the role of antecedent infections and anti-ganglioside antibodies in the pathogenesis and heterogeneity of GBS and MFS.

activation, membrane attack complexes, opsonization and infiltration of macrophages and subsequent demyelination and/or axonal degeneration. The site of action and pathogenicity of these antibodies will highly depend on their fine-specificity, and on the function, distribution and accessibility of the target antigens in the peripheral nerve system. The variety of ganglioside-like structures in *C. jejuni* LPS will partly determine the spectrum of anti-ganglioside antibody fine-specificities and thereby the clinical heterogeneity of GBS and its variants.

5.4 FUTURE RESEARCH

Future research should focus on further investigating the underlying mechanisms which lead to the immune mediated nerve damage, as well as on improving treatment for patients with GBS. The studies described in this thesis illustrate that research on these two fields can support each other.

We identified the cause of the antecedent infection in 56% of GBS patients, and found anti-ganglioside antibodies in 32%. Infections with other agents and antibodies against other glycoconjugates may be present in the 'negative' GBS patients. Although several lines of evidence indicate that some of the anti-ganglioside antibodies play a pathogenetic role in GBS and MFS, little is known about the immunological characteristics and pathophysiological effects of these antibodies. Research in this field will be stimulated by further improvement and standardization of the techniques used to detect these antibodies. Determining the fine-specificity and affinity of the antibodies to gangliosides and neural tissues may allow us to discriminate between pathological and normal anti-ganglioside antibodies. These studies will also further clarify the relation with neurological deficits. Recently developed techniques like the mouse phrenic nerve/diaphragm preparation will enable us to determine the pathophysiological effects of anti-ganglioside antibodies. Sofar, only a limited number of anti-ganglioside antibodies has been evaluated in these models. The studies on the specificity and pathogenicity of anti-ganglioside antibodies were hampered by the lack of monoclonal IgG antibodies from patients with GBS and MFS. New approaches, like constructing phage-display libraries from B-cells from GBS and MFS patients, will support this research.

The demonstration of ganglioside-like structures in *C. jejuni* LPS emphasized the role of infections in the pathogenesis of GBS. Future research should investigate the presence of these structures in other *C. jejuni* strains and in other micro-organisms. Animal models are needed to determine if these structures actually induce cross-reacting anti-ganglioside antibodies leading to peripheral nerve dysfunction. These animal models may also be used to investigate the possible role of T-cells and ganglioside-binding receptors and carrier proteins.

The studies in this thesis give support to the hypothesis that GBS consists of different subgroups of patients, characterized by specific antecedent infections and anti-ganglioside antibodies. In these subgroups distinct pathogenetic mechanisms may play a role. Elucidating these mechanisms will make it possible to give causal and individual treatment to patients with GBS.

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APPENDIX 1. DIAGNOSTIC CRITERIA FOR GUILLAIN-BARRÉ SYNDROME (1)

Features required for diagnosis

1. Progressive motor weakness of more than one limb
2. Areflexia
3. Absence of another identifiable cause

Features strongly supporting the diagnosis

Clinical

1. Progression of less than 4 weeks
2. Relative symmetry
3. Mild sensory symptoms and signs
4. Cranial nerve involvement
5. Recovery beginning 2 weeks to months after progression ceases
6. Autonomic dysfunction
7. Absence of fever at onset

Cerebrospinal fluid (CSF)

1. Elevated concentration of protein after the first week
2. Less than 10 mononuclear leukocytes per μl CSF

Electrodiagnostic

1. Conduction slowing or block

Features casting doubt on the diagnosis

1. Marked, persistent asymmetry of weakness
2. Persistent bladder or bowel dysfunction
3. Bladder or bowel dysfunction at onset
4. More than 50 mononuclear leukocytes per μl CSF
5. Presence of polymorphonuclear leukocytes in CSF
6. Sharp sensory level
7. Onset with respiratory failure disproportionate to limb weakness

1. Adapted from the criteria supported by the National Institute of Neurological and Communication Disorders and Stroke. Asbury AK, Cornblath DR. Assessment of diagnostic criteria for Guillain-Barré syndrome. Ann Neurol 1990(suppl);27:21-24.

APPENDIX 2. NOMENCLATURE AND STRUCTURE OF GANGLIOSIDES

Nomenclature of gangliosides

Gangliosides are sialic acid containing glycosphingolipids. Glycosphingolipids are glycolipids composed of an oligosaccharide attached to a ceramide. The ceramide is formed by *N*-acylation of sphingosine, a long chain aliphated amine of variable length. Characteristic for gangliosides is the variation in the oligosaccharide moiety. The oligosaccharide portion consists of a variable sequence of hexoses which forms the oligosaccharide core, and a variable number of sialic acids. Based on the oligosaccharide core, gangliosides are classified into 5 subgroups: the ganglio, lacto, gala, hemato and globo series. Gangliosides of the ganglio and lacto series predominate in the human peripheral nerve system. Sialic acid is the generic name for *N*-acyl-neuraminic acid, the acyl group being acetyl (*N*-acetyl-neuraminic acid) or glycolyl (*N*-glycolyl-neuraminic acid). In the human nervous system *N*-acetylneuraminic acid is the predominant sialic acid. Sialic acids are attached to other sialic acid residues, or to hexoses in the oligosaccharide core.

The nomenclature of the gangliosides used in this thesis, is based on the classification of the oligosaccharide moiety according to Svennerholm (1), and the IUPAC-IUB Commission on Biochemical Nomenclature (2).

1. The first letter refers to the hexose sequence of the oligosaccharide core:
   G :ganglio series (including *N*-acetyl-galactosamine)
   L :lacto series (including *N*-acetyl-glucosamine)
2. The second letter refers to the number of sialic acids in the oligosaccharide moiety: M (mono), D (di), T (tri), Q (quad).
3. The Arabic numeral and lower case letter refer to the migration of the ganglioside in thin-layer chromatography. In general, gangliosides with more hexoses and sialic acids in the oligosaccharide moiety migrate more slowly than the smaller gangliosides. Arabic numbers can also be calculated as five minus the number of hexoses in the oligosaccharide core (3). The lower case letters refer to isomer forms.

For example, GD1b is a ganglioside of the ganglio series [G], with two sialic acids [D] and an oligosaccharide core of four [1] hexoses, which has a slower migration in chromatography [b] than its isomere GD1a.

1. Svennerholm L. Chromatographic separation of human brain gangliosides. J Neurochem 1963;10:613-623.
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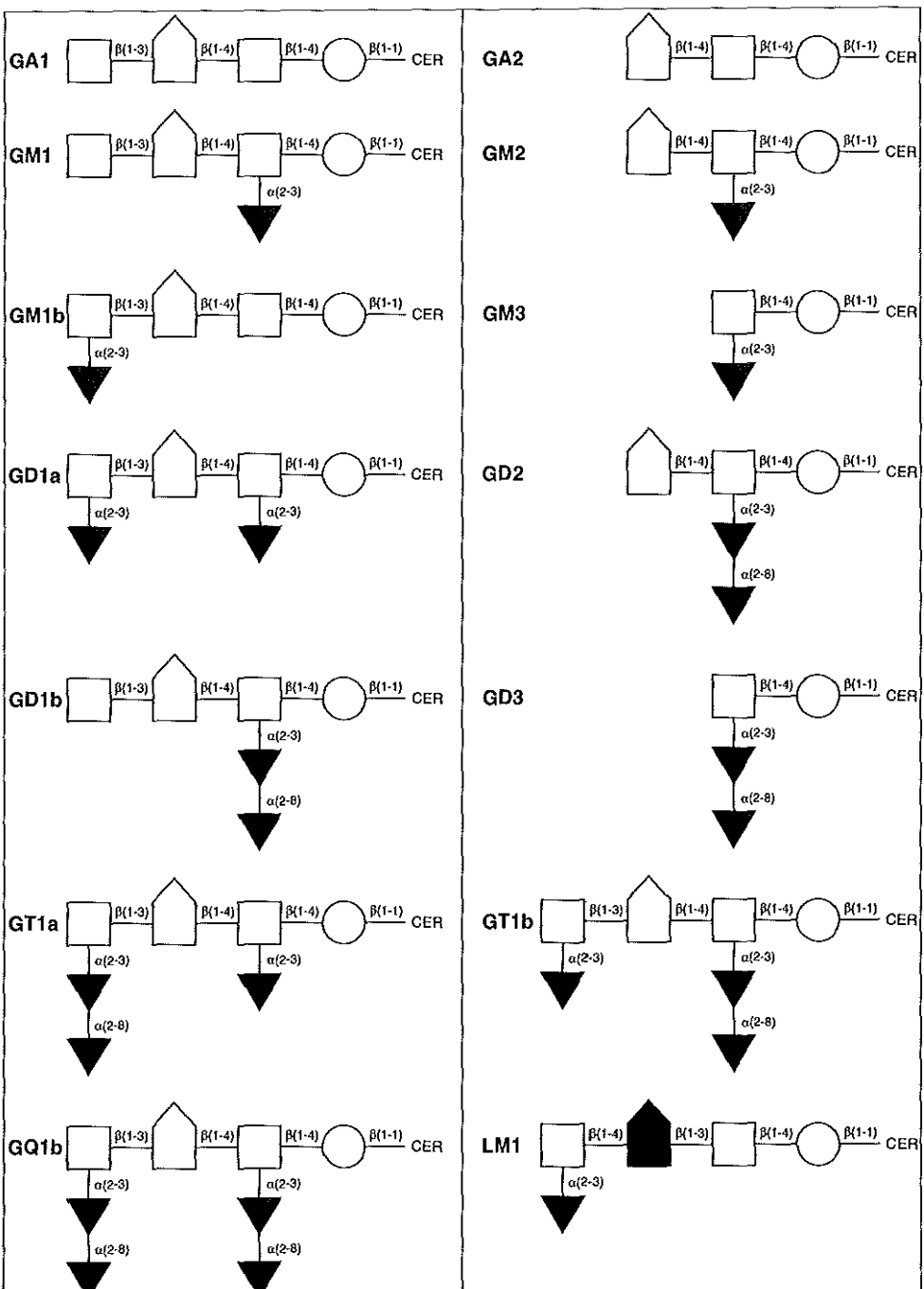
3. Alberts B, Bray D, Lewis J, Rass M, Roberts K, Watson JD. Plasma membrane (Chapter 6). In: Molecular biology of the cell, 2nd edition, Garland Publishing Inc., New York, 1989.

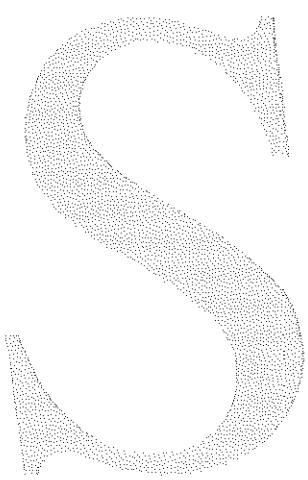
Structure of gangliosides



□, galactose, ▫, *N*-acetyl galactosamine, ○, glucose, ▼, *N*-acetyl neuraminic acid (sialic acid), ◆, *N*-acetyl glucosamine, CER, ceramide. GA1 (asialo-GM1) and GA2 (asialo-GM2) do not belong to the gangliosides since they lack the sialic acids.

SUMMARY

SAMENVATTING



SUMMARY

The Guillain-Barré syndrome (GBS) is the most common form of acute neuromuscular paralysis in The Netherlands. The GBS is characterized by demyelination and axonal degeneration of peripheral nerves, leading to progressive paralysis with areflexia. GBS patients show a remarkable variation in clinical symptoms, electrodiagnostic manifestations and underlying pathological defects. The presence of complement, immunoglobulins, and macrophages in the peripheral nerves of these patients suggests that the immune system is involved in the development of GBS. However, it is unknown which mechanism triggers the immune system to attack the peripheral nerves, and what the precise structures are to which the immune response is directed.

The immune response may be directed against gangliosides in peripheral nerves, because antibodies to various gangliosides are demonstrated in a number of patients with GBS. In patients with other forms of neuropathy, similar antibodies with a pathophysiological effect on peripheral nerves were demonstrated. Gangliosides form an extensive family of sialic acid containing glycolipids, which are particularly abundant in the membranes of neural cells. If the anti-ganglioside antibodies also play a role in the pathogenesis of GBS, the neurological deficit will reflect the distribution and function of these gangliosides in peripheral nerves.

In most GBS patients, the neurological deficit is preceded by an infection. We hypothesize that this infection triggers the production of anti-ganglioside antibodies in GBS patients. Micro-organisms may have ganglioside-like structures against which anti-ganglioside antibodies are induced, which cross-react with gangliosides in peripheral nerves. In this way, infections will determine the specificity of the anti-ganglioside antibodies, and thereby the neurological deficit in the patient. The reported clinical heterogeneity may partly result from the diversity of the antecedent infections and anti-ganglioside antibodies in patients with GBS. The aim of the studies described in this thesis was to test this hypothesis by investigating (i) if specific antecedent infections and anti-ganglioside antibodies are related to distinct clinical and electrodiagnostic subgroups of GBS, (ii) if anti-ganglioside antibodies are related to antecedent infections in GBS, and (iii) if anti-ganglioside antibodies can cross-react with *Campylobacter jejuni* bacteria, the predominant cause of antecedent infections in GBS.

To determine the relation between antecedent infections, anti-ganglioside antibodies and clinical manifestations (**Chapters 2 and 3**), pretreatment serum samples from 154 clinically and electrodiagnostically well-defined GBS patients were used who participated in two therapeutic studies. These samples were tested for the presence of IgM, IgG and IgA antibodies against the major peripheral nerve gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b and LM1 by enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatography (TLC) overlay. In addition, these samples were used to determine the serology of 16 acute viral or bacterial infections.

In a case-control study with these GBS patients and 154 age-, sex-, and season-matched controls with other neurological diseases, we investigated which infections were specifically associated with GBS (**Chapter 2**). The predominant cause of the antecedent infection in GBS patients was *C. jejuni* (32%), followed by cytomegalovirus

(CMV) (13%), Epstein-Barr virus (EBV) (10%), and *Mycoplasma pneumoniae* (5%). These infections occurred significantly more frequent in GBS patients than in the controls. In addition, infections with herpes simplex virus (1%), varicella zoster virus (1%), adenovirus (1%), influenza A virus (1%), hepatitis B virus (1%), and parainfluenza 1 virus (1%) were identified, although they did not occur more frequently than in the control group.

In **Chapter 3.2** the association between recent *C. jejuni* infection and the presence of anti-GM1 antibodies in GBS patients is described. The patients with recent *C. jejuni* infection and/or anti-GM1 antibodies more often suffered from a rapidly progressive and severe neuropathy with predominantly distal distribution of weakness, without sensory disturbances or cranial nerve deficits. **Chapter 3.3** describes that this subgroup of patients showed a poor recovery after plasma exchange compared to treatment with intravenous immunoglobulins. These studies suggest that *C. jejuni* infections and anti-GM1 antibodies are related to a pure motor variant of GBS which may preferentially be treated with intravenous immunoglobulins.

An association between antecedent CMV infections and anti-GM2 IgM antibodies in GBS patients is described in **Chapter 3.4.** The patients with CMV infections and anti-GM2 antibodies also had antibodies to asialo-GM2, but not to GM1, suggesting that the antibodies were directed to the shared GalNAc(β1-4)Gal epitope on GM2 and asialo-GM2. The patients with a CMV infection and anti-GM2 antibodies suffered from globally distributed moderate to severe limb weakness with facial palsy, severe sensory loss, paresthesias and respiratory insufficiency. The clinical presentation of these patients clearly differed from that of patients with a *C. jejuni* infection and anti-GM1 antibodies.

Chapter 3.5 reports the frequency and specificity of the other anti-ganglioside antibodies in the group of GBS patients. Antibodies against at least one of the eight above mentioned gangliosides were detected in 29% of the GBS patients, compared to 13% of 63 patients with other neurological diseases, and 2% of 50 normal controls. Most patients with anti-GD1b antibodies had additional anti-GM1 antibodies, suggesting that the antibodies recognized the common Gal(β1-3)GalNAc-epitope on GM1 and GD1b. Anti-GD1b antibodies were also associated with the pure motor variant of GBS, but only in the presence of additional anti-GM1 antibodies. Patients with anti-GD1a antibodies suffered from more severe weakness. Anti-GQ1b antibodies were specifically related to the involvement of the oculomotor nerves. On the other hand, antibodies to LM1, the predominant ganglioside in peripheral nerves, and to GM3 and GT1b, were not related to a specific clinical presentation.

In **Chapter 3.6**, the relation between the electrodiagnostic findings in GBS patients and the presence of antibodies against GM1 and GQ1b is described. The electrodiagnostics enabled further localization of the lesions in specific nerve fibres. Anti-GM1 antibodies were associated with low distal compound muscle action potential amplitudes and relatively preserved compound sensory nerve action potentials amplitudes, indicating a severe and selective dysfunction of motor nerve fibres. Axonal dysfunction was frequently found in patients with anti-GM1 antibodies. These findings are in accordance with the clinical presentation of patients with anti-GM1 antibodies, and with the high concentration of GM1 in the myelin of motor nerves and axons. In patients with anti-GQ1b antibodies compound sensory nerve action potentials could

not be detected, indicating a severe dysfunction of sensory nerves.

The studies in **Chapter 3** demonstrate the presence of a wide spectrum of anti-ganglioside antibody specificities, which is partly associated with the clinical heterogeneity of GBS. This finding is in accordance with the hypothesis that GBS consists of subgroups of patients with distinct patterns of nerve damage, caused by specific anti-ganglioside antibodies.

These studies also support the hypothesis that anti-ganglioside antibodies are induced by specific antecedent infections, possibly by molecular mimicry between micro-organisms and gangliosides. In **Chapter 4.2** the binding of anti-GM1 antibodies from GBS patients with whole *C. jejuni* bacteria was investigated by inhibition ELISA. Anti-GM1 IgG antibodies from nine of 11 GBS patients were found to cross-react with *C. jejuni* isolates from GBS patients and controls. In **Chapter 4.3** a similar investigation on *C. jejuni* isolates from three patients with the Miller Fisher syndrome (MFS) was described. MFS is considered to be a variant of GBS, and is characterized by ataxia, areflexia, and ophthalmoplegia. MFS is highly associated with the presence of anti-GQ1b antibodies. The anti-GQ1b antibodies in these MFS patients were found to cross-react with the MFS associated *C. jejuni* isolates, but not with isolates from GBS patients or controls. Further studies, presented in **Chapter 4.4**, demonstrated that the responsible structures are present in the lipopolysaccharide (LPS) fraction of the *C. jejuni*. Anti-GQ1b antibodies cross-reacted with sialidase sensitive epitopes of the LPS from the three MFS associated isolates. These structures were not present in LPS from other *C. jejuni* isolates. Anti-GM1 antibodies bound with LPS structures that were resistant to sialidases, but were recognized by cholera toxin. The control experiments with sialidases and cholera toxin clearly indicate that *C. jejuni* LPS contains structures which show high similarity with gangliosides. The experiments further indicate that the cross-reactivity of antibodies to LPS and gangliosides is not due to aspecific binding. The anti-GM1 antibody binding epitopes were also demonstrated in LPS of *C. jejuni* isolates from GBS patients and controls without anti-GM1 antibodies. Therefore, other factors in addition to molecular mimicry will determine the induction of anti-GM1 antibodies.

In **Chapter 4.5** the *C. jejuni* LPS were further examined with monoclonal IgM antibodies against disialosyl moieties on gangliosides, purified from the serum of two patients with chronic ataxic neuropathy. The monoclonal antibodies specifically reacted with *C. jejuni* LPS from the three MFS patients, one GBS patient and one control, as was demonstrated in direct ELISA and TLC overlay. In addition, using immunofluorescence, these antibodies bound to human dorsal root ganglion neurons, which are damaged in patients with chronic ataxic neuropathy. These studies indicate that *C. jejuni* LPS and dorsal root ganglion neurons share common epitopes which are recognized by anti-ganglioside antibodies in patients with neuropathies related to these antibodies.

The humoral immune response against *C. jejuni* LPS in GBS and MFS patients was further investigated in **Chapter 4.6**. In GBS and MFS patients long-lasting high titres of IgG1 and IgG3 antibodies against LPS were demonstrated, characteristic of a T-cell dependent antibody formation. These antibodies against LPS had the same titre course and isotype as the detected anti-ganglioside antibodies. The antibody activity

was predominantly directed against cholera toxin or sialidase sensitive epitopes in LPS, suggesting that the ganglioside-like structures in LPS are immunodominant epitopes.

The studies described in **Chapter 4** support the hypothesis that part of the anti-ganglioside antibodies in GBS patients with antecedent *C. jejuni* infection are induced by ganglioside-like structures in the LPS of *C. jejuni*. However, the formation of anti-ganglioside antibodies also depends on other factors than molecular mimicry. In **Chapter 5**, we discussed the mechanisms by which infections may induce cross-reactive antibodies, and by which these antibodies may interfere with nerve conduction in patients with GBS and MFS. Based on the results of the studies in the thesis, a model was proposed for the role of *C. jejuni* infections and anti-ganglioside antibodies in the pathogenesis of GBS. Future research is necessary to further elucidate the role of molecular mimicry and other bacterial and host factors in this model. Moreover, the pathogenesis of GBS in patients without anti-ganglioside antibodies remains to be clarified.

SAMENVATTING

Het syndroom van Guillain-Barré (GBS) is de meestvoorkomende vorm van acute neuromusculaire verlamming in Nederland. Het GBS wordt gekenmerkt door demyelinisatie en axonale degeneratie van perifere zenuwen, welke leidt tot een progressieve verlamming met areflexie. Patiënten met GBS vertonen een opmerkelijke variatie in klinische symptomen, electrodiagnostische manifestaties en onderliggende pathologische afwijkingen. De aanwezigheid van complement, immunoglobulinen en macrofagen in de perifere zenuwen van deze patiënten suggereert dat het immuunsysteem betrokken is bij het ontstaan van GBS. Het is echter onbekend door welk mechanisme het immuunsysteem wordt geactiveerd om de perifere zenuwen aan te vallen, en tegen welke structuren de immuunreactie precies is gericht.

De immuunreactie is mogelijk gericht tegen gangliosiden in perifere zenuwen, aangezien antistoffen tegen diverse gangliosiden zijn aangetoond bij een deel van de patiënten met GBS. Bij patiënten met andere vormen van neuropathie werden soortgelijke antistoffen aangetoond met een pathofysiologisch effect op perifere zenuwen. Gangliosiden vormen een uitgebreide familie van siaalzuurbevattende glycolipiden, die met name talrijk zijn in de membranen van neurale cellen. Indien antiganglioside-antistoffen ook een rol spelen bij de pathogenese van GBS zal de neurologische uitval overeenkomen met de verdeling en functie van deze gangliosiden in perifere zenuwen.

Bij de meeste GBS-patiënten wordt de neurologische uitval voorafgegaan door een infectie. Onze hypothese is dat deze infectie de productie van antiganglioside-antistoffen teweegbrengt bij GBS-patiënten. Micro-organismen zouden ganglioside-achtige structuren kunnen hebben waartegen antistoffen worden opgewekt, die kruisreageren met gangliosiden in de perifere zenuwen. Op deze manier bepalen infecties de specificiteit van de antiganglioside-antistoffen en daarmee de neurologische uitval bij de patiënt. De beschreven klinische heterogeniteit is mogelijk deels het gevolg van de variatie in de voorafgaande infecties en antiganglioside-antistoffen bij patiënten met GBS. Het doel van de studies beschreven in dit proefschrift was om deze hypothese te toetsen door te onderzoeken (i) of specifieke voorafgaande infecties en antiganglioside-antistoffen zijn gerelateerd aan bepaalde klinische en electrodiagnostische subgroepen van GBS, (ii) of antiganglioside-antistoffen zijn geassocieerd met voorafgaande infecties bij GBS, en (iii) of antiganglioside-antistoffen kunnen kruisreageren met *Campylobacter jejuni* bacteriën, de voornaamste oorzaak van voorafgaande infecties bij GBS.

Om de relatie tussen voorafgaande infecties, antiganglioside-antistoffen en klinische manifestaties te bepalen (**Hoofdstukken 2 en 3**), werden serummonsters van vóór de behandeling gebruikt, van 154 klinisch en electrodiagnostisch goedbeschreven patiënten met GBS die deelnamen aan twee therapeutische studies. Deze monsters werden getest op de aanwezigheid van IgM-, IgG- en IgA-antistoffen tegen de belangrijkste gangliosiden in perifere zenuwen, GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b en LM1, met behulp van 'enzyme-linked immunosorbent assay' (ELISA) en 'thin-layer chromatography (TLC) overlay'. Daarnaast werden deze monsters gebruikt

om de serologie te bepalen van 16 acute virale of bacteriële infecties.

In een case-controlstudie met deze GBS-patiënten en 154 leeftijd-, geslacht-, en seizoen-gematchte controles met andere neurologische aandoeningen onderzochten we welke infecties specifiek zijn geassocieerd met GBS (**Hoofdstuk 2**). De meestvoorkomende oorzaak van de voorafgaande infectie bij patinten met GBS was *C. jejuni* (32%), gevolgd door cytomegalovirus (CMV) (13%), Epstein-Barrvirus (EBV) (10%), en *Mycoplasma pneumoniae* (5%). Deze infecties vonden significant vaker plaats bij GBS-patiënten dan bij de controles. Daarnaast werden infecties met herpes-simplexvirus (1%), varicella-zostervirus (1%), adenovirus (1%), influenza-A-virus (1%), influenza-B-virus (1%), en parainfluenza-1-virus (1%) aangetoond bij GBS-patiënten, ofschoon deze niet vaker voorkwamen dan in de controlegroep.

In **Hoofdstuk 3.2** wordt de associatie beschreven tussen recente infecties met *C. jejuni* en de aanwezigheid van anti-GM1-antistoffen bij GBS-patiënten. De patiënten met *C. jejuni*-infecties en/of anti-GM1-antistoffen leden vaker aan een snel-progressieve en ernstige neuropathie met voornamelijk distale verdeling van de zwakte, zonder sensibele stoornissen of uitval van hersenzenuwen. **Hoofdstuk 3.3** beschrijft dat deze subgroep van patiënten een slecht herstel vertoonde na plasma-uitwisseling vergeleken met intraveneuze toediening van immunoglobulinen. Deze studies suggereren dat *C. jejuni*-infecties en anti-GM1-antistoffen gerelateerd zijn aan een puur-motore variant van GBS, die mogelijk bij voorkeur door intraveneuze toediening van immunoglobulinen behandeld zou moeten worden.

Een associatie tussen voorafgaande CMV-infecties en anti-GM2-IgM-antistoffen in GBS-patiënten wordt beschreven in **Hoofdstuk 3.4**. De patiënten met CMV-infecties en anti-GM2-antistoffen hadden tevens antistoffen tegen asialo-GM2, maar niet tegen GM1, suggererend dat de antistofactiviteit gericht was tegen het gemeenschappelijke GalNAc(β1-4)Gal-epitoop op GM2 en asialo-GM2. De patiënten met een CMV-infectie en anti-GM2-antistoffen leden aan een globaalverdeelde matige tot ernstige zwakte met aangezichtsverlamming, ernstige sensore uitval, paresthesieën en respiratoire insufficiëntie. Het klinische beeld van deze patiënten verschilde duidelijk van dat van de patiënten met een *C. jejuni*-infectie en anti-GM1-antistoffen.

Hoofdstuk 3.5 vermeldt de frequentie en specificiteit van de andere antiganglioside-antistoffen in de groep van GBS-patiënten. Antistoffen tegen tenminste één van de bovengenoemde acht gangliosiden werden gevonden bij 29% van de GBS-patiënten, vergeleken met 13% van de 63 patiënten met andere neurologische aandoeningen, en 2% van de 50 normale controles. De meeste patiënten met anti-GD1b-antistoffen hadden tevens anti-GM1-antistoffen, suggererend dat de antistoffen het gemeenschappelijke Gal(β1-3)GalNAc-epitoop op GM1 en GD1b herkenden. Anti-GD1b-antistoffen waren ook geassocieerd met de puur-motore variant van GBS, maar alleen in aanwezigheid van anti-GM1-antistoffen. Patiënten met anti-GD1a-antistoffen leden aan een ernstigere zwakte. Anti-GQ1b-antistoffen waren specifiek gerelateerd aan uitval van de oculomotore zenuwen. Daarentegen waren antistoffen tegen LM1, het voornaamste ganglioside in het myeline van perifere zenuwen, en tegen GM3 en GT1b, niet gerelateerd aan een specifieke klinische presentatie.

In **Hoofdstuk 3.6** wordt de relatie beschreven tussen de electrodiagnostische bevindingen bij GBS-patiënten en de aanwezigheid van antistoffen tegen GM1 en GQ1b. De electrodiagnostiek maakte het mogelijk om de lesies in specifieke zenuwvezels nader te localiseren. Anti-GM1-antistoffen waren geassocieerd met lage distale

amplitudes van de samengestelde spier-actiepotentialen en relatief gespaarde amplitudes van de samengestelde sensibele-zenuwactiepotentialen, ten teken van een ernstige en selectieve dysfunctie van de motore-zenuwvezels. Axonale dysfunctie werd vaak gevonden bij patiënten met anti-GM1-antistoffen. Deze bevindingen sluiten aan bij het klinisch beeld van de patiënten met anti-GM1-antistoffen en bij de hoge concentratie GM1 in het myeline van motore vezels en axonen. Bij patiënten met anti-GQ1b-antistoffen konden de samengestelde sensibele-zenuwactie-potentialen niet worden opgewekt, passend bij ernstige dysfunctie van de sensibele zenuwen.

De studies in **Hoofdstuk 3** tonen aan dat er een breed spectrum van antiganglioside-antistofspecificiteiten aanwezig is bij GBS-patiënten, welke deels is gerelateerd aan de klinische heterogeniteit van het GBS. Deze bevinding is in overeenstemming met de hypothese dat GBS bestaat uit subgroepen van patiënten met bepaalde patronen van zenuwbeschadiging, veroorzaakt door specifieke antiganglioside-antistoffen.

Deze studies ondersteunen ook de hypothese dat antiganglioside-antistoffen worden geïnduceerd door de specifieke voorafgaande infecties, mogelijk ten gevolge van een moleculaire gelijkenis tussen micro-organismen en gangliosiden. In **Hoofdstuk 4.2** werd met behulp van een inhibitie-ELISA onderzocht of anti-GM1-antistoffen van GBS-patiënten konden binden aan *C. jejuni*-bacteriën. Anti-GM1-IgG-antistoffen van negen van de 11 GBS-patiënten bleken te kunnnen kruisreageren met *C. jejuni*-isolaten van GBS-patiënten en controles. In **Hoofdstuk 4.3** wordt een soortgelijk onderzoek beschreven met *C. jejuni* isolaten van drie patiënten met het syndroom van Miller Fisher (MFS). Het MFS wordt beschouwd als een variant van het GBS, en wordt gekenmerkt door ataxie, areflexie en ophthalmoplegie. Het MFS is sterk geassocieerd met de aanwezigheid van anti-GQ1b-antistoffen. De anti-GQ1b-antistoffen van deze MFS-patiënten bleken te kunnen kruisreageren met de MFS-geassocieerde *C. jejuni*-isolaten, maar niet met isolaten van GBS-patiënten of controles. Nader onderzoek, weergegeven in **Hoofdstuk 4.4**, toonde aan dat de verantwoordelijke structuren aanwezig zijn in de lipopolysaccharide-(LPS)fractie van de *C. jejuni*. Anti-GQ1b-antistoffen kruisreageerden met sialidase-gevoelige epitopen van het LPS van de MFS-geassocieerde isolaten. Deze structuren waren niet aanwezig in het LPS van andere *C. jejuni*-isolaten. Anti-GM1-antistoffen bonden aan structuren in het LPS die resistent waren voor sialidases, maar die werden herkend door choleratoxine. De controle-experimenten met sialidases en choleratoxine toonden duidelijk aan dat in LPS van *C. jejuni* structuren aanwezig zijn die een sterke gelijkenis vertonen met gangliosiden. De experimenten toonden verder aan dat de kruisreactiviteit van de antistoffen met LPS en gangliosiden niet berust op aspecifieke binding. De epitopen die anti-GM1-antistoffen kunnen binden werden ook aangetoond in het LPS van *C. jejuni*-isolaten van GBS-patiënten en controles zonder anti-GM1-antistoffen. De inductie van anti-GM1-antistoffen wordt derhalve naast de moleculaire mimicry, ook bepaald door andere factoren.

In **Hoofdstuk 4.5** werden de *C. jejuni*-LPS nader onderzocht met behulp van monoclonale IgM-antistoffen tegen disialosylstructuren op gangliosiden, welke werden gezuiverd uit het serum van twee patiënten met een chronische atactische neuropathie. Met behulp van directe ELISA en TLC-overlay kon worden aangetoond dat de monoclonale antistoffen specifiek reageerden met de LPS van *C. jejuni*-isolaten van de drie MFS-patiënten, één GBS patiënt en één controle. Daarnaast bleken deze antistoffen in immunofluorescentiestudies te binden aan humane dorsale ganglioncellen,

welke bij patiënten met chronische atactische neuropathie zijn beschadigd. Deze studies tonen aan dat *C. jejuni*-LPS en dorsale ganglioncellen gemeenschappelijke epitopen hebben, die herkend kunnen worden door antiganglioside-antistoffen bij patiënten met neuropathieën die gerelateerd zijn aan deze antistoffen.

De humorale immuunreactie tegen *C. jejuni*-LPS bij GBS- en MFS-patiënten werd nader onderzocht in **Hoofdstuk 4.6**. In GBS- en MFS-patiënten werden langdurig hoge titers van IgG1- en IgG3-antistoffen tegen LPS aangetoond, welke karakteristiek zijn voor een T-celafhankelijke antistofvorming. Deze antistoffen tegen LPS vertoonden hetzelfde titerbeloop en isotype als de aangetoonde antistoffen tegen gangliosiden. De antistofactiviteit bleek voornamelijk te zijn gericht tegen choleratoxine- en sialidasegevoelige epitopen in het LPS, suggererend dat de ganglioside-achtige structuren in LPS immunodominante epitopen zijn.

De onderzoeken die beschreven worden in **Hoofdstuk 4** ondersteunen de hypothese dat een deel van de antiganglioside-antistoffen in GBS-patiënten met een voorafgaande *C. jejuni*-infectie wordt geïnduceerd door ganglioside-achtige structuren in het LPS van *C. jejuni*. De vorming van antiganglioside-antistoffen is echter ook afhankelijk van andere factoren dan moleculaire mimicry. In **Hoofdstuk 5** werden de mogelijke mechanismen beschreven waarop infecties kruisreagerende antistoffen zouden kunnen induceren, en hoe deze antistoffen de zenuwgeleiding bij patiënten met GBS en MFS zouden kunnen verstoren. Gebaseerd op de resultaten van de studies in dit proefschrift werd een model opgesteld voor de rol van *C. jejuni*-infecties en antiganglioside-antistoffen bij de pathogenese van GBS. Toekomstig onderzoek is noodzakelijk om de rol van moleculaire mimicry en andere bacteriële en gastheerfactoren nader te verklaren. Bovendien moet de pathogenese van GBS bij patiënten zonder antiganglioside-antistoffen nog worden opgehelderd.

LIST OF ABBREVIATIONS

AIDP	:	acute inflammatory demyelinating polyneuropathy
ALS	:	amyotrophic lateral sclerosis
AMAN	:	acute motor axonal neuropathy
AMSAN	:	acute motor sensory axonal neuropathy
AU	:	*Arthrobacter ureafaciens*
BNB	:	blood-nerve barrier
BSA	:	bovine serum albumin
CANOMAD	:	chronic ataxic neuropathy with ophthalmoplegia, M-protein, agglutination, and disialosyl antibodies
CB	:	conduction block
CD	:	cluster of differentiation/cluster of designation
CF	:	complement fixation
CIDP	:	chronic inflammatory demyelinating polyneuropathy
CJ	:	*Campylobacter jejuni*
CJC	:	*Campylobacter jejuni* infected controls
C. jejuni	:	*Campylobacter jejuni*
CMAP	:	compound muscle action potential
CMV	:	cytomegalovirus
CMVC	:	cytomegalovirus infected controls
CP	:	*Clostridium perfringens*
CSF	:	cerebrospinal fluid
CSNAP	:	compound sensory nerve action potential
CT	:	cholera toxin
CV	:	conduction velocity
CVA	:	cerebrovascular accident
DML	:	distal motor latencies
DRG	:	dorsal root ganglion
EBV	:	Epstein-Barr virus
ECL	:	enhanced chemiluminescence
E. coli	:	*Escherichia coli*
ELISA	:	enzyme-linked immunosorbent assay
EMG	:	electrodiagnostic examination
F	:	female
GA1	:	asialo-GM1
GA2	:	asialo-GM2
Gal	:	galactose
GalNAc	:	*N*-acetyl galactosamine
GBS	:	Guillain-Barré syndrome
Glc	:	glucose
GlcNAc	:	*N*-acetyl glucosamine
HIV	:	human immunodeficiency virus
HLA	:	human leukocyte antigen
IEF	:	isoelectric focusing

IF	:	immunofluorescence
IgA	:	immunoglobulin A
IgG	:	immunoglobulin G
IgM	:	immunoglobulin M
IVIg	:	intravenous immunoglobulins
LIO	:	Lior system
LLN	:	lower limit of normal
LPS	:	lipopolysaccharide
M	:	male
MFS	:	Miller Fisher syndrome
MHC	:	major histocompatibility complex
MMN	:	multifocal motor neuropathy
MP-IVIg	:	methyl-prednisolon and intravenous immunoglobulins
M. pneumoniae	:	*Mycoplasma pneumoniae*
MRC	:	Medical Research Council
MS	:	multiple sclerosis
NC	:	normal controls
NeuAc	:	*N*-acetyl neuraminic acid
nt	:	not tested
OD	:	optical densities
OND	:	other neurological diseases
PBS	:	phosphate-buffered saline
PE	:	plasma exchange
PEN	:	Penner system
PNP	:	polyneuropathy
RBC	:	red blood cells
rbc-M	:	M-protein purified by eluation on red blood cells
SD	:	standard deviation
SLE	:	systemic lupus erythematosus
TLC	:	thin-layer chromatography
ULN	:	upper limit of normal
URTI	:	upper respiratory tract infection

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Bart

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- 1987-1988	:	student-assistent op afdeling Psychiatrie (Academisch Ziekenhuis, Rotterdam); onderzoek naar puerperaal psychose (o.l.v. Dr. J.-L. Klompenhouwer)
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1992-1997	:	werkzaam op afdelingen Neurologie (afdelingshoofd: Prof. dr. F.G.A. van der Meché) en Immunologie, Erasmus Universiteit/Academisch Ziekenhuis, Rotterdam, met als taken: - promotieonderzoek naar de rol van *Campylobacter jejuni* infecties en antiganglioside-antistoffen in de pathogenese van het Guillain-Barré syndrome (o.l.v. Prof. dr. F.G.A. van der Meché en Dr. P.A. van Doorn) - consulent t.b.v. de neuroimmunologische diagnostiek - onderwijs in de klinische immunologie aan studenten geneeskunde
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