Host-pathogen Interactions in Guillain-Barré Syndrome

Gastheer-pathogeen interacties in het Guillain-Barré syndroom

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1

Introduction

GUILLAIN-BARRÉ SYNDROME

Guillain-Barré syndrome (GBS) is a neurological illness in which patients become rapidly paralyzed and require long-term rehabilitation. At present, it is the world's most frequent cause of acute ascending paralysis in those countries where poliomyelitis has been eradicated (1). GBS is a post-infectious immune-mediated disease and in the last twenty years much progress has been made in elucidating the immune response to infections and peripheral nerves, the types of infection and the mechanism of nerve damage (2). As a consequence, GBS is regarded a model disease for other post-infectious diseases.

The aim of the following paragraphs is to present a comprehensive framework for reading the remaining chapters of this thesis. It will focus on the role of infections, antibodies to neural antigens and the presumed immunological and molecular factors that determine why some individuals may develop GBS after infections whereas most do not. A general introduction about the history and clinical aspects of GBS and related disorders will be presented first. Next, the pathogenesis of GBS will be addressed in more detail. The role of serum anti-neural antibodies in the diagnosis of GBS and other immune-mediated neuropathies will be discussed separately.

Central to this thesis is the thought that interactions between patients (the host) and microorganisms (pathogens) contribute to the development of this post-infectious syndrome. At the end of this chapter the outline and aims of the studies described in this thesis will be presented.

History

When modern medicine roots were shaped during the 19th century different authors have described cases resembling GBS. The most detailed description of "acute ascending paralysis" during that time was written by J. B. Landry de Thézillat in 1859 (3). His superior suggested a relationship with preceding diphtheria in such patients, a common cause for gastroenteritis. During the First World War, G. Guillain and J.-A. Barré were serving doctors in the French army and performed lumbar punctures in two soldiers who had developed an acute partial paralysis that resembled Landry's ascending paralysis. In 1916 the radiologist and physicist A. Strohl described in a French journal that both soldiers had reduced tendon reflexes in electrophysiological examination (4). He also found an elevated protein content in the cerebrospinal fluid (CSF) of these patients but without pleiocytosis. Together with Guillain and Barré they called this phenomenon the "*dissociation albuminocytologique*" and published this observation in the same edition of the journal (5). At the time these were relevant discoveries since they discriminated the syndrome from poliomyelitis and other prominent febrile causes of paralysis.

At a meeting in 1927, H. Draganesco and J. Claudian introduced the eponym Guillain-Barré syndrome, neglecting Landry and Strohl in the discovery of this syndrome (6). Today GBS is divided into different subgroups with different acronyms and is considered to be an immune-mediated polyneuropathy.

Clinical features

Diagnosis

Clinical symptoms of GBS

In essence, GBS is an acute generalized polyradiculopathy affecting limbs proximally and distally that commonly spreads to cranial nerves (7). The initial hallmark of GBS is progressive muscle weakness with or without numbness. In some patients progressive shortness of breath indicates involvement of respiratory muscles, which can lead to respiratory failure. Double vision and difficulties with swallowing indicate cranial nerve involvement. Sensory symptoms include numbness, paresthesia, pain and ataxia. Pain particularly occurs at the beginning of the disease and is more frequently present in children. In some patients autonomic dysfunction causes blood pressure disturbances and cardiac arrhythmias. In neurological examination a symmetrical distribution of sensory and motor deficits is usually found although asymmetric distribution of symptoms does not rule out the diagnosis. Tendon reflexes are low or absent in affected muscles.

An increased protein level in CSF is usually found by the second week of disease. Electrophysiology may demonstrate signs of a demyelinating or axonal polyneuropathy and polyradiculopathy and is also best performed in the second week (8). Diagnostic criteria have been proposed that are generally used for inclusion of GBS patients in clinical trials and summarize these characteristics (9) (Appendix 1).

Disease course and prognosis

A trigger precedes the onset of GBS, which is usually an infection in the days or weeks before (Figure 1). Subsequently, symptoms progress within days or weeks but sometimes within hours. The maximum level of weakness is reached within 4 weeks after onset of symptoms and is followed by a plateau phase, which can persist for weeks or months. Usually spontaneous recovery will take place that is often incomplete and may take years. In principle, GBS is a monophasic disorder although clinical fluctuations sometimes occur termed treatment-related fluctuations, which suggest a relationship with a temporarily response to treatment (10). A retrospective study showed that secondary deterioration nine weeks after onset of symptoms is suggestive for chronic inflammatory demyelinating polyneuropathy (CIDP) instead of GBS (11).

Several prognostic factors are associated with poor outcome, including increased age (12-14), artificial ventilation (13), rapid onset of symptoms (15), absent motor responses and axonal involvement in initial electromyography (12-14), previous diarrhea or *Campylobacter jejuni* (*C. jejuni*) infection and antecedent cytomegalovirus (CMV) infection (13). Recently a clinically easy to use prediction model based on these prognostic factors was proposed that could be used to inform patients in an early stage (16). Retrospective analysis reveals that most patients will eventually function independently. However, 15-20% of patients are still unable to walk unaided after 6 months. In addition, 4-15% of patients die, mostly due to complications such as pneumonia, although this also depends on demographical factors such as geography and social economic factors. Residual symptoms constitute a challenge for patients and a lasting fatigue occurs in 70% of patients after stabilization (17).

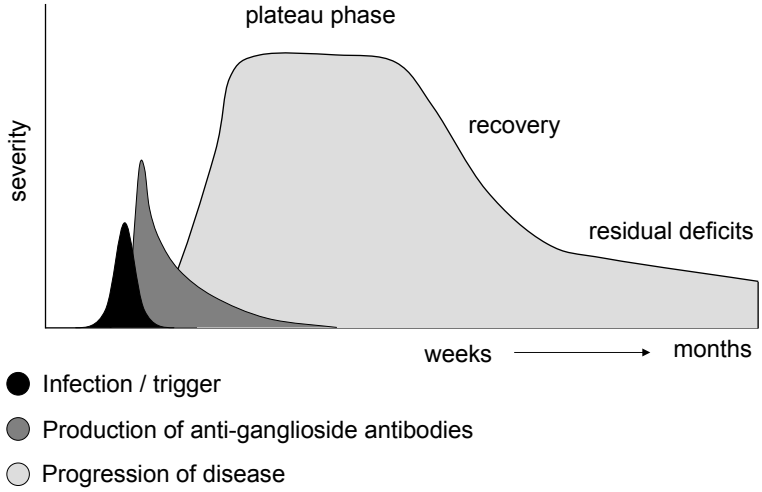


Figure 1. Disease course of GBS. The subsequent phases of disease are shown in relation to the severity and time course of GBS.

Treatment

The most important cornerstone for GBS treatment is supportive care, which generally requires a multidisciplinary approach (18). Regular monitoring of vital capacity, cardiac arrhythmias, blood pressure, urinary retention, pain, ileus and deep vein thrombosis are particularly important. Monitoring in an intensive care unit is indicated in patients with rapid progressive disease course, respiratory failure and patients with autonomic dysfunction. Intravenous immunoglobulin (IVIg) is the first choice of treatment and is similarly effective as plasmapheresis, but easier to be administered, better tolerated and is associated less often with complications (19). Addition of intravenous corticosteroids to IVIg may be beneficial in a selection of patients but is ineffective as monotherapy (20,21). In some patients a second course of IVIg may be given when patients deteriorate after an initial improvement. In developing countries therapies are hardly implemented presumably because they are expensive and only shorten disease duration of a self-limiting disease. However, prognosis of GBS is poor in more than 20% of patients; more effective, cheaper and more easily manufactured drugs are therefore desirable. New promising trials will investigate the beneficial use of complement inhibitors and additional courses of IVIg in a selection of patients with poor prognosis. Furthermore, many patients benefit from patient support groups such as the international GBS/CIDP foundation and the Dutch Vereniging Spierziekten Nederland (VSN).

Classification and heterogeneity

A syndrome is often defined as a spectrum of symptoms that characterizes a disease without reference to its cause. Accordingly, GBS is considered a syndrome with several distinct

subtypes and not a unitary disorder. The different features that determine this heterogeneity are illustrated in Figure 2. This heterogeneity is presumably caused by different host genetic and environmental factors.

Accordingly, GBS can be classified in various ways. The most commonly used classification is based on electrophysiological findings, which are supposed to reflect the histological damage and is related to geographical areas. Acute inflammatory demyelinating polyneuropathy (AIDP) is the predominant form of GBS in Europe and in Northern America (22). In 1993, a collaborative study from Chinese and American researchers reported a primary axonal form of GBS occurring as an epidemic in a rural area in Northwest China (23). This form was called acute motor axonal neuropathy (AMAN) when a pure motor syndrome was found (without sensory symptoms) and acute motor and sensory axonal neuropathy (AMSAN) when sensory and motor involvement were both found. AMAN later appeared to be the most common type of GBS in China, Japan, Central and South America (23-25) and was highly associated with preceding *C. jejuni* infections and anti-ganglioside antibodies.

In 1956, Charles Miller Fisher reported three patients with acute ophthalmoplegia, ataxia and areflexia. This clinical triad was subsequently termed the Miller Fisher syndrome (MFS)(26). The clinical symptoms in MFS are often supplemented by bulbar or oropharyngeal signs.

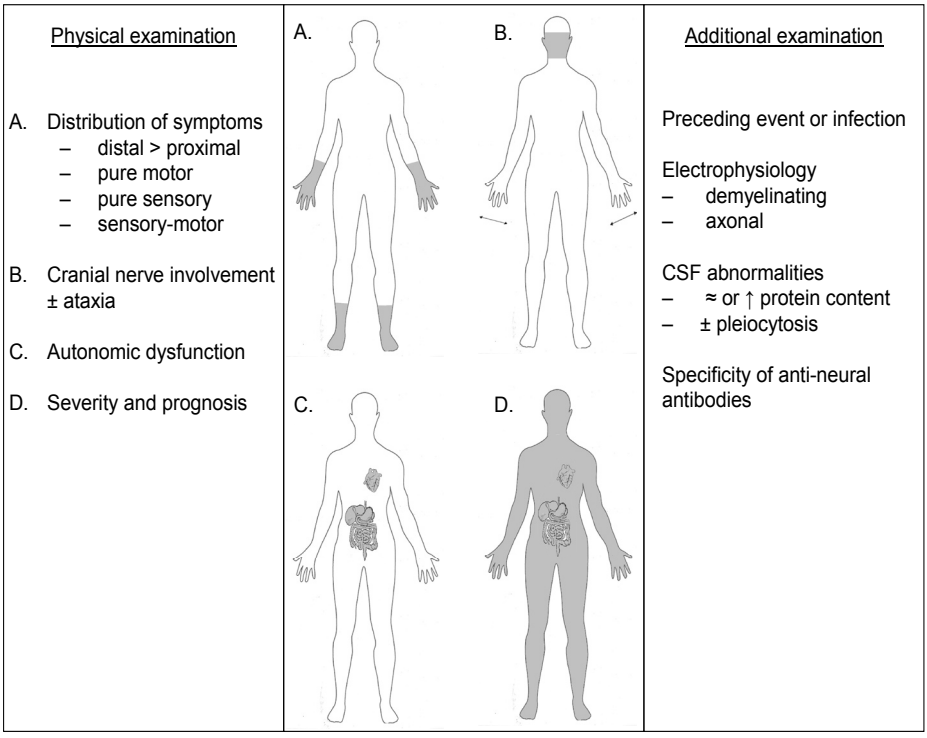


Figure 2. Heterogeneity in GBS in clinical practice. The variability of several factors in relation to clinical deficits and additional examination determines the heterogeneity in GBS.

Some extent of muscle weakness is occasionally found in which cases it is sometimes referred as an overlapping form of GBS with ophthalmoplegia. In the original publication, Miller Fisher already considered the triad a variant of GBS as the clinical course of MFS is also very similar to GBS. In the current literature it is generally accepted that the syndromes are associated with each other.

Table 1. Classification of most important variants of GBS.

Classification	Clinical syndrome	Electrophysiology	Geographical relative frequency
AIDP	Pure motor or Sensory motor	Demyelinating	Western countries: 80% Asian countries: < 20%
AMAN / AMSAN	Pure motor / Sensory motor	Axonal	Western countries: 5-10% Asian countries: 30-50%
MFS	Ophthalmoplegia Ataxia Areflexia	Absent SNAP in SFEMG	Western countries: <5% Japan: 10-15%
Overlapping forms	Ophthalmoplegia Sensory motor	Various patterns	<5%

Abbreviations: AIDP: acute inflammatory demyelinating polyneuropathy, AMAN: acute motor axonal neuropathy, AMSAN: acute motor and sensory axonal neuropathy, MFS: Miller Fisher syndrome, SNAP: sensory nerve action potential, SFEMG: single fiber electrophysiology.

Epidemiology

Extrapolation of available data from 40 years of research reveals an estimated annual incidence between 0.8 and 2.0 GBS cases per 100.000 individuals (15). The syndrome is found worldwide with possibly a slightly higher incidence of MFS in Japan. Generally, in most studies more males are observed with an estimated ratio of 3:2 (male: female). GBS occurs more frequently in higher age categories with a smaller second peak between 20-30 years of age (15,27). A seasonal fluctuation of GBS incidence with more cases during summer and early winter has been reported in several countries (15,27,28). In countries like China, this pattern of increased incidence during summer is related to the seasonal fluctuation of *C. jejuni* infections, being more prevalent during warmer periods (28,29). Endemic forms of *C. jejuni*-related GBS have been reported in China, Curaçao and Bangladesh (1, 28,30).

Pathogenesis

Histopathology

Only limited autopsy studies in GBS cases are available but the results of individual cases showed that AMAN and AIDP presumably have different histopathological characteristics. Macrophages appear to play a critical role in both but act at different anatomical localizations.

In AIDP, macrophages invade the Schwann cell basement membrane and strip off the abaxonal Schwann cell cytoplasm (7,31) In AMAN, macrophages are located at the nodes of Ranvier where they insert their processes in between the axon and the Schwann cell axolemma (31) (Figure 3). The myelin initially appears untouched, but secondary demyelination as well as Wallerian degeneration is found at later stages of the disease and when the ventral root is affected, the entire axon is destroyed (22,31). Interestingly, T-cell infiltration is not observed in AMAN patients suggesting a different pathophysiology from AIDP (31). Both in AMAN and AIDP complement activation plays a prominent role (32).

Anti-ganglioside antibodies in GBS

Anti-ganglioside antibodies were first described in patients with a paraproteinaemic polyneuropathy (33) but soon also in cases of GBS patients (34). Antibodies to several other types of gangliosides including GD1a, GD1b, GalNAc-GD1a and GQ1b were subsequently reported in large case-control studies that confirmed the association with GBS (35).

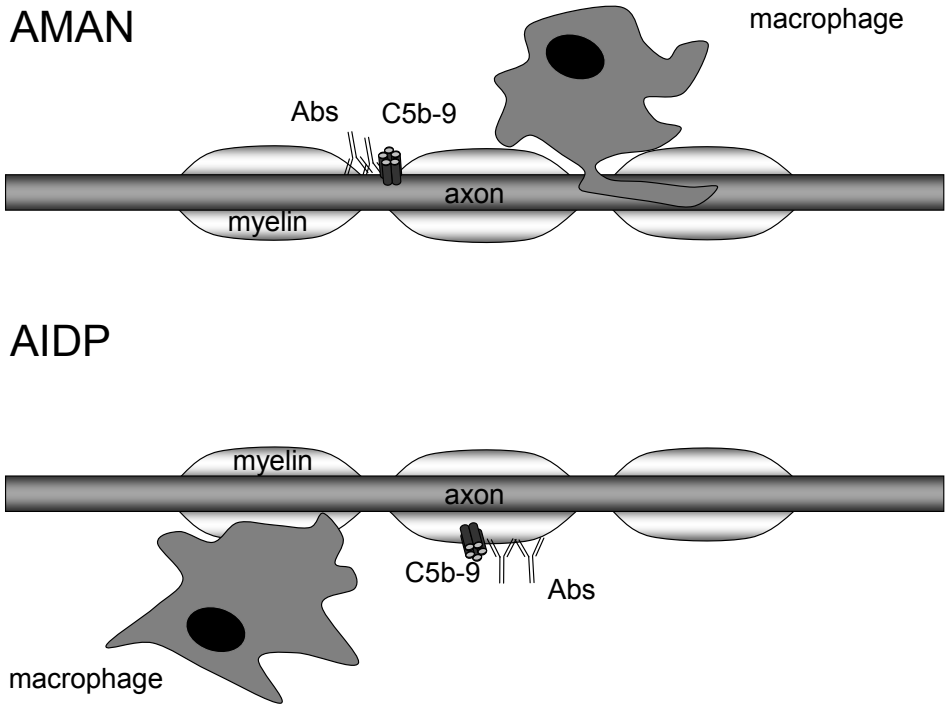


Figure 3. Histopathology of GBS and role of macrophages.
Histological examination in patients with AMAN (acute motor axonal neuropathy) and AIDP (acute inflammatory demyelinating polyneuropathy) variants showed the presence of macrophages at different localizations. Abs: antibodies, C5b-9 complement complex.

The term gangliosides refers to a group of glycosphingolipids that express sialic acid. The nomenclature of gangliosides is based on the number of carbohydrate molecules in the oligosaccharide chain and the position and number of sialic acid residues (36). The lipid tail of gangliosides is a ceramide that is immersed into the lipid membrane. The carbohydrate part is hereby exposed extracellularly and serves as an autoantigen for antibodies. The molecular structure of relevant gangliosides and glycolipids is given in Table 2.

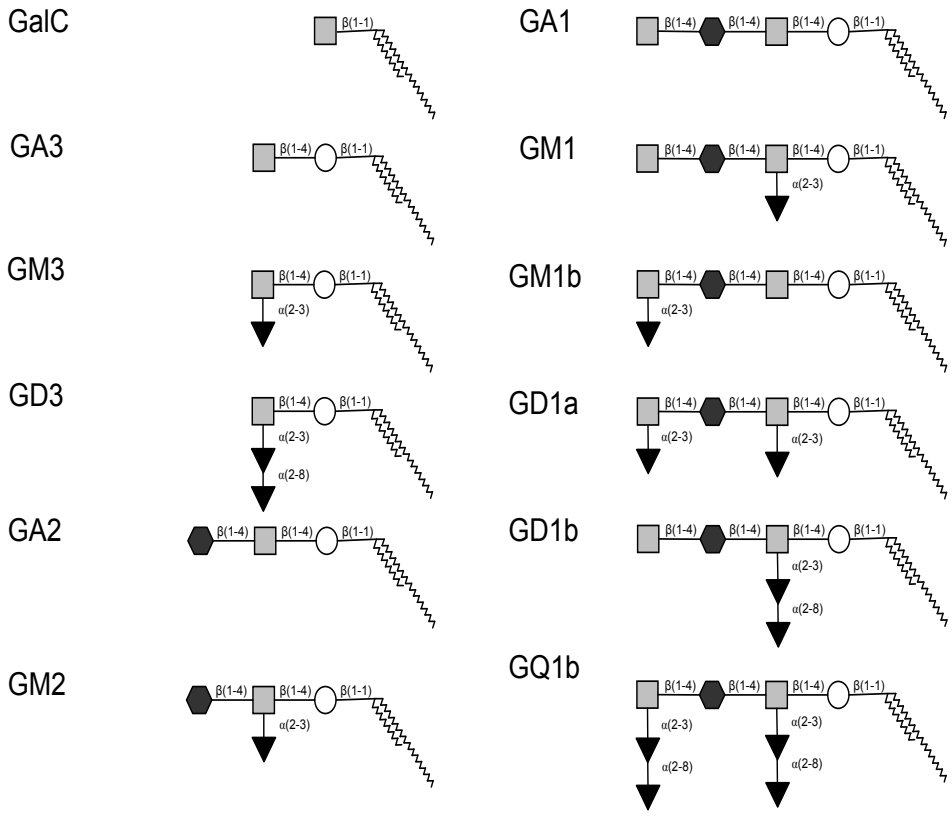
Gangliosides can be found in so called glycosynapses; organized molecular assemblies present in microdomains of the cellmembrane that are involved in cell adhesion, signal transduction events and altering cell phenotypes. Gangliosides are present in almost any cell type of the body but are highly concentrated in myelin sheaths and the axolemma of the nervous system. For example, GM1 is particularly concentrated in peripheral motor nerve myelin (37) whereas GQ1b is found in high concentrations in the oculomotor nerve (38). The distribution of the neurological deficits found in GBS patients shows a remarkable association with antibody specificity and the distribution of these glycoconjugates. Antibodies to GM1 and GD1a are typically associated with motor deficits in GBS patients, whereas anti-GQ1b antibodies are highly associated with oculomotor deficits (35).

With current techniques, in around 40-50% of GBS patients serum antibodies to one or more gangliosides are detected but in the remaining patients the target antigens are unknown. In 2004, a new concept was proposed that opens a new field of research for this seronegative group of patients. Gangliosides are lipid molecules that accumulate in specialized domains of the cell membrane called lipid rafts (39). The fluidity of this environment generates the possibility that single gangliosides may form complexes with other gangliosides when they reside in each other's proximity. Accordingly, it was hypothesized that GBS patients without detectable anti-ganglioside antibodies may have antibodies to ganglioside complexes. This was demonstrated in a GBS patient having antibodies to a complex formed by GD1a and GD1b (40). The serum of this patient did not react to GD1a or GD1b alone in thin-layer chromatography but clearly showed a band present in between these lipids. Antibodies to other ganglioside complexes have been described in other GBS as well as in MFS patients (41). The clinical correlate of anti-ganglioside complex antibodies in GBS is under current investigation.

Pathogenicity of anti-ganglioside antibodies in GBS

In concept, the presence of anti-ganglioside antibodies in GBS and other immune-mediated neuropathies was first linked to the presence of high amounts of gangliosides in peripheral nerves. However, antibodies found in GBS patients could in theory be induced secondary to peripheral nerve damage and represent an epiphenomenon. Instead, compelling evidence indicates that anti-ganglioside antibodies are induced during antecedent infection and in GBS directly cause peripheral nerve damage (35,42). Antibody deposits are found in peripheral nerves of GBS patients and are neurotoxic for mouse perisynaptic Schwann cells, nodes of Ranvier, peripheral nerve endings and neuromuscular junctions (43-46). Disruption of these structures is mediated by local complement activation (Figure 4). Anti-ganglioside antibodies may induce leucocyte degranulation via Fc-receptors (47,48). Passive transfer of serum from GBS patients with anti-GQ1b antibodies results in nerve dysfunction *in vitro* as well as neurological symptoms in mice, which can be prevented by addition of IVIg and complement

Table 2. Schematic representation of relevant glycolipids in thesis and GBS.



Legend: galactose, sialic acid, glucose, N-acetyl-galactosamine, ceramide tail, GA1: asialo-GM1, GA2: asialo GM2, GA3: asialo-GM3, GalC: galactocerebroside.

inhibitors (49,50). Furthermore, immunization of rabbits with gangliosides and *C. jejuni* results in anti-ganglioside antibody responses and neurological symptoms resembling GBS in mice and rabbits (42,51). In GBS patients, there is a significant association between the specificity of antibodies and clinical deficits that further suggests a crucial role of anti-ganglioside antibodies in the pathogenesis of GBS (52-54). Collectively these observations have firmly established the concept that anti-ganglioside antibodies are pathogenic and induce GBS. However, GBS patients have antibodies to different forms of gangliosides, of which the pathogenicity has not yet been demonstrated in all cases. In addition, in about half of GBS patients no serum antibodies to gangliosides can be detected at all.

Preceding infections and other triggers

One to three weeks prior to development of GBS, symptoms of an upper respiratory tract infection (URTI) or flu or diarrhea are reported in two thirds of GBS patients. Initial case reports

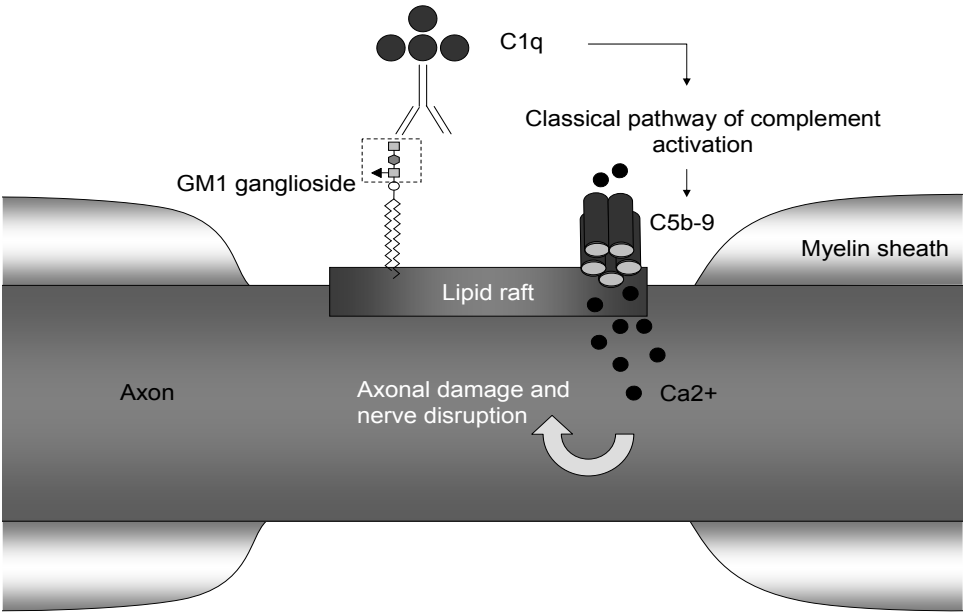


Figure 4. Anti-ganglioside antibodies induce complement-mediated nerve injury.
Gangliosides residing in lipid rafts in axons serve as targets for antibody binding. Subsequently, C1q complement binds to the Fc-part of antibodies and is activated through the classical pathway resulting in the formation of the C5b-9 or membrane attack complex (MAC). This leads to disruption of sodium channels, Ca^{2+} influx and axonal nerve injury.

suggested *C. jejuni* infections as a cause for the antecedent diarrhea in GBS patients (55). Large case control studies using serological techniques confirmed that around 30 to 40% of GBS patients have recent infections caused by *C. jejuni* and also indicated that other pathogens are associated with GBS such as CMV, Epstein-Barr virus (EBV) and *Mycoplasma pneumoniae* (Table 3) (56,57). *Haemophilus influenzae* is frequently isolated from GBS patients but is also isolated from controls (58,59). Nevertheless, indirect evidence suggests that *H. influenza* may be the precipitating factor in a similar proportion as those having antecedent *C. jejuni* infections (60). Several other types of infections have been associated with GBS in single cases or small case series of which the significance is unclear.

It is an intriguing question what triggers GBS in the remaining one third of patients not reporting infections. Most likely, a considerable proportion of these are caused by subclinical infections. In addition to subclinical infections other factors may also provoke GBS. For example, an increased incidence of GBS after vaccination programs for rabies (61) and swine-flu (62) was reported during the 1970s and 1980s although the significance of this association has been debated (63). Other possible triggers suggested by case reports include intravenous ganglioside mixture therapy, pregnancy, surgery and cancer, of which some may be related to an infection as well (64). GBS thus appears to be a typical post-infectious syndrome with or without a defined trigger. In the next paragraphs, the role of *C. jejuni* as most important pathogen for GBS will be discussed further.

Table 3. Preceding infections and their relative frequency in GBS.

Pathogen	Estimated frequency
C. jejuni	30-40%
Cytomegalovirus	15%
Epstein-Barr virus	5-10%
M. pneumoniae	10%

The role of C. jejuni infections in GBS

C. jejuni infection is the leading bacterial cause of diarrhea in the industrialized world and is the most frequent cause of infection preceding the development of GBS (65). Reported frequencies in GBS range between 30 to 40% (66). Based on current serological techniques, the annual incidence of *C. jejuni*-related GBS cases for The Netherlands with around 17 million inhabitants has been estimated 60 cases per year (67). From this, it has been deduced that the risk of getting GBS after a *C. jejuni* enteritis is around 1 per 1.300 (68). Since only about 60-75% of culture confirmed cases of *C. jejuni* gastroenteritis also have positive serology, this may still be an underestimation. It is important to know which risk factors determine the susceptibility of developing GBS after *C. jejuni*-related gastroenteritis and why the large majority of individuals do not develop this disabling sequel. Comparing the demography of *C. jejuni*-related gastroenteritis and GBS may identify predisposing factors for development of GBS after *C. jejuni* infection.

C. jejuni infections induce cross-reactive anti-ganglioside antibodies

A breakthrough in GBS research was made in 1993 when the mechanism by which anti-ganglioside antibodies arise was postulated to occur through molecular mimicry with microbial lipo-oligosaccharide structures (LOS) present in the outer cell wall of *C. jejuni* (Figure 5). Chemical and structural analysis of *C. jejuni* LOS purified from the strain that infected a GBS patient with anti-GM1 antibodies, demonstrated a carbohydrate structure resembling GM1 (2). This suggested that an immune response was induced, in which serum antibodies cross-react to *C. jejuni* LOS and peripheral nerves. Other observations further supported the idea that anti-ganglioside antibodies in GBS arise in response to infections by pathogens expressing ganglioside-like structures. These observations include:

1. The presence of GD1a, GD3, and GT1a-like moieties in LOS of *C. jejuni* strains isolated from other GBS patients with anti-ganglioside antibodies (69,70).
2. Serum reactivity to gangliosides was inhibited by pre-incubation with purified *C. jejuni* LOS containing ganglioside-like structures demonstrating that these antibodies cross-react (71,72).
3. Ganglioside-like structures in LOS were more frequently found in GBS-associated *C. jejuni* strains compared to strains isolated from uncomplicated *C. jejuni* enterits patients (52).
4. Rabbits repeatedly immunized with *C. jejuni* LOS in the presence of adjuvant showed similar clinical, electrophysiological and histopathological findings as found in AMAN patients (51).

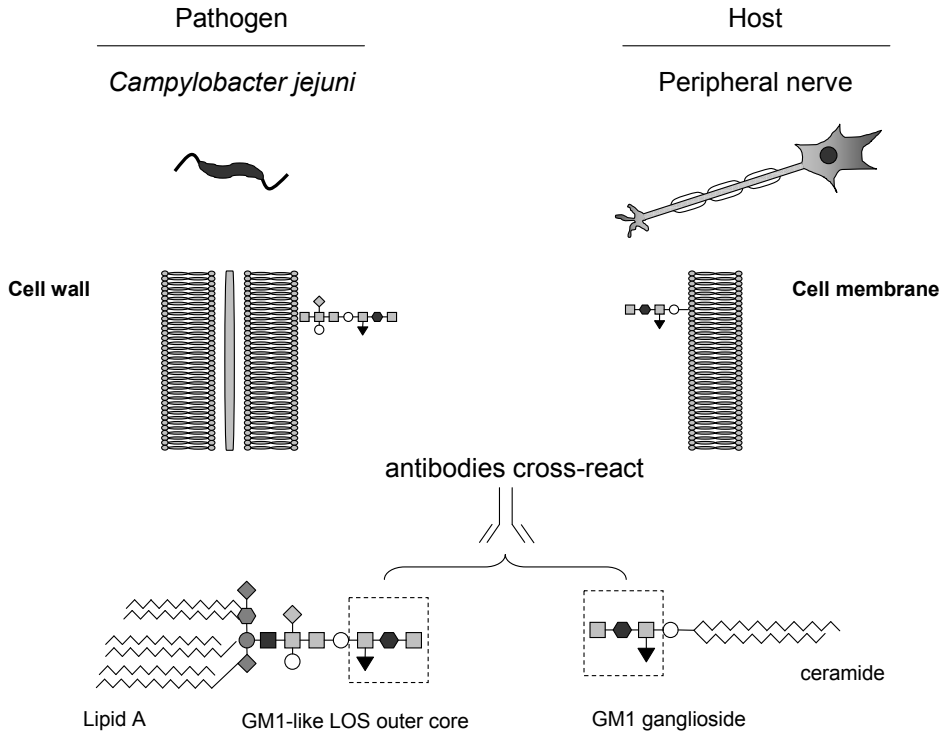


Figure 5. The concept of molecular mimicry in GBS.
Carbohydrate structures in the cell wall of *C. jejuni* resemble gangliosides present in the cell membrane of host peripheral nerves. This mimicry is thought to induce cross-reactive antibodies that cause GBS.

Molecular mimicry has been suggested to be the critical pathogenic mechanism for other types of infections in GBS although this has not been demonstrated as convincingly as for *C. jejuni* and gangliosides. For example, *H. influenza* isolated from a GBS patient was associated with a GM1-like epitope in LOS and cross-reacted with IgM, IgG and IgA of other GBS patients with predominantly preceding respiratory tract infections (73). In addition, serum reactivity to GM2 was inhibited by incubation with fibroblasts infected with a GBS-associated CMV strain (74). Furthermore, serum antibodies to galactocerebroside, GM1b and GM1 cross-reacted to *M. pneumoniae* antigens in patients with preceding *M. pneumoniae* infections (75-77). These examples suggest a more common pathway for the immunopathogenesis of GBS of which the structural nature is unclear.

Host-pathogen interactions

Characterization of the post-infectious autoimmune response in GBS
In GBS, an aberrant humoral immune response in patients (the hosts) is triggered by a pathogen that results in anti-neural antibody response and nerve damage. This process is subject to

interactions between host and pathogen and the factors that determine the outcome of these interactions are unknown. To further reflect on the mechanism involved in this molecular mimicry driven autoimmunity, the following observations in GBS may be relevant:

a) Antibody titers to gangliosides rapidly decay in weeks to months.
b) The anti-ganglioside antibodies arise in response to carbohydrate antigens such as *C. jejuni* LOS.
c) Anti-ganglioside antibodies are mainly of IgA and IgG isotypes. The IgG subclass distribution is predominantly IgG1 and IgG3.
d) Anti-ganglioside antibodies are affinity matured antibodies.

Based on immunological observations in mice, it has been suggested that *C. jejuni* LOS functions as a thymus-independent antigen activating B-1 and marginal zone (MZ) B-cells (78). Some of the above mentioned observations could be explained by this hypothesis, such as the absence of immunological memory and isotype and subclass distribution. To class-switch to IgG however, B-1 or MZ cells require accessory signals from other cell types. Theoretically, in the mucosal compartment this help could come from T-cells, dendritic cells (DCs), epithelial cells or other adjacent cells. In the next paragraphs, the potential role of T-cells and DCs will be discussed.

The possible role of T-cells in GBS

Different T-cell subsets exist that each have specific functions in adaptive immunity. A highly variable pattern of activated T-cell subsets in peripheral blood and CSF samples from GBS patients was found (79-86). Anti-ganglioside specific T-cells have not been isolated from GBS patients thus far. In one cohort of GBS patients a restricted usage of Vβ chain genes in activated T-cells suggested a response to a common antigen but this could not be reproduced in a different GBS cohort (87). Sural nerve biopsies of GBS patients showed the presence of $CD8^+$ as well as γδ-T-cells, although the latter observation was not disease-specific since these cells were also found in biopsies from vasculitic neuropathy patients (88,89). γδ-T-cells show a limited diversity of receptors that are encoded by a few common rearrangements. γδ-T-cells probably constitute an ontogenetic early form of T-cells that function to defend mucosal surfaces. This location makes them an interesting candidate cell to interact with e.g. B-1 cells. They do not generally recognize peptides presented by MHC molecules, but rather seem to recognize their target antigens directly or via CD1 molecules. They have the capacity to respond to glycolipid antigens and expand in vitro to infection with *C. jejuni* in healthy controls but not in GBS patients (90,91). This interesting finding however, was only studied in a small cohort of GBS patients and has not been confirmed by others. The results of studies addressing the role of T-cells in GBS do not demonstrate a consistent pattern yet to give direction for further exploring the role of T-cells.

Although a role for T-cell mediated autoimmunity in GBS cannot be excluded, there are other observations that suggest T-cells unlikely are involved as a central factor in GBS pathogenesis. Adaptive T-cell mediated immunity generally induces immunological memory. For GBS, the low recurrence rate of less than 5% and the high yearly rate of encountering potential pathogens able to induce GBS indicate that generation of immunological memory is unlikely. Second, T-cell mediated autoimmune diseases are often genetically linked to HLA-class II polymorphisms, which could not be demonstrated for GBS (92). Third, monotherapy

with corticosteroids especially suppresses T-cell function and is an ineffective treatment when given as monotherapy. A role for T-cell mediated immunity in GBS pathogenesis is unclear and therefore it may be interesting to focus first on other cell types interacting with B-cells.

Dendritic cell function and their potential role in GBS

In this thesis the role of DCs in the human immune response to GBS-associated *C. jejuni* will be addressed as a candidate cell to interact with B-cells. In the next paragraph an immunological introduction about DCs will be given.

DCs are professional antigen presenting cells (APC) and are abundantly present in the gut directly underneath the epithelial cell layer (93). Here they generate networks of extending dendrites scavenging for microbes. DCs sample luminal antigens through these dendrites by opening tight junctions that hold together the epithelial cell layer (93). They are specialized in uptake of antigens through various pattern-recognition receptors (PRRs) including Toll-like receptors (TLRs), C-type lectins, sialic-acid Ig binding lectin's (Siglec's), complement receptors and Fc-receptors (94). Amongst others, the binding of antigens to these receptors results in the release of cytokines and chemokines such as IL-8 that recruit neutrophils and monocytes from the bloodstream to the site of infection. In addition, antigen uptake by DCs initiates the migration of DCs to organized lymphoid tissues where adaptive immune responses are coordinated.

Protein antigens from degraded pathogens within DCs are loaded on specialized antigen presenting molecules such as MHC-I and MHC-II. When these antigens are recognized in the context of MHC by antigen specific T-cells, this induces adaptive and highly specific immune responses. CD1 molecules expressed on DCs share the β2-microglobulin with MHC molecules but have a much longer antigen-binding groove that fits the long fatty-acid tails of self- and foreign lipid antigens. Lipid antigens such as gangliosides are thus presented through CD1 and are recognized by γδ-T-cells and NK-T cells (95,96). Interestingly, signalling through PRRs on DCs may alter the type of these adaptive immune responses. PRRs scavenge antigens (Figure 6) and induce the release of specific cytokine profiles by DCs that may skew the immune response to either humoral immune responses (Th2), cellular immune responses (Th1) or other newer concepts of regulatory immune responses (Th3, Th17). Thus, DCs play a pivotal role as bridge between innate and adaptive immunity (94,97).

Interestingly, in the last decade various reports have indicated important interactions occuring between DCs and B-cells, which may have implications for understanding the cross-reactive anti-carbohydrate antibody response in GBS (98-101). DCs have the capacity to retain antigens in their native state and directly present these for engagement of the B-cell receptor (101). Thymus independent antigens opsonized by DCs can enhance humoral responses in FcγRIIB and complement dependent fashion (102). They additionally produce several cytokines and chemokines influencing B-cells (99,102). DCs can also synthesize entire antigens and hereby increase the epitope density that is required for optimal B-cell activation (103). Furthermore, DCs can induce direct class-switching in B-cells via expression of B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) and skew adaptive immune responses by TLR-dependent mechanisms (101,104). Whether B-cell responses are influenced by DCs in relation to *C. jejuni* LOS or other GBS-associated pathogens, however, remains to be established. Because DCs are localized in the gut they are likely one of the

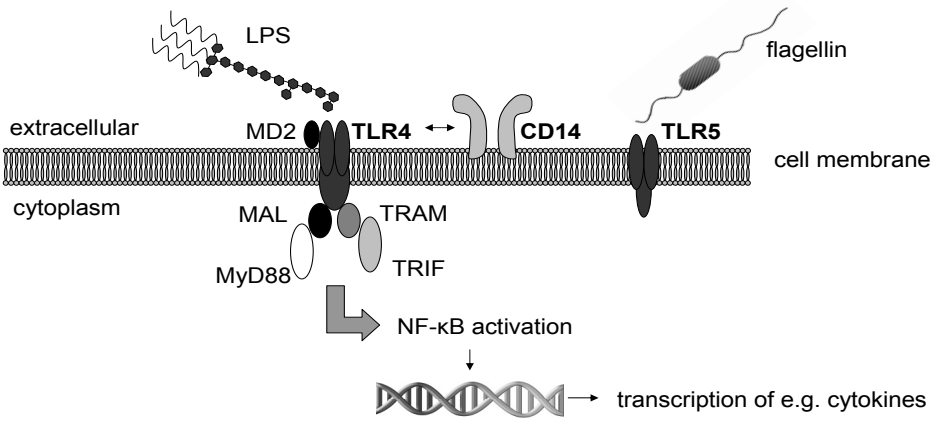


Figure 6. Host-pathogen molecular interactions at the cellular surface.
Pattern recognition receptors (PRRs) recognize evolutionary conserved repetitive structures such as lipid A in LPS and flagellin present in various micro-organisms. Various adaptor molecules downstream of these PRRs are involved, depending on the type of LPS (smooth or rough LPS) that result in NF-κB activation and cell activation. LPS: lipopolysaccharide, MD2: MD-2 protein also referred to as lymphocyte antigen 96, TLR4: toll-like receptor 4, TLR5: toll-like receptor 5, MAL: MyD88 adaptor-like, TRAM: TRIF-related adaptor molecule, TRIF: TIR domain-containing adaptor-inducing IFN-β, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

first immune cells responding to *C. jejuni.* In fact, it has been shown that DCs are activated by *C. jejuni* and produce several types of pro-inflammatory cytokines (105). Various host- or pathogen-related factors may influence the outcome of these initial steps that eventually lead to development of GBS. It is of specific relevance to further assess DC interactions with GBS-associated *C. jejuni* isolates to obtain an immunological model for GBS immunopathogenesis that may enable the development of new therapeutic or preventative strategies.

Host related genetic factors
Several families were reported in which clustering of GBS occurred that suggested GBS to be a complex genetic disorder (106). In a case report of a family outbreak of *C. jejuni* gastroenteritis, only one family member developed GBS with high titres of anti-ganglioside antibodies (107). As all family members were likely infected with the same *C. jejuni* strain, host-related factors may have played an important role. Furthermore, in an estimated 1 to 5% of GBS patients recurrences occur, suggesting a common general (host-related) factor within these episodes (108).

More specific genetic associations for GBS have been published that were the result of candidate gene studies for single nucleotide polymorphisms (SNPs) in GBS cohorts. SNPs are mutational variations in genes, are widely distributed throughout the genome and are present in at least 1% of the general population (109). SNPs in the pattern recognition receptor *MBL2* were associated on the allele and the haplotype level with susceptibility to develop GBS and severity of GBS patients (110). Moreover, severity of GBS expressed as muscle weakness was

associated with high MBL concentrations and high MBL complex activity in sera from GBS patients (110). Two SNPs in *CD1A* and *CD1E* have also been associated with susceptibility to develop GBS. The functional implication of these SNPs in GBS immunopathogenesis was not demonstrated but may be theoretically feasible (111). Various other SNPs (Table 4) have been studied in relation to GBS susceptibility, production of cross-reactive antibodies, severity of GBS and outcome of GBS (112). Although promising results have been reported in these studies, finding a general genetic factor is difficult because of the many possible simultaneously active pathways, heterogeneity of the disease and unknown interactions between pathogen- and host (Table 4 and Figure 7).

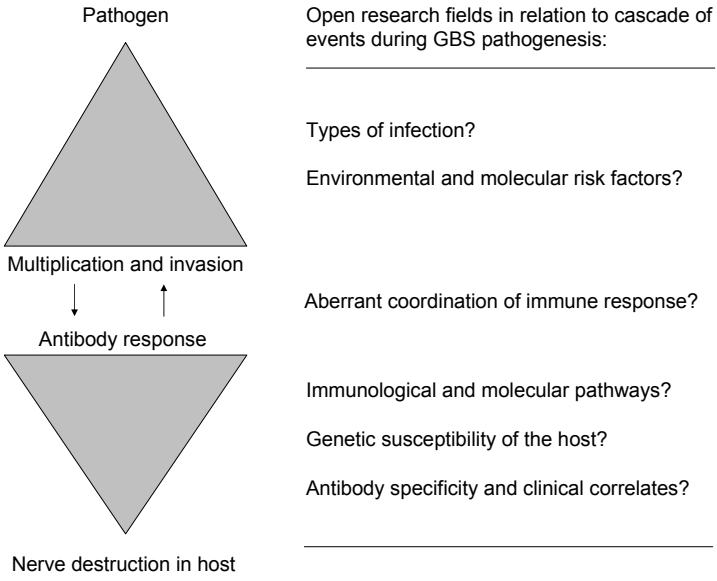


Figure 7. Selection of open research fields in GBS pathogenesis dealing with interactions between pathogen- and host.

Pathogen related factors

C. jejuni strains can be serotyped according to their heat-stable antigens being LPS or LOS, which is denominated as the O serotyping or Penner serotyping system. Several reports suggest that specific serotypes are associated with GBS (66). This led to the concept that specific clones of *C. jejuni* strains were associated with post-infectious development of GBS. However, collections of *C. jejuni* strains within restricted timeframes and geographic areas displayed extensive diversity of clonality. This may indicate that associating strains or clones with GBS could lead to an underestimation of the capacity of strains to induce GBS (113,114).

The interest in the heat-stable antigen as the basis for serotyping different strains consequently led to more insight in the chemical structure of *C. jejuni* LOS. First, the presence

Table 4. Host and pathogen-related factors implicated in GBS.

C. jejuni-related factors	GBS patient-related factors
Clonality (66)	Demography (age, gender, geography)
Presence of sialylated LOS (35,115)	Cross-reactive anti-ganglioside antibody reponse (35)
Presence of *C. jejuni* LOS biosynthesis locus class A (117,118)	SNPs in *HLA-DR1* and mechanical ventilation (92)
Transcription and presence of sialyltransferase (*cst-II* gene*)* in GBS-associated strains (116,117)	SNPs in *MBL2* and severity and susceptibility of GBS (110)
SNPs Asn51 and Thr51 in *cst-II* gene (120)	SNPs in immune response genes *CTLA4, CD40, Fc-γ III, TNF-α, Fas* and *MMP-9* (122-124)
Presence of anti-ganglioside antibodies	Severity of GBS and muscle weakness
Clinical deficits in GBS patients	Presence of anti-ganglioside antibodies
Infection with specific serotypes including O:19, O:4, O:41 (66)	SNPs in *CD1A* and *CD1E* and GBS susceptibility (111)

of *N*-acetylneuraminic acid (Neu5Ac), also termed sialic acid, in LOS of *C. jejuni* was discovered (115). This supported the concept of molecular mimicry since sialic acid residues are also present in gangliosides (2). However, serotypes with ganglioside-like moieties were also described in *C. jejuni* isolates from uncomplicated enteritis patients, suggesting that other pathogen or host-related factors possibly determines the development of GBS.

Next, the focus of research moved towards the genes encoding the enzymes responsible for the biosynthesis of LOS. By comparative genomics technologies, the c*st-II* gene was discovered and was found to be related to the presence of GQ1b epitopes in *C. jejuni* LOS and development of MFS (116). This gene encodes the sialyltransferase that attaches sialic acid residues to the LOS backbone. *C. jejuni cst-II* mutant knock-out strains have been developed for further immunological studies. Mice immunized with mutant *C. jejuni* c*st-II* LOS were unable to mount anti-ganglioside antibody responses in contrast to mice immunized with wild-type LOS (117). Interestingly, the presence and functionality of the *cst-II* gene in *C. jejuni* strains is also dependent on horizontal transfer of genes from other sources which can lead to substantial variation in LOS structures (118). In addition, polymorphisms in the *cst-II* gene influence the presence of either GM1 or GD1a mimics in LOS (119-121). These observations may at least partially explain why ganglioside-expressing strains in enteritis patients generally do not lead to GBS development. It remains to be established to what extent LOS variation is related to clinical variants and neurological deficits.

DIAGNOSTIC VALUE OF ANTI-NEURAL ANTIBODIES IN IMMUNE-MEDIATED NEUROPATHIES

The previous paragraphas concerned the role of antibodies induced by infections in GBS. The next paragraphs discuss the role of anti-neural antibodies in GBS and other polyneuropathies in clinical practice.

The prevalence of polyneuropathy is estimated around 1-7% of the general population and depends on clinical definitions (125). The prevalence increases with age and both sexes can be affected. Many causes and associated conditions have been identified. Underlying diabetes is most frequently found. Other frequent causes for polyneuropathy include excessive use of alcohol, hereditary causes, metabolic disturbances, use of toxic drugs or substances, vitamin deficiency and the presence of paraproteins (125).

In an outpatient clinic around 5-12% of all polyneuropathy patients have an immune-mediated cause for their neuropathy (125). Recognition of immune-mediated neuropathies in an early stage is clinically relevant because this group of patients may well respond to immunosuppressive therapy.

Anti-neural antibodies in immune-mediated neuropathies

In 1980 Latov *et al.* reported the first patient with a demyelinating neuropathy and an IgM-κ gammopathy in which serum antibodies reacted with myelin associated glycoprotein (MAG) (127). Ganglioside antibodies were first described in a case of IgM paraproteinaemic neuropathy in 1985 (33). Currently, more than 20 glycoconjugates have been identified and characterized as target antigens for serum antibodies in mainly demyelinating neuropathies (35) (Tables 5 and 6). In clinical practice, the diagnostic value of the antibodies in most of these polyneuropathies is uncertain.

Table 5. Classification of immune-mediated polyneuropathies according to clinical course (35,126).

Onset of disease	Disease	Association with autoantibodies
Acute		
	Vasculitis (systemic or non-systemic)	ANA, ANCA, ENA, dsDNA
	GBS and variants	Gangliosides
	Paraneoplastic	Hu
Chronic		
	MMN	GM1
	IgM MGUS polyneuropathy	MAG, gangliosides, SGPG
	CANOMAD	GQ1b
	Pareneoplastic	Hu
	CIDP	Sulfatide
	Chronic sensory neuropathy	Sulfatide
	MADSAM	
	DADS	

Abbreviations: GBS, Guillain-Barré syndrome, CIDP, chronic inflammatory demyelinating polyneuropathy, MMN, multifocal motor neuropathy, IgM MGUS, IgM monoclonal gammopathy of unknown significance, MADSAM, multifocal acquired demyelinating sensory and motor neuropathy, DADS, distal acquired demyelinating sensory neuropathy, CANOMAD, chronic ataxic neuropathy, ophthalmoplegia, IgM paraproteinemia, cold agglutinin and disialosyl antibodies.

Table 6. Characteristics of anti-neural antibodies in immune-mediated neuropathies.

Clinical syndrome	Antigen	Frequency	Main isotype
GBS	GM1	20-30%	IgG > IgM
	GD1a	10-20%	IgG > IgM
	GM2	5-10%	IgG > IgM
GBS with ophthalmoplegia	GQ1b, GT1a	90-100%	IgG > IgM
	GQ1b/GD1a complex	<5 %	IgG > IgM
Motor axonal form of GBS	GM1/GD1b complex	5-10 %	IgG > IgM
MFS	GQ1b, GT1a	95-100%	IgG > IgM
Bickerstaff encephalitis		60-70%	
CANOMAD		100%	IgM
MMN	GM1	50%	IgM
MGUS polyneuropathy	GM1	< 5%	IgM > IgG
	MAG	50%	IgM
	SGPG, SGLPG	50%	IgM
Chronic ataxic neuropathy	GD1b, GD2, GD3, GT1b, GQ1b	unknown	IgM
Chronic sensory neuropathy	Sulfatide	0.7-25%	IgM > IgG

Abbreviations: GBS, Guillain-Barré syndrome, CIDP, chronic inflammatory demyelinating polyneuropathy, MMN, multifocal motor neuropathy, MGUS, IgM monoclonal gammopathy of unknown significance, MFS, Miller Fisher syndrome, CANOMAD, Chronic Ataxic Neuropathy, Ophthalmoplegia, IgM paraproteinemia, cold Agglutinin and Disialosyl antibodies.

Antibodies to myelin associated glycoprotein

MAG is a cell membrane molecule (also called Siglec-4) that functions as a receptor for sialic acid containing moieties such as gangliosides. It is expressed in peripheral nerve myelin sheaths and Schwann cells and is involved in the process of myelination. Antibodies to MAG react with the HNK-1 carbohydrate epitope that is shared with the peripheral nerve acidic glycolipid sulfate-3-glucuronyl paragloboside (SGPG) (128). Anti-MAG antibodies also cross-react with other myelin glycoproteins such as P0 and PMP22 (129).

Patients with an anti-MAG polyneuropathy are a homogeneous subgroup of polyneuropathy patients predominantly occurring in the elderly (>65 years). Characteristically, the patients have a slowly progressive distal form of mixed motor-sensory polyneuropathy, but generally with more sensory symptoms and frequently also sensory ataxia. This form of neuropathy is associated with the presence of an IgM- or biclonal IgM/IgG gammopathy. In most patients the malignant cause for the gammopathy is not found and subsequently denominated gammopathty of unknown significance (MGUS). Testing for these antibodies may be clinically relevant since patients with an anti-MAG neuropathy tend to respond poorly to immunosuppressive

therapy. When patients with an IgM MGUS polyneuropathy suddenly deteriorate, when the IgM paraprotein further increases or when anti-MAG antibody titers suddenly increase it is important to consult the hematologist and again search for any possible malignancy (130).

The pathogenicity of anti-MAG antibodies is suggested by several observations. IgM deposits in peripheral nerve sections of patients with a chronic and distal sensorimotor form of polyneuropathy have been demonstrated that co-localize with antibodies to MAG (131). Moreover, passive immunization experiments with anti-MAG IgM lead to prominent widening of myelin lamellae in chickens, which was a typical finding in human biopsies of patients with these antibodies (132). Recently, cats immunized with MAG developed clinical symptoms resembling those of anti-MAG neuropathy patients (133). It is suggested that bacterial antigens may recruit somatically mutated autoreactive B cells, causing the monoclonal gammopathy and subsequent polyneuropathy (134). More research is needed to establish the pathogenicity of anti-MAG antibodies and to confirm whether preceding infections lead to development of anti-MAG related neuropathies.

Antibodies to glycosphingolipids

This group can be subdivided into patients with antibodies to GM1, disialylated gangliosides and sporadic forms of glycosphingolipids.

A high prevalence of IgM anti-GM1 antibodies is particularly found in multifocal motor neuropathy (MMN). This chronic progressive neuropathy exclusively involves motor nerves and has a patchy asymmetrical and distal distribution. In electrophysiological examination the presence of conductance block is suggestive and mandatory for diagnosis. IVIg therapy stabilizes progression and is currently the only proven effective therapy (135). Some patients with an IgM MGUS polyneuropathy may also have IgM antibodies to GM1 (136).

Antibodies to disialylated gangliosides such as GD1b, GD3, GT1b and GQ1b are particularly found in patients with sensory symptoms, ataxia and in patients with acute ophthalmoplegia. These symptoms and antibodies may be found in the rare chronic ataxic neuropathy ophthalmoplegia IgM paraprotein cold agglutinins and disialyl-antibodies syndrome (CANOMAD). The syndrome may have a relapsing remitting course and is associated with a lymphoproliferative disorder producing an IgM-κ or IgM-λ gammopathy (137). Case reports suggest that IVIg therapy may be beneficial.

Antibodies that are less frequently observed include anti-sulfatide antibodies and anti-GM2 antibodies. The clinical spectrum of anti-sulfatide antibodies include sensory axonal forms of neuropathies that typically present with pain (138). Anti-GM2 antibodies may be found in subgroups of GBS patients or chronic motor forms of polyneuropathy.

Variation in assay techniques for anti-neural antibodies

Despite the significant interest in anti-neural antibodies the past twenty years, inconsistencies in the techniques used for detection of these antibodies may have resulted in discrepancies of reported frequencies in diseases. Western blotting and ELISA techniques are usually used to detect antibodies to MAG and gangliosides. Standardization of the ELISA assay was proposed by the European Inflammatory Neuropathy Cause and Treatment group (INCAT) (139). The diagnostic value of this INCAT ELISA, however, requires validation in a large group of neuropathy patients and controls. Comparative studies of ELISA and western blotting for

anti-MAG serology have led to the recommendation by the EFNS/PNS taskforce to consider positive western blotting as definite proof and positive ELISAs as suggestive proof. However, golden standards are lacking and newer ELISAs have been developed that should be clinically validated.

SCOPE AND OUTLINE OF THESIS

The working hypothesis in the following studies is that **host-pathogen interactions** trigger the induction of an aberrant immune response to peripheral nerves that will lead to the development of GBS. Previous studies identified several **host-related factors** associated with GBS, including demographic features, genetic polymorphisms in immune response genes and presence of serum antibodies to gangliosides. Other studies found **pathogen-related factors** involved in the pathogenesis of GBS, including distinct types of antecedent infection, such as *Campylobacter jejuni*, and of molecular mimicry with gangliosides. The mechanisms by which these host and pathogen factors interact to induce GBS and the clinical relevance of these factors are unknown. More specifically the studies in this thesis investigated the following topics:

- demography of antecedent infections in relation to GBS
- effect of *C. jejuni* genotype and LOS structure on antibody specificity and GBS phenotype
- CD1 gene polymorphisms and the susceptibility to develop GBS
- modulation of dendritic cells and B-cell activation by *C. jejuni* lipo-oligosaccharides (LOS)
- validation of diagnostic methods to detect serum antibodies to peripheral nerve antigens

In **Chapter 2** the epidemiology of *C. jejuni* infections was compared in patients with GBS *versus* uncomplicated gastroenteritis to identify demographic features that may predispose to GBS. Additionally, serum and cerebrospinal fluid samples from GBS patients were screened for recent infections with CMV.

Chapter 3 describes the effect of the pathogen antigenic structure on the antibody specificity and clinical phenotype in GBS. First, the genotype of 26 GBS related *C. jejuni* isolates was defined to determine the relation with the molecular structure of the LOS, specificity of the cross-reactive antibodies and clinical deficits in GBS. Second, experimental support was provided for the hypothesis that serum antibodies to combinations of gangliosides or complexes in GBS are also induced by molecular mimicry of *C. jejuni* LOS.

Chapter 4 focuses on the role of host cellular activation and antigen presentation by pathogens in the development of GBS. We hypothesized that DCs are activated by *C. jejuni* and orchestrate the aberrant immune response leading to GBS. DCs may present *C. jejuni* LOS by CD1, which is a glycolipid antigen-presenting molecule abundantly present on DCs and genetically polymorphic. First, variation in CD1 genes was determined in GBS patients and healthy controls to assess if these polymorphisms are a susceptibility factor in developing GBS. Second, the activation of human DCs to *C. jejuni* LOS and subsequent proliferation of B-cells was defined. These experiments showed that the sialylation of *C. jejuni* LOS modulates the immune activation and may predispose to GBS.

Chapter 5 describes the clinical validation of current techniques to detect serum antibodies to the ganglioside GM1 and to MAG in the diagnosis of patients with immune-

mediated neuropathies. An illustrative case is reported in which anti-GQ1b antibodies helped to distinguish the cause of a relapsing ophthalmoplegia and ataxia.

In **Chapter 6** the observations from the studies in chapters 2-5 are summarized and discussed in relation to current literature. In addition, recommendations for future research are proposed that succeed the observations described in this thesis.

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2

Preceding infections in Guillain-Barré syndrome (GBS)

2.1

Presence or absence of cytomegalovirus in the cerebrospinal fluid of patients with Guillain-Barré syndrome?

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To the editor - Steininger *et al.* (1) reported the presence of cytomegalovirus (CMV) DNA in cerebrospinal fluid (CSF) of patients with Guillain-Barré syndrome (GBS). In the understanding of the pathogenesis of GBS as a post-infectious disorder, this observation may have important consequences. In their study, CMV DNA was demonstrated by a polymerase chain reaction (PCR) assay in CSF of 13 (31%) and serum of 14 (33%) of 42 GBS patients. These observations would indicate that CMV is a predominant preceding infection in GBS and that serological studies, in which usually less than 15% of patients are considered to be CMV positive, underestimates the frequency of this infection (2). In addition, serology studies may be biased to certain types of CMV as suggested by Steininger *et al.* (1). This would also have implications for further delineating GBS patients in pathogenic subgroups, since some reports indicate that patients with positive CMV serology are thought to be younger of age with a clinical variant of severe involvement of motor, sensory and cranial nerves and a prolonged progressive active phase and have cross-reactive antibodies to the ganglioside GM2 (3,4). The presence of CMV in CSF may also indicate that endogenous reactivation of CMV infection within the central nervous system compartment occurs and that perhaps these patients may profit from antiviral therapy (1). The interesting finding of Steiniger *et al.* (1) and the possible consequences for pathogenic research prompted us to investigate the presence of CMV DNA by PCR in our own collection of CSF samples of GBS patients.

We have collected acute phase, pre-treatment CSF samples from 170 (43%) of 397 GBS patients participating in the multicenter Dutch GBS trials (5,6). These patients did not differ clinically from the remaining patients. All patients fulfilled the diagnostic criteria for GBS, were in their acute phase of disease (less than 2 weeks between first signs of weakness and randomization) and were unable to walk independently (6). The patients we studied were comparable to those studied by Steininger *et al.* (1) with regard to age, frequency of positive CMV IgM serology (2), (estimated) time-interval to lumbar puncture and CSF protein level (Table). Serological evidence of a recent CMV infection was detected in 22 (14 %) of 159 patients (Table). In contrast, we could detect CMV DNA in CSF from only a single GBS patient. This patient also had serum CMV-specific IgM antibodies.

The discrepancy with the results of Steininger *et al.* (1) are remarkable since a method with similar assay characteristics (as determined by quality control proficiency testing) was used by the two reference laboratories. Random errors in our study are less likely considering the large number of included patients. Patient selection cannot be excluded although both study groups were similar with respect to relevant clinical characteristics and frequency of positive CMV IgM serology. Steininger *et al.* (1) only tested patients for CMV DNA in CSF and serum when CMV-specific IgG or IgM antibodies was present, which was a selection of 42 out of 65 identified GBS patients. Our patients may have been more severely affected, as indicated by the larger proportion of ventilated patients in our group and the inclusion criteria requiring that patients were unable to walk independently. Furthermore, the time interval between onset of GBS and lumbar puncture were defined slightly different. However, the CSF protein content and the observed time intervals were not significantly different between the groups, indicating that the CSF was obtained at about the same time in the acute phase of the disease. Steininger *et al.* (1) reported that in none of the five patients with positive CMV IgM serology CMV DNA was present in CSF. Of all their sera tested positive for CMV DNA, only 2 (15%) had CMV-specific IgM antibodies. Furthermore, in only 3 (7%) of their patients

CMV DNA could be detected in both CSF and serum. The lack of demonstrable CMV DNA in our patients, invokes the question whether there are geographical differences in the incidence of CMV related GBS. More data from other laboratories are required to clarify the role of intrathecal CMV in the pathogenesis of GBS and especially to suggest the beneficial use of antiviral therapy.

Table. Comparison of baseline and clinical characteristics, cytomegalovirus (CMV) serology and polymerase chain reaction (PCR) results in two groups of Guillain-Barré syndrome (GBS) patients.

	Present study	Steiniger *et al.* (1)
Number of patients	170	42
Age, mean (SD), years	50 (19.5)	47 (22.7)
CSF protein, mean (g/l)	1.48	1.72
Ventilation required no. (%) of patients	50 (29)	4 (10%)
Interval between onset of GBS and lumbar puncture, geomatric mean, days		
CMV DNA present in CSF	2	3.3
CMV DNA absent in CSF	4.4	6.0
Positive CMV IgM serological test result (serum), no. positive/ total tested (%)	22 / 159 (14)[a]	5 / 42 (12)
Positive CMV PCR (CSF), no. positive/total tested (%)	1 / 170 (<1)	13 (31)

NOTE SD, standard deviation; CSF, cerebrospinal fluid

[a]Serological results were partially published previously (2).

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2.2

Epidemiology of *Campylobacter*-related Guillain-Barré syndrome in the Netherlands

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ABSTRACT

BACKGROUND: *Campylobacter jejuni* infections are the predominant cause of bacterial gastroenteritis in the industrialised world. The most perilous complication of *C. jejuni* gastroenteritis is the Guillain-Barré syndrome (GBS), an immune mediated neuropathy that occurs in about 1 in 1,000 to 5,000 persons infected. Serological evidence for a recent *C. jejuni* is found in nearly 40% of GBS patients, although *C. jejuni* isolation is often negative because of the delay between infection and onset of weakness. Risk factors for developing GBS after a *C. jejuni* infection are unknown.

AIM OF STUDY: The aim of this study was to compare the epidemiology of *C. jejuni*-related gastroenteritis and culture- and serology proven *C. jejuni*-related GBS to identify new risk factors for development of GBS after *C. jejuni*-related gastroenteritis.

PATIENTS: Demographic, clinical and serological data were available from 406 GBS patients that participated in clinical trials during 1986 and 2005. In addition, demographic data were available from 16,621 culture proven *C. jejuni*–related gastroenteritis patients from the Dutch Laboratory Surveillance between 2002-2007 and 1,107 sera from the general population collected between 1999 and 2001.

RESULTS: A recent *C. jejuni* infection was demonstrated by culture in 24 of 369 (7%) GBS patients and by serology in 128 of 369 (35%) patients. GBS patients with a positive serology and culture differed on the same clinical characteristics to the serology negative group such as preceding diarrhoea, clinical course and presence of anti-ganglioside antibodies. However, serologically proven *C. jejuni*-related GBS patients were slightly older, had less frequently diarrhoea, a pure motor GBS or anti-ganglioside antibodies compared to culture proven *C. jejuni*-related GBS. *C. jejuni*-related GBS tended to be less frequent during spring and more frequent during winter in GBS patients whereas *C. jejuni* gastroenteritis peaked during summer months. GBS patients of 61-70 years had the highest relative rate of preceding *C. jejuni* infection.

CONCLUSION: The relative risk of developing GBS following *C. jejuni* infection is increased in older patients and during the Northern hemisphere winter season, which may be related to increased susceptibility of older people for circulating infections during winter or other environmental factors.

INTRODUCTION

Campylobacter jejuni is the worlds' most common cause of bacterial gastroenteritis and is associated with the post-infectious development of the Guillain-Barré syndrome (GBS), a severe inflammatory neuropathy characterized by progressive muscle weakness (1,2). The frequency of antecedent infection caused by *C. jejuni* in GBS ranges between 20 to 40%, and is considered the predominant infection preceding GBS (3). *C. jejuni* infections are associated with a specific subgroup of GBS characterized by pure motor involvement, severe weakness and a poor prognosis (4). Approximately one in 1,000 to 5,000 patients with *C. jejuni* infections develop GBS, but the risk factors that determine the onset of GBS after a *C. jejuni* infection are unknown (5-7).

The seasonality of *C. jejuni*–related gastroenteritis depends on regional differences (8-12). Higher temperatures and daily hours of sunlight increase the chance to become infected by *C. jejuni* in temperate regions of the world (13-16). In general, males are more often infected by *C. jejuni*, except for the 20-to-29-year-old age group in which females are (17,18). Directly comparing the seasonality and demography of *C. jejuni*-related gastroenteritis and GBS in well described patients may lead to identification of new risk factors for development of GBS.

For epidemiological studies of *C. jejuni*-related GBS a reliable method to identify *C. jejuni*-related GBS patients is required. The disadvantage of demonstrating antecedent *C. jejuni* infection in GBS patients by selective culturing techniques is the poor sensitivity due to the interval between neurological symptoms and gastrointestinal infection and possible intermediate treatment with antibiotics. Recently, a capture ELISA assay was validated specifically for patients with GBS and reactive arthritis (19). It is unknown whether clinical differences exist between culture and serology proven *C. jejuni*-related GBS. Comparing the clinical characteristics between these groups is relevant in order to study the epidemiology of *C. jejuni*-related GBS because serology can be easily performed in a large number of patients. In contrast, prospectively collecting culture-proven *C. jejuni*-related GBS cases is extremely time-consuming for obtaining a sufficient number of patients.

In this study the demography and seasonality of *C. jejuni*-related gastroenteritis and GBS were compared to identify risk factors that increase the chance of developing GBS after a *C. jejuni* infection. *C. jejuni* infections were defined by both serological and culture studies. In addition, we assessed the patient characteristics related to a positive culture or serology for *C. jejuni* infection.

MATERIALS AND METHODS

Patients and *C. jejuni* isolates

Demographic, clinical and serological data were available from 406 GBS patients collected in previous prospective clinical trials and surveys conducted in The Netherlands between 1986 and 2005 (20-22). Because paediatricians did not participate in these trials and survey studies but do primarily see children with GBS in The Netherlands, 37 patients younger than 21 years were left out of analysis in order to minimize selection bias. This resulted in

369 GBS patients available for analysis. Demographic and seasonal data were also available from 16,621 culture-proven *C. jejuni*–gastroenteritis patients that are part of Dutch national surveillance studies (11,23). In addition, serum from 1,107 healthy individuals with known date of sampling between 1999 and 2001 was available (11,24).

In total our laboratory has obtained 24 *C. jejuni* isolates from GBS patients or patients with the Miller-Fisher syndrome (MFS), a clinical variant of GBS with opthalmoplegia, ataxia and areflexia. These strains were isolated between 1991 and 2005. *C. jejuni* was not isolated from GBS patients between 1986 and 1991 as *C. jejuni* cultures were only performed on routine basis starting from 1991. Eighteen patients had GBS, 4 patients MFS and two patients an overlapping form of GBS with opthalmoplegia. From 19/24 of these patients serum was available obtained during onset of disease. Fourteen of the 24 patients had participated in one of the Dutch clinical trials or survey studies. The remaining *C. jejuni* isolates were obtained from 10 patients seen by neurologists in various hospitals in The Netherlands. Diagnosis in these patients was retrospectively confirmed from patient records according to standard diagnostic criteria (25). Demographic and clinical data were available from these patients.

Disease severity was defined by the GBS disability score (F-score), ranging from 0 (no symptoms) to 6 (dead), and the Medical Research Counsil (MRC) sumscore, ranging from 0 (tetraplegia) to 60 (normal) at nadir (26,27). Time to nadir was expressed as the number of days before reaching the highest disability score or lowest MRC sumscore during follow-up.

C. jejuni ELISA

Serological screening for most common antecedent infections and anti-ganglioside antibodies was performed using standard procedures (28). Serology for recent *C. jejuni* infection was performed by an IgM and IgA capture ELISA and indirect ELISA for IgG and was recently validated for use in GBS (19). Our criteria for a recent infection with *C. jejuni* were the presence of either elevated IgA or elevated IgM in serum. The cut-off values used for determining positivity were determined in a previous study (19). In this previous study, we found that an IgA ratio of >0.6 and/or IgM ratio >1.0 cut-off value, yielded a 93% specificity level and sensitivity levels of 82% in uncomplicated culture proven *C. jejuni*-enteritis patients and 96% in culture proven *Campylobacter*-related GBS cases (19).

Statistical analysis

The relative rate per month concerned the fraction of all GBS cases found in that month divided by the fraction of all gastroenteritis cases found in that month. 95% confidence intervals of the relative rates were determined using a bootstrap method (29) that involved sampling both GBS and gastroenteritis datasets by resampling with replacement 10,000 times and recalculation of the monthly relative rates after each round. The 95% confidence interval of a month concerns the interval between the 2.5th and 97.5th percentile of the 10,000 calculated relative rates for that month. For comparison of groups Chi-square tests were used. For comparison of groups for age and MRC-sumscores the non-parametric Kruskal-Wallis test was used. For calculating confidence intervals and tests a *P*-value of 0.05 was used.

RESULTS

Frequency and clinical correlates of *Campylobacter* infection in GBS

Using the revised criteria for ELISA, 152/369 (41%) of GBS patients had evidence for a recent *C. jejuni* infection. All culture positive cases tested (n=24, 7%) were also positive in serology (Table I).

To ensure that our results using data based on ELISA results were valid, we investigated whether the clinical characteristics of *C. jejuni* serology-positive GBS patients, differed from *C. jejuni* culture-positive GBS patients (Table I). Although GBS patients from whom *C. jejuni* was isolated in stool cultures were younger (p<0.01), more frequently had antibodies against anti-GD1a (p<0.03) or anti-GQ1b (p<0.003) or a pure motor variant (p<0.04) than serology positive patients, the clinical characteristics of both were similar to *C. jejuni*-related GBS cases described in earlier studies (30-32). Moreover, the serology positive patients had lower MRC sumscores at nadir (p<0.002) than the culture positive group. In addition, patients with positive *C. jejuni* serology more often had diarrhoea and less often upper-respiratory tract infections, lower MRC sum-scores at nadir and more frequently had anti-ganglioside antibodies than serology negative GBS patients (Table I). In consideration of these differences it was decided to further study the culture- and serology positive group in combination.

Age distribution of *C. jejuni*-related gastroenteritis and GBS patients

In older age categories of the total GBS cohort, the number of GBS patients was generally higher than in the younger age categories (Figure 1A). Above the age of 70, these numbers decreased which reflects the distribution of age in the general population. The number of GBS patients peaked within the 61-70-age category. The fraction of *C. jejuni*-related patients (50%) was higher in this group compared to other age categories. Within the *C. jejuni*-related GBS cases, 26% of patients were also between 61-70 years of age.

In contrast, uncomplicated *C. jejuni*-related gastroenteritis occurrence generally declined with age and the fraction of patients with uncomplicated *C. jejuni*-related gastroenteritis between 21-30 years was highest (23%) compared to the other age groups (3-19%, Figure 1B). The odds-ratio (OR) for the 21-30-age group to develop GBS after a *C. jejuni* infection was 0.64 (95% CI 0.39-1.04, p<0.07) whereas the OR was 3.83 (95% CI 2.69-5.58, p <0.0001) for the age group of 61-70 years (Figure 1C).

Seasonality of *C. jejuni*-related gastroenteritis and GBS patients

GBS occurred throughout the year in the Netherlands, with a slight preponderance in the winter months. As in other countries in non-tropical regions such as the Netherlands, uncomplicated *C. jejuni*-related gastroenteritis cases peak in summer (8,11). Surprisingly, besides August the highest numbers of *C. jejuni*-related GBS cases were found in November, December and January (Figure 2). The percentage of *C. jejuni*-related cases of GBS varied from 13% in November (OR 1.89 compared to *C. jejuni*-related gastroenteritis, p<0.007) to 4% in May (OR 0.58 compared to *C. jejuni*-related gastroenteritis, not significant). This pattern was also reflected in the fraction of *C. jejuni*-related GBS cases per month and height of IgA, IgM and IgG serum reactivity to *C. jejuni* in ELISA (Figure 2B and 3A). No seasonal fluctuation of antibody reactivity to *C. jejuni* in ELISA was observed in healthy controls (N=1,107, Figure 3B).

Table I. Demographic and clinical characteristics of patients with culture proven *C. jejuni* infection and serological evidence of *C. jejuni* infection.

C. jejuni	Culture +	Serology + (culture -)	Serology – (culture-)		
	N=24	*N*=128	*N*=217	*p*-value[a]	*p*-value[b]
Demography					
Sex (Male:Female ratio)	3,8 : 1	1,4 : 1	1,3 : 1	0.03	-
Mean age (y)	45	54	53	0.03*	-
Season					
- Winter	7 (29)	40 (32)	69 (32)	-	-
- Spring	1 (4)	20 (16)	50 (23)	0.03	0.098
- Summer	11 (46)	25 (20)	43 (20)	0.004	-
- Autumn	5 (21)	42 (33)	55 (25)	-	0.08
Symptoms of preceding infections					
Diarrhea	16 (73)	43 (37)	24 (13)	<0.0001	<0.0001
Upper respiratory tract	1 (5)	27 (24)	87 (47)	<0.0001	<0.0001
Serology for other recent infections					
CMV	0 (0)	11 (9)	30 (16)	-	-
EBV	0 (0)	11 (9)	28 (15)	-	-
Mycoplasma pneumoniae	0 (0)	9 (8)	5 (3)	-	0.04
Clinical features					
Cranial nerve deficits	11 (55)	46 (39)	73 (38)	-	-
Pure motor	13 (62)	44 (38)	60 (32)	0.007	-
Mechanical ventilation	5 (25)	38 (36)	45 (25)	-	0.06
F-score (median, 95%CI))					
- At entry	4 (2.8-3.8)	4 (3.8-4.0)	4 (3.8-3.9)	-	-
- At nadir	4 (3.1-4.4)	4 (4.2-4.5)	4 (4.1-4.3)	-	0.05
MRC-sum score (median, 95%CI)	52 (39-58)	34 (28-36)	42 (36-42)	-	0.06*
- at entry					
- at nadir	52 (32-58)	22 (17-26)	38 (31-37)	0.049*	<0.0001*
Prognosis					
- Improvement 1 point in F-score during 1st four weeks	3 (25)	56 (48)	118 (62)	-	0.02
- F-score at 6 months (median, 95%CI)	1 (0.8-2.1)	1 (1.5-2.1)	1 (1.1-1.5)	-	0.003
- F<3 at 6 months	12 (80)	81 (71)	162 (88)	-	<0.0001
Anti-ganglioside serology					
GM1	6 (33)	39 (33)	23 (12)	0.01	<0.0001
GD1a	5 (29)	8 (7)	9 (5)	<0.0001	-
GQ1b	10 (59)	9 (8)	10 (5)	<0.0001	-
GM1 and/or GD1a and/or GQ1b	15 (89)	48 (41)	36 (19)	<0.0001	<0.0001

In columns the number of patients from available data are shown for each row characteristic with percentages in brackets, for F-scores and MRC sumscores median values with 95% confidence intervals (95% CI) are shown. [a] Compared between culture positive only and serology and culture negative, [b] compared between serology positive and culture negative and serology and culture negative., F-score: GBS disability score, MRC sumscore: Medical Research Counsil sumscore, - : $p > 0.1$, * Kruskall-Wallis test.

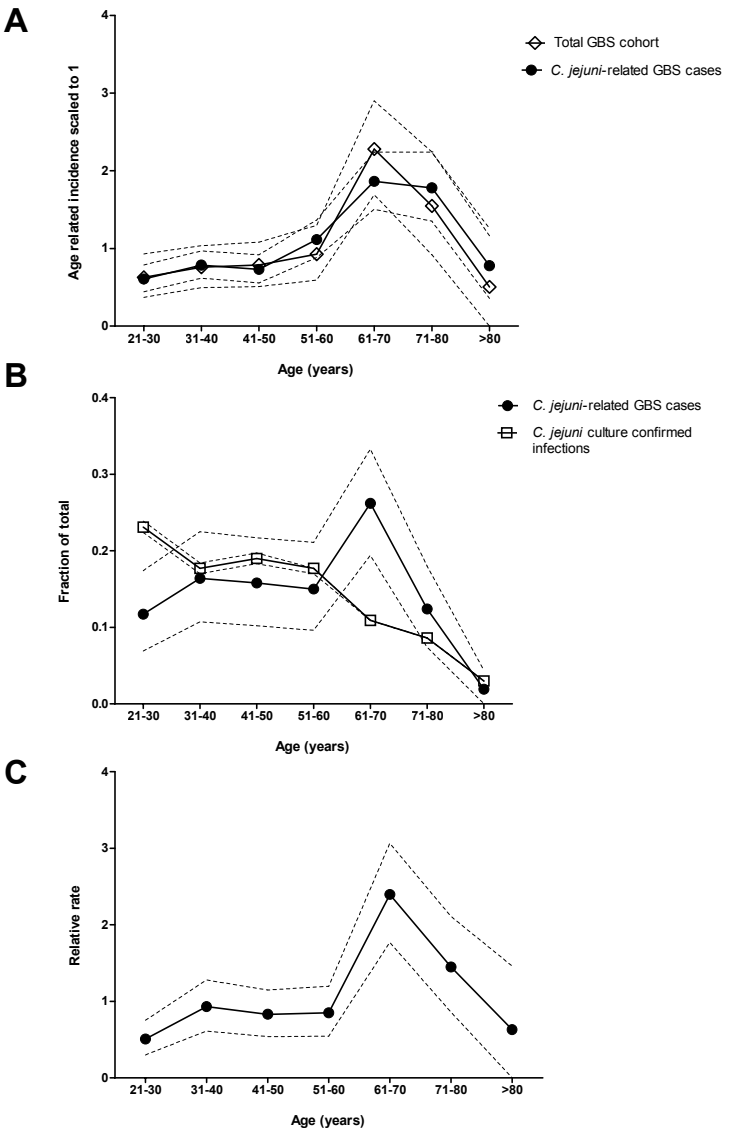

Figure 1.

(A) Age related incidence of GBS patients and *C. jejuni*-related cases. The incidence of GBS cases 20 years and older is standardized to 1 and per age category is expressed relative to this overall incidence. This was done as the real number of Dutch GBS cases in the study period is an unknown multiple of the cases collected. The fraction of all GBS cases collected per age category however can be assumed to be representative for that age category and hence can be compared to the fraction of the Dutch population at risk for that age. Dotted lines represent 95% confidence intervals after bootstrap analysis. **(B)** Age distribution in *C. jejuni*-related GBS and gastroenteritis patients. The fraction of each age decade from the total *C. jejuni*-related GBS cohort (n=152) or *C. jejuni* gastroenteritis cohort (n=16,621) is shown after bootstrap analysis. **(C)** Relative rate of *C. jejuni*-related GBS versus gastroenteritis in relation to age. The ratio of fractions from *C. jejuni*-related GBS and gastroenteritis was calculated for bootstrap adjusted fractions for each age decade.

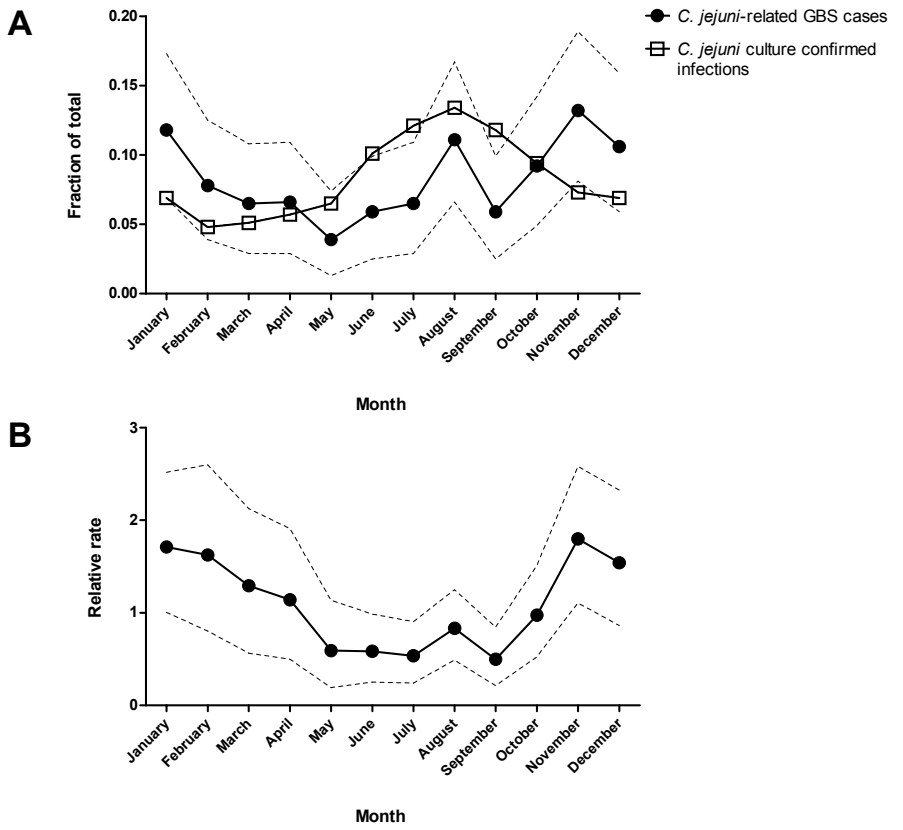

Figure 2.
(A) Seasonal distribution of *C. jejuni*-related GBS and gastroenteritis. The fraction of each month from the total *C. jejuni*-related GBS cohort (*n*=152) or *C. jejuni* gastroenteritis cohort (*n*=16,621) is shown. **(B)** Relative rate of *C. jejuni*-related GBS and gastroenteritis in relation to season. The ratio of fractions from *C. jejuni*-related GBS and gastroenteritis was calculated for bootstrap adjusted fractions for each month.

DISCUSSION

This study shows that the demography and seasonality of patients that develop GBS after *C. jejuni* infection differs from patients experiencing an uncomplicated *C. jejuni*-related gastroenteritis. *C. jejuni*-related GBS occurred more frequently during the winter months. In addition, *C. jejuni*-related GBS was more often observed in older patients, especially between 61 and 70 years. The relationship of GBS with age and season is the inverse of that of uncomplicated gastroenteritis due to *C. jejuni.* This observation suggests that a *C. jejuni* infection in the Netherlands is more frequently followed by GBS during the winter months and at older age.

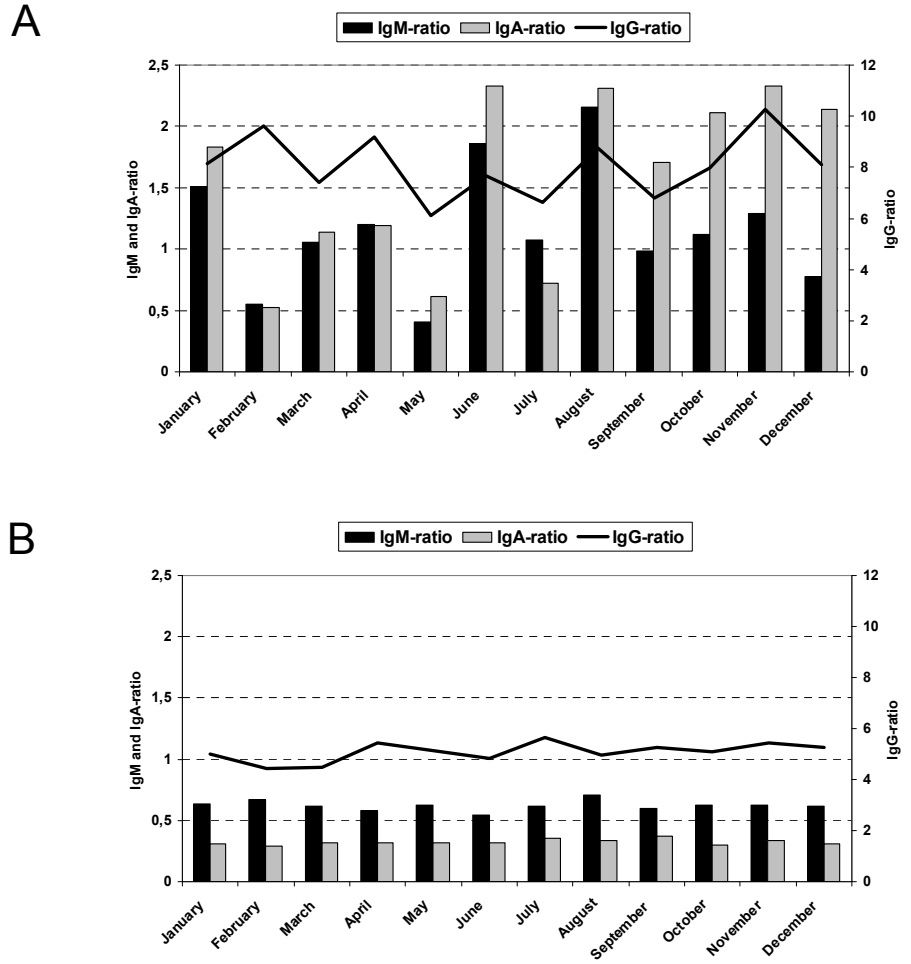

Figure 3.
(A) Seasonal fluctuation of serum reactivity (IgM, IgG and IgA) to *C. jejuni* in GBS patients (*n*=294) between 1986 and 2005. IgM, IgA and IgG ratio was determined in a *C. jejuni* specific capture ELISA recently validated for GBS. (**B)** Seasonal fluctuation of serum reactivity (IgM, IgG and IgA) to *C. jejuni* in healthy individuals (*n*=1,107) between 1999 and 2001.

The frequency of occurrence of *C. jejuni*-related gastroenteritis generally declined with age whereas an opposite pattern was found in *C. jejuni*-related GBS. A slightly different pattern has been reported in a Japanese epidemiological study of *C. jejuni* isolates from GBS patients, in which *C. jejuni*-related-GBS occurred most frequently in patients younger than 30 with a second peak in the 50 to 59 age category (33). A recent nationwide study in England showed that only among those below 36 years of age, numbers of weekly GBS hospitalizations were associated with an increase in weekly *C. jejuni* reports 4 to 5 weeks earlier (34). This observed association does however not provide a direct link between individual GBS

patients and *C. jejuni* positive cultures. In the present study, patients under the age of 18 years did not routinely participate in the clinical trials or survey studies and therefore we cannot directly compare the age group below 36 years of age. The results of this study suggest that, at least in The Netherlands, older individuals are at increased risk for developing GBS after a *C. jejuni* infection. Although it has been reported that elderly patients may be more prone to infections with rare *C. jejuni* serotypes, it is unknown whether these serotypes are more common in GBS as is the case for reported serotypes such as HS:41, HS:4 and HS:19 (35). Alternatively, sensitization to common *C. jejuni* strains may determine this increased risk. The immunological background and the involved host- and pathogen-related factors that explain the age distribution of GBS in relation to *C. jejuni* need to be studied further.

The results of this study emphasize that seasonality of GBS may not always follow the seasonality of GBS-related infections. During winter months, the fraction of GBS patients with preceding *C. jejuni* infection was higher compared to the other seasons whereas in patients with *C. jejuni*-related gastroenteritis a striking opposite pattern was found. Endemic forms of *C. jejuni*-related GBS have been reported in China and Curaçao in which a rise in *C. jejuni* infections correlated with GBS incidence but seemed not related to season (30,36,37). In Japan, seasonality of *C. jejuni* is less pronounced as one study showed no clear seasonality of *C. jejuni*-related GBS whereas others reported a higher incidence of *C. jejuni*-related GBS during spring (33,38). Preponderance of GBS incidence in the winter months was observed in France but could not be attributed to serologically proven *C. jejuni* infections (39). Seasonal fluctuations of *C. jejuni* laboratory reports in the general population are however common. Increases of incidence between June and August have been published in both European countries as well as New Zealand at the opposite hemisphere (16,40,41). A direct comparison of the seasonality of *C. jejuni*-related gastroenteritis and GBS was not reported previous. Given the inverse seasonal pattern of *C. jejuni*-related gastroenteritis and GBS observed in this study, this indicates that *C. jejuni* infections that occur during winter in The Netherlands are more likely to cause post-infectious sequelae such as GBS. Interestingly, 4 (3%) patients had symptoms of both diarrhoea and an upper respiratory tract infection, which all developed GBS during winter months (November n=1, February n=3). In Japan, 16 of 107 (15%) GBS and MFS patients with positive *C. jejuni* culture also had both diarrhoea and symptoms of upper respiratory tract infection (33). This may suggest that at least in a proportion of GBS patients, multiple infections in a double hit fashion could play a role.

Limitations related to the comparisons made in this study are associated with the recruitment of patients and determination *C. jejuni* infections. By using bootstrap analysis as resampling method a comparison could be made that takes into account the difference in number of patients within the *C. jejuni*-related gastroenteritis and GBS cohort. The ELISA used in this study is a specifically validated method to demonstrate *C. jejuni* infections in GBS patients (19). However, it should be recognized that patients might have a different clinical phenotype when ELISA demonstrates the infection and culture confirmation is not available. This confounding by selection can not be completely excluded although similar differences between patients without evidence of *C. jejuni* infection (culture- and serology negative) and patients with culture or serology proven *C. jejuni* infection were observed. To further exclude the possibility that the observed patterns are due to inadequate interpretation of ELISA test values, we performed the analyses with more stringent cut-off values, resulting in the same

seasonal and age-related patterns (not shown). Non-specific seasonal variability of the ELISA was neither likely given the absence of seasonal fluctuation of serum reactivity in healthy individuals (Figure 3B). We encourage new developments in techniques for sensitive and reproducible testing of antecedent infections that may have already been cleared from the host such as in GBS patients (42,43).

In conclusion, *C. jejuni*-related GBS is more likely to occur in older individuals and during the European winter period. Given the fact that only a very small proportion of patients with *C. jejuni*-related gastroenteritis will develop GBS, these results suggest that both environmental and host-related risk factors may determine the outcome of *C. jejuni* infections.

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3

Campylobacter jejuni and molecular mimicry in GBS

3.1

Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndrome

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ABSTRACT

Molecular mimicry between lipooligosaccharides (LOS) of *Campylobacter jejuni* and gangliosides in peripheral nerves plays a crucial role in the pathogenesis of *C. jejuni*-related Guillain-Barré syndrome (GBS). We have analyzed the LOS outer core structures of 26 *C. jejuni* strains associated with GBS and its variant the Miller Fisher syndrome (MFS) by capillary-electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS). Sixteen out of 22 (73%) GBS-associated and all 4 (100%) MFS-associated strains expressed LOS with ganglioside mimics. GM1a was the most prevalent ganglioside mimic in GBS-associated strains (10/22, 45%) and in 8 of these strains GM1a was found in combination with GD1a mimics. All 7 strains isolated from patients with ophthalmoplegia (GBS or MFS) expressed disialylated (GD3 or GD1c) mimics. Three out of 22 GBS-associated strains (14%) did not express sialylated ganglioside mimics because their LOS locus lacked the genes necessary for sialylation. Three other strains (14%) did not express ganglioside mimicks because of frame-shift mutations in either the *cstII* sialyltransferase gene or in the *cgtB* galactosyltransferase gene. It is not possible to determine if these mutations were already present during the *C. jejuni* infection. This is the first report in which mass spectrometry combined with DNA sequence data was used to infer the LOS outer core structures of a large number of neuropathy-associated *C. jejuni* strains. We conclude that molecular mimicry between gangliosides and *C. jejuni* LOS is the presumable pathogenic mechanism in most cases of *C. jejuni*-related GBS. However, our findings suggest that in some cases other mechanisms may play a role. Further examination of the disease etiology in these patients is mandatory.

INTRODUCTION

Gastro-enteritis caused by *Campylobacter jejuni* is the most common infection preceding the Guillain-Barré syndrome (GBS), an acute immune-mediated neuropathy (1,2). Molecular mimicry between lipooligosaccharides (LOS) in the *C. jejuni* cell wall and gangliosides in peripheral nerves plays a crucial role in the pathogenesis of GBS (3). Gangliosides are membrane glycolipids that are highly enriched in the nervous system. They are composed of a highly variable oligosaccharide core containing one or more sialic acid molecules and a ceramide tail inserted in the cell membrane. Acute phase sera of most patients with *C. jejuni*-associated GBS contain high titers of antibodies to various gangliosides that cross-react with *C. jejuni* LOS (4,5). The specificity of these anti-ganglioside antibodies relates to specific antecedent infections and different clinical presentations of GBS. For example, anti-GM1 antibodies have been associated with a preceding *Campylobacter* infection and with a severe, pure motor form of GBS (6). The Miller Fisher syndrome (MFS), a variant of GBS with oculomotor weakness and ataxia, is strongly associated with the presence of anti-GQ1b antibodies (7).

Since the first report in 1993, several studies have demonstrated ganglioside-like structures in the LOS outer core of *C. jejuni* strains isolated from GBS and MFS patients (8). Mass spectrometry (MS) and NMR analysis of individual strains has revealed the presence of GM1a, GD3, GD1a and GT1a mimics in GBS-associated strains and of GD3 mimics in MFS-associated strains (9-13). Serological studies on larger collections of isolates have confirmed and extended these findings (5,14). However, serological assays are not suitable to determine the exact chemical structure of the LOS outer core.

Detailed knowledge of the biosynthesis and structures of LOS outer cores in neuropathy-associated *C. jejuni* isolates may help to further elucidate the role of microbial factors in the pathogenesis of GBS, especially since *C. jejuni* displays considerable structural variation in its LOS outer core. Several genetic mechanisms responsible for this variation have been described (15). First, there is extensive variation in the gene content of the LOS biosynthesis gene locus ("LOS locus"). In addition, variation in homopolymeric tracts, single base deletions, insertions and mutations can lead to gene inactivations or glycosyltransferases with different acceptor specificities, resulting in the expression of different LOS structures. Previously, we analyzed the LOS locus of a collection of Dutch neuropathy-associated and control enteritis *C. jejuni* isolates (16). We found that the class A LOS locus was associated with GBS and the expression of GM1-like structures, whereas the class B LOS locus was associated with MFS and the expression of GQ1b-like structures. The presence of GM1-like and GQ1b-like structures was determined with serological assays and the exact LOS structures were not known. The development of new MS methods combined with serotyping and preliminary genetic knowledge to predict LOS structures allows quick screening of many strains (17). In the current study, we used this method to infer the LOS outer core structures of 26 GBS- and MFS-associated *C. jejuni* strains. Furthermore, we analyzed the genetic mechanisms responsible for the observed variation in these structures and we related the different LOS structures to clinical symptoms in the corresponding patients.

MATERIALS AND METHODS

C. jejuni strains

Twenty-two GBS-associated and 4 MFS-associated *C. jejuni* isolates were isolated from patients from The Netherlands, Belgium and the Netherlands Antilles between 1991 and 2000 (Table 1). GB13 and GB14, and GB26 and GB27, were cultured from the diarrheal stools of family members of two GBS patients (18). In both families, there was an outbreak of *C. jejuni* enteritis whereas only one family member developed GBS. From both GBS patients, we were unable to culture *C. jejuni*, despite the serological evidence that the GBS patients had also been infected with *C. jejuni*. These paired isolates were found to be highly related by various genotyping methods (19, 20), suggesting that family members had been infected with the

Table 1. *C. jejuni* strains and patient characteristics.

Strain	HS serotype[a]	Origin	Patient	GenBank Accession No[b]
GB1	1	The Netherlands	GBS	EF066651
GB2	UT	The Netherlands	GBS	DQ813306
GB3	19	The Netherlands	GBS	DQ906040
GB4	37	The Netherlands	GBS	AY943308
GB5	4, 64	The Netherlands	GBS	AY854153
MF6	4, 64	The Netherlands	MFS	AY422196
MF7	35	The Netherlands	MFS	DQ140270
MF8	23, 36	The Netherlands	MFS	DQ102714
GB11	2	The Netherlands	GBS	AY422197
GB13	2	The Netherlands	enteritis, family GBS	EF101695
GB14	2	The Netherlands	enteritis, family GBS	EF101696
GB15	5, 34	The Netherlands	GBS	AY423554
GB16	13, 66	Belgium	GBS (with ophthal moplegia)	EF07670
GB17	4, 13, 64	The Netherlands	GBS	EF094857
GB18	19	The Netherlands	GBS	DQ868320
GB19	4, 50	The Netherlands	GBS (with ophthal moplegia)	DQ357237
MF20	2	The Netherlands	MFS	EF064287
GB21	13, 65	The Netherlands	GBS	EF076704
GB22	13, 64	Netherlands Antilles	GBS	EF091821
GB23	4, 13, 43	The Netherlands	GBS	EF107518
GB24	31	The Netherlands	GBS	AY573819
GB25	2	The Netherlands	GBS (with ophthal moplegia)	EF064288
GB26	1, 44	The Netherlands	enteritis, family GBS	DQ351737
GB27	1, 44	The Netherlands	enteritis, family GBS	EF095404
GB28	19, 38	Netherlands Antilles	GBS	DQ906041
GB31	13, 50	Netherlands Antilles	GBS	DQ518908

[a] HS = heat stable (Penner serotyping system).

[b] GenBank accession numbers are given for the partial DNA sequences within the LOS locus.

same *C. jejuni* strain. The degree of sub-culturing was kept to a minimum, but 6 to 8 passages were necessary for isolation, storage, transport and preparing cells for mass spectrometry analysis.

Determination of the LOS locus class by PCR

The LOS locus class was determined as described previously (16). To distinguish between class D and class F, an additional primer set for the detection of orf17d (specific for class D) was included (21).

Mass spectrometry analysis

Confluent overnight growths from one agar plate (Mueller-Hinton medium) were treated as described by Szymanski *et al.* except that we used proteinase K at 60 mg/mL, RNase A at 200 mg/mL and DNase I at 100 mg/mL (17). The *O*-deacylated LOS samples were analyzed by capillary-electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS) as described by St. Michael *et al.* (22). All CE-ESI-MS and CE-ESI-MS/MS were performed using a crystal Model 310 capillary electrophoresis instrument (ATI Unicam, Boston, MA, USA) coupled to an API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) via a microIon-spray interface.

DNA sequencing

Genomic DNA was isolated using a DNeasy Tissue kit (Qiagen). Long PCR products were generated using an Advantage 2 PCR kit (Clontech Laboratories). The PCR products were sequenced using custom-made primers that were used previously to sequence the LOS locus in multiple strains (15). DNA sequencing was performed using an Applied Biosystems model 373 automated DNA sequencer (Montreal, Canada) and the manufacturer's cycle sequencing kit. See supplementary Table XXVII for additional details.

Cloning and expression of the *cst-II* gene from *C. jejuni* GB26

The GB26 *cst-II* gene was amplified using *Pwo* polymerase (Roche Diagnostics, Laval, Canada) and the following primers: CJ-131 (5' CTTAGGAGGT*CATATG*AAAAAAGTTATTATTGC-TGGAAATG 3', 41 mer, *Nde*I site in italics) and CJ-764 (5' TTTAGG*GTCGAC*T-CAAAGATTAAAATTTTTTGAG 3', 34 mer, *Sal*I site in italics). These two primers amplified the region encoding amino acids 1 to 260 of *cst-II* from *C. jejuni* GB26. The PCR product was digested with *Nde*I and *Sal*I and cloned in pCWori+(-*lacZ*) (23) giving construct CST-125. *E. coli* AD202 containing construct CST-125 was grown in 2 YT medium containing 150 μg/mL ampicillin. The culture was incubated at 37°C until A_{600} = 0.35, induced with 1 mM IPTG, and then incubated 7 h at 37°C. The cells were broken using an Avestin C5 Emulsiflex cell disruptor (Avestin, Ottawa, Canada). α-2,3- and α-2,8-sialyltransferase activities were assayed as described previously (15).

Statistical analysis.

Differences in frequencies between groups were analyzed with the Fisher's exact test using InStat version 3.0 (Graphpad Software, San Diego, CA, USA). Differences were considered significant at $P < 0.05$ after two-sided testing.

RESULTS

Determination of the LOS outer core structures

We used CE-ESI-MS on *O*-deacylated *C. jejuni* LOS to propose LOS outer core structures for the 26 GBS- and MFS-associated isolates (Figures 1 and 2, Table 2, supplementary Tables I to XXVI). The CE-ESI-MS procedure did not provide linkage information but provided information about the sugar composition of the LOS outer core. The glycosyltransferase variants present in the LOS locus of each strain (Table 3) were used to help interpret the data obtained by CE-ESI-MS (see supplementary appendixes A and B). For several strains, we could only determine sugar composition of the LOS outer core and no structure. In classes A and B strains, the presence of a two-domain Cj1135 (glucosyltransferase) suggests that both heptoses are substituted with glucose while the presence of a one-domain Cj1135 suggests that only HepI is substituted with glucose (24). The extension of the outer core from HepII

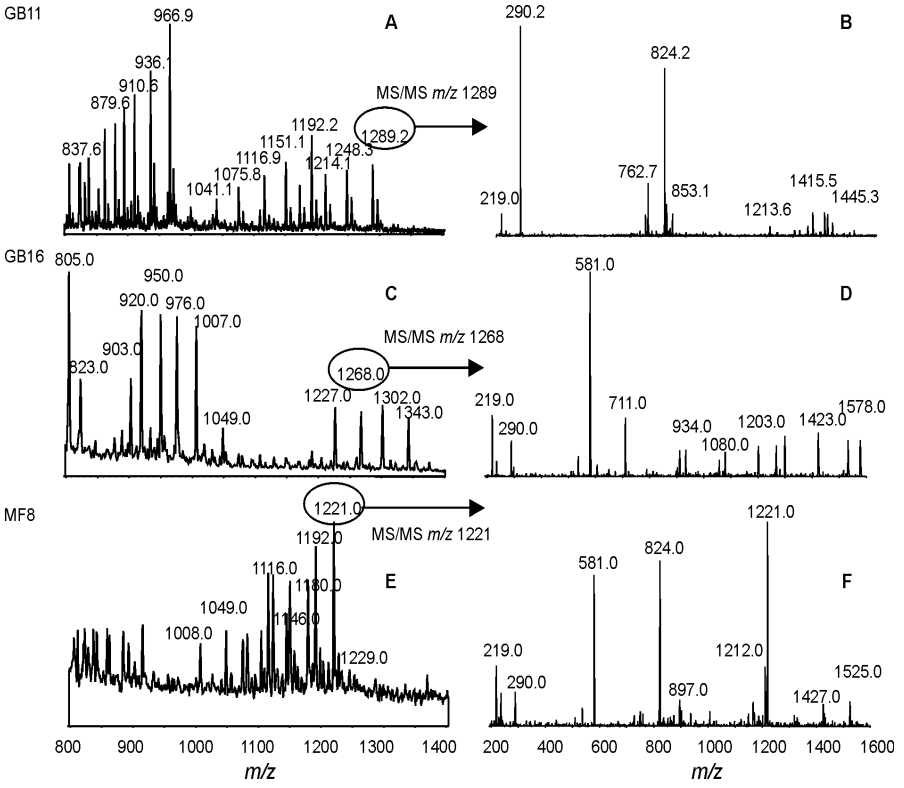


Figure. 1. Mass spectrometry analysis of *O*-deacylated LOS from repesentative *C. jejuni* strains with sialylated LOS outer cores.

Panels A and B: *C. jejuni* GB11, Panels C and D: *C. jejuni* GB16, Panels E and F: *C. jejuni* MF8. Panels A, C and E show extracted mass spectra from CE-MS. Panels B, D and F show MS/MS of a representative peak from each CE-MS spectrum.

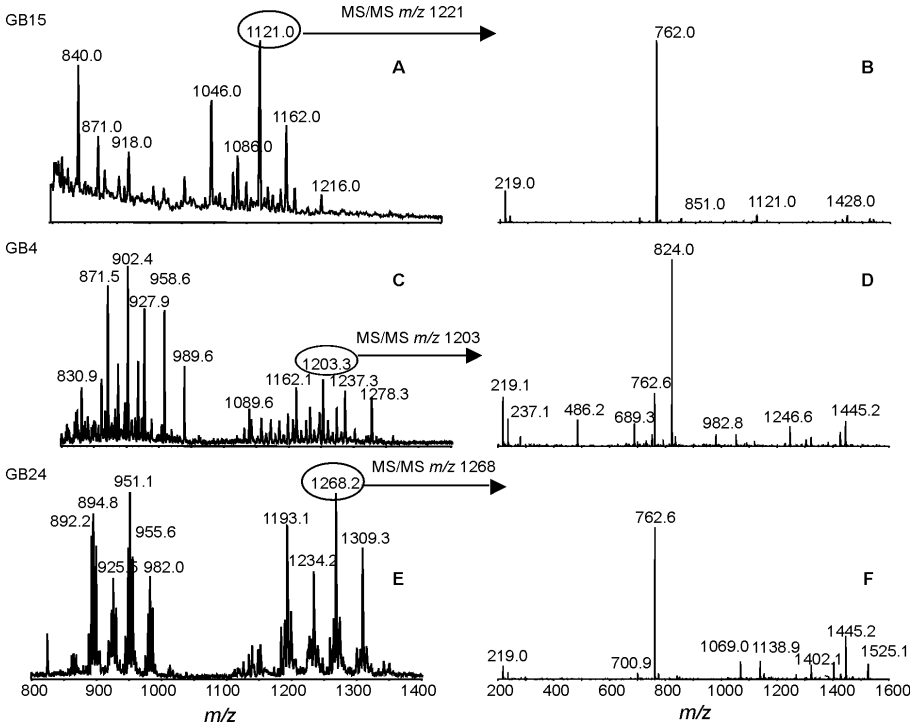


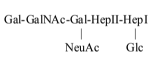
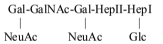
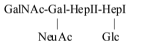
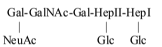
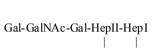
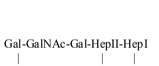
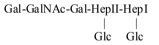
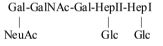
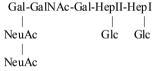
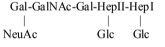
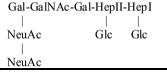
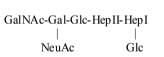
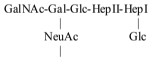
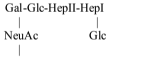
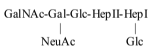
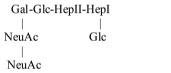
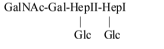
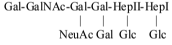
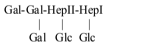
Figure 2. Mass spectrometry analysis of *O*-deacylated LOS from repesentative *C. jejuni* strains with non-sialylated LOS outer cores.

Panels A and B: *C. jejuni* GB15, Panels C and D: *C. jejuni* GB4, Panels E and F: *C. jejuni* GB24. Panels A, C and E show show extracted mass spectra from CE-MS. Panels B, D and F show MS/MS of a representative peak from each CE-MS spectrum.

is proposed for the strains that have an active Cj1136 variant while the two strains (MF7 and MF8) that have an inactive Cj1136 variant due to frame-shift mutations (see GenBank DQ140270 and DQ102714) are proposed to have an outer core extended from the glucose substituted to HepII. Based on our previous observations with strains whose LOS outer core structures were completely determined (24), we propose that the inner galactose is substituted with a sialic acid in the strains that have no glucose on HepII and that have CgtA and CgtB variants that are specific for a sialylated acceptor. We propose that the inner galactose is not substituted with sialic acid in the case of classes A and B strains that have a glucose on HepII, an active Cj1136 variant and CgtA/CgtB variants that are specific for non-sialylated accceptors.

Fifteen different outer core structures were identified among the 26 strains that were analyzed and 14 strains expressed a mixture of at least 2 different outer core structures. It was not possible to quantify the proportions of the different outer core structures because their different sialic acid contents result in different ionization efficiencies, which then have an impact on observed peak intensities. Several strains harboring the same LOS locus expressed different LOS structures. Within the class A strains, 5 different (mixtures of) LOS structures

Table 2. Proposed LOS outer core structures expressed by GBS- and MFS-associated strains. The brackets indicate the portions of the LOS outer cores that are mimicking gangliosides. The HepI of the inner core in linked to a Kdo residue that is linked to the lipid A portion of the LOS (11).

Strain(s)	LOS class	Structure	Ganglioside mimic
GB2, GB3, GB11, GB18, GB21, GB22, GB28, GB31	A		GM1a
			GD1a
GB23	A		GM2
GB26	A		GM1b
GB27	A		none (GA1)
GB16, GB19	A		GD1c
GB17, GB25	B		none (GA1)
			GM1b
			GD1c
MF6, MF20	B		GM1b
	B		GD1c
MF7	B		GM2
			GD2
			GD3
MF8	B		GM2
			GD3
GB5	B		none (GA2) [a]
GB13, GB14	C		GM1a
GB1	C		none
GB24	K	$Hex_6\,HexNAc_1\,Hep_2$	none
GB4	P	$Hex_3HexNAc_3QuiNAc_1Hep_2$ [b]	none
GB15	F	$Hex_4\,HexNAc_1\,Hep_2$	none

[a] The exact structure could not be deduced from the observed masses, but the composition of the sugar residues was consistent with an "asialo-GM2"-like (GA2) structure.

[b] The observed masses suggest that this LOS structure is related to the LOS outer core structure of strain ATCC 43431 (HS:3 type strain)(26)

were detected. Clearly, knowledge of the LOS locus class is not sufficient to predict the LOS structure. It is also necessary to sequence the key glycosyltransferases to determine the variants involved and whether they encode complete or truncated products.

Expression of ganglioside mimics in the LOS

Sixteen of 22 (73%) GBS-associated isolates and all 4 (100%) MFS-associated isolates expressed LOS with ganglioside mimics including GM1a, GM1b, GM2, GD1a, GD1c, GD2 and GD3. Ganglioside mimics were only detected in strains with a class A, B or C LOS locus (presence of ganglioside mimics in class A/B/C vs. other classes: 20/23 vs. 0/3, $P<0.01$). In GBS-associated strains, GM1a was the most prevalent ganglioside mimic, present in 10 out of 22 strains (45%). Interestingly, in all 8 GBS strains with a class A LOS locus and GM1a mimicry (36% of all GBS strains), the GM1a mimic was present as part of a GM1a/GD1a mixture (presence of GM1a/GD1a in class A vs. non-A: 8/13 vs. 0/13, $P<0.01$). All 7 strains isolated from patients with MFS or GBS with ophthalmoplegia expressed structures with a terminal di-NeuAc-Gal (GD3 and GD1c), versus only 1/19 other GBS-associated strains (5%, $P<0.01$). These mimics were predominantly found in strains with a class B LOS locus (presence of GD1c or GD3 in class B vs. non-B: 6/7 vs. 2/19, $P<0.01$). Both class A strains with a GD1c mimic were isolated from GBS patients with ophthalmoplegia.

LOS outer core structures without ganglioside mimics

Ganglioside mimics could not be detected in the LOS of 6 out of 22 GBS-associated isolates (27%): GB1, GB4, GB5, GB15, GB24 and GB27 (Table 2). These strains were further analyzed to explain the absence of ganglioside mimics. The class C LOS locus of strain GB1 contains all genes necessary to synthesize sialylated LOS. However, we found a 5-base deletion in the *cst-III* gene of GB1 (GenBank accession number EF066651), resulting in a truncated Cst-III (219 aa instead of 294 aa). This will prevent the transfer of sialic acid and subsequent addition of the terminal GalNAc to the LOS backbone. Strain GB5 also contains a LOS locus (class B) that is essentially capable of directing the synthesis of sialylated LOS, but single base deletions in the *cgtB* and *cst-II* genes result in the expression of a truncated LOS outer core without sialic acid (25). Although GB26 and GB27, isolated from two family members of a GBS patient, were indistinguishable by various pheno- and genotyping methods (16,20), mass spectrometry revealed that only GB26 expresses sialylated LOS. We detected a poly-G tract in the *cst-II* gene that leads to a frame shift and premature translation stop in GB27 (10-G tract) and mostly a complete translation product in GB26 (9-G tract).

We sequenced the entire LOS biosynthesis locus of GB4, GB15 and GB24 and found that all three strains lack the genes necessary for sialylation of the LOS (GenBank accession numbers AY943308, AY423554 and AY573819, respectively). These three LOS loci do not contain either the sialyltransferase gene (*cst-II* or *cst-III*) or the genes (*neuA*, *neuB* and *neuC*) necessary for the synthesis of sialic acid and its activated donor, CMP-NeuAc. GB15 has a class F LOS locus (24), whereas both GB4 and GB24 contained novel LOS loci (classes P and K, respectively, Parker *et al.*, manuscript in preparation). The exact LOS outer core structures of strains GB4 and GB24 could not be deduced from the mass spectrometry data, but the former structure is related to the LOS outer core of strain ATCC 43431 (HS:3 type strain), which does not contain a ganglioside mimic (26). The mass spectrometry profile suggests

Table 3. Variants of the glycosyltransferases involved in the synthesis of the LOS outer core structures in *C. jejuni* strains with class A or B LOS locus.[a]

Strain	LOS class	Cj1135	Cj1136	CgtAI	CgtAII	CgtB	Cst-II [b]
GB2	A	One-domain	on	Mono-sialyl.[c]	N/A [d]	Mono-sialyl.	Mono-
GB3	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB5	B	Two-domain	on [e]	Non-sialyl.[f]	Mono/di-sialyl.[g]	off [h]	off
MF6	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
MF7	B	Two-domain	off	off	Mono/di-sialyl.	off	Bi-
MF8	B	Two-domain	off	off	Mono/di-sialyl.	Mono-sialyl.	Mono-
GB11	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB16	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	Bi-
GB17	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
GB18	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB19	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	Bi-
MF20	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
GB21	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB22	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB23	A	One-domain	on	Mono-sialyl.	N/A	off	Mono-
GB25	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
GB26	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	Mono-
GB27	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	off
GB28	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB31	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-

[a] Assignment of the glycosyltransferase variants is based on amino acid sequence comparisons with variants of known specificities (15,24).

[b] Cst-II variants: mono-: monofunctional, Cst-II has α-2,3-sialyltransferase activity, bi-: bifunctional, Cst-II has both α-2,3-sialyltransferase and α-2,8-sialyltransferase activity.

[c] Mono-sialyl.: the glycosyltransferase is specific for mono-sialylated acceptor.

[d] N/A: Not applicable.

[e] on: indicates that a gene has no frame-shift mutation.

[f] Non-sialyl.: the glycosyltransferase is specific for non-sialylated acceptor.

[g] Mono/di-sialyl.: the glycosyltransferase can use a mono- or a di-sialylated acceptor.

[h] off: indicates that a gene is inactive because of a frame-shift mutation.

that the LOS outer core of GB15 is composed of 4 hexoses and 1 HexNAc. It is possible that the LOS outer core of GB15 mimics a human glycolipid of the globo- or isoglobo- series. However, none of the LOS outer cores of GB4, GB15 and GB24 contains sialic acid, as shown by the absence of the diagnostic ion (*m/z* 290) in CE-MS/MS spectra of *O*-deacylated LOS samples from theses strains (Figure 2). This was further confirmed by precursor ion scan experiments, in which no glycoforms were detected with a precursor ion at *m/z* 290 (data not shown).

Cst-II variants and LOS structure

Polymorphism in the *cst-II* gene determines the extent of sialylation of the LOS (15).

Therefore, we determined the correlation between Cst-II variants and LOS outer core structure. We have previously shown that amino acid residues 51 and 53 affect the level of activity and specificity of Cst-II (15). Most of the variants with Asn51 express disialylated LOS outer cores (bifunctional Cst-II; α-2,3- and α-2,8-sialyltransferase activity) while most of the variants with Thr51 express LOS outer cores with only α-2,3-linked sialic acids (monofunctional Cst-II; only α-2,3-sialyltransferase activity). Cst-III, the Cst-II homologue present in class C strains, is always monofunctional and was therefore not analyzed in this study. We detected 8 different variants among the 20 classes A and B neuropathy-associated strains (Figure 3), 5 of which had Asn51. Seven out of 10 strains (70%) with an Asn51 variant expressed disialylated LOS, as opposed to 1 out of 10 strains (10%) with a Thr51 variant (P=0.02). However, the correlation between Cst-II variants and LOS structure was not perfect. MF8 has the Thr51 variant and expresses a mixture of mono- and di-sialylated LOS outer cores, while GB26 has Asn51 and expresses only mono-sialylated LOS outer core. The LOS loci of MF8 and HS:36 are identical except for a difference in the length of the G-tract in *cgtA* (15). Although speculative, it is possible that a *cgtA* mostly in an "off status" enables Cst-II with very low α-2,8-sialyltransferase activity to add a second sialic acid, since the lack of GalNAc addition preserves the acceptor for Cst-II. We cloned and expressed Cst-II from GB26 and found that it has only α-2,3-sialyltransferase activity (using *in vitro* assays, supplementary Figure 1) although it has Asn51. Cst-II from GB26 has the sequence that diverges most from the other Cst-II sequences (Figure 3) and it is possible that one (or several) amino acid substitution(s) has(ve) inactivated the α-2,8-sialyltransferase activity. We observed that the Asn51 variant was present in 6/7 class B strains (86%), whereas Thr51 was the most prevalent variant in class A strains (9/13, 69%, P=0.06). Likewise, the Asn51 variant was present in 6/7 (86%) of MFS-associated strains and strains associated with GBS and ophthalmoplegia, whereas the Thr51 variant was primarily found in the other GBS-associated strains (9/13, 69%, P=0.06).

DISCUSSION

Ganglioside mimicry is considered to be a crucial factor in the pathogenesis of *C. jejuni*-associated GBS (3). Detailed knowledge of the bacterial components mimicking human structures, the genetic mechanisms responsible for the observed variation in these structures and their relation to cross-reactive auto-antibodies and clinical features may provide a better understanding of the role of molecular mimicry in post-infectious neuropathy. For the first time, MS combined with DNA sequence data was used to determine the LOS outer core structures of a large number of neuropathy-associated *C. jejuni* strains. Our data confirm that ganglioside mimicry is the most likely pathogenic mechanism underlying the majority of *C. jejuni*-associated GBS cases, but that in some GBS patients mimicry towards microbial structures other than ganglioside-like LOS or other mechanisms may lead to the neurological damage.

Various ganglioside mimics were found in the LOS of neuropathy-associated strains. GM1a was the most prevalent ganglioside mimic in GBS strains and it was predominantly present in combination with GD1a mimics (36% of all GBS strains). Although GM1a mimics were found in both class A and class C strains, the GM1a/GD1a mixture was only present in

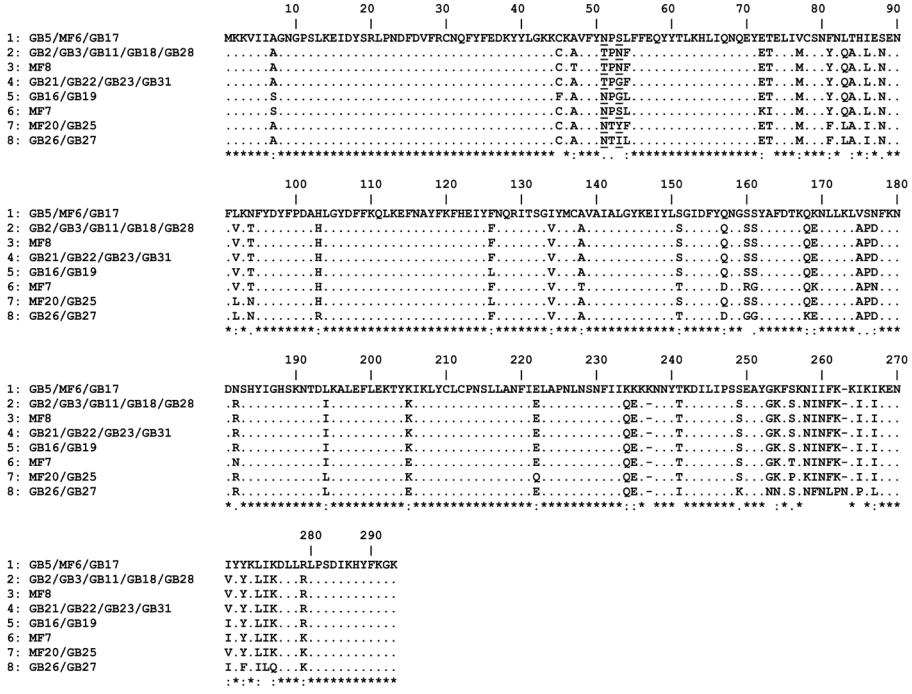


Figure 3. Alignment of the Cst-II sialyltransferase amino acid sequences from the GBS and MFS *C. jejuni* strains.

Only variable residues are shown in addition to the consensus sequence. The "*" indicate conserved residues, the ":" indicate strongly similar residues and the "." weakly similar residues. The amino acids that were shown to influence the activity and specificity of Cst-II are underlined (residues 51-53).

strains with a class A LOS locus, which has previously been associated with GBS (16,27). Because the prevalence of a class A LOS locus in enteritis-associated strains is 14-17% (16, 27), it is expected that a maximum of 14-17% of these strains have the GM1a/GD1a mixture. The high prevalence of a GM1a/GD1a mixture in GBS-associated strains suggests that a cluster or complex of these two ganglioside mimics may be the target antigens in a subgroup of GBS, rather than single ganglioside mimics. This finding is consistent with the results of Koga *et al.*, but in contrast with those of Nachamkin *et al.*, who found that expression of GD1a and not GM1 was associated with GBS (14, 27). Furthermore, our results are in agreement with recent observations that ganglioside complexes are important target antigens in GBS as well as in MFS (28, 29).

MFS and GBS with ophthalmoplegia (GBS/MFS overlap) have been associated with the presence of anti-GQ1b antibodies and with the presence of GQ1b-like LOS as determined with serological assays (5, 7). This association may be explained by the enrichment of GQ1b in the nerves that innervate the oculomotor muscles (30). Up to now, MS analysis has not demonstrated true GQ1b-like structures in *C. jejuni* LOS. The detection of structures with a

terminal di-NeuAc-Gal in 7 out of 8 (87%) strains associated with ophthalmoplegia suggests that in these patients, pathogenic antibodies are raised against the disialylated, GD3- or GD1c-like LOS and cross-react with GQ1b in the human nerves.

There are several possible explanations for the observation that 6 GBS-associated strains did not express ganglioside mimics in their LOS. We have previously demonstrated that a GBS patient had been co-infected with two *C. jejuni* strains while only one strain could be linked to GBS (25). In such cases, it is possible that a co-infecting strain, possibly a strain without ganglioside mimics, is isolated from the stool sample and wrongfully regarded as "GBS-associated" strain. This may also have occurred in patients described here related to strains that lacked ganglioside mimics. However, it is also possible that the expression of ganglioside mimics vanished during the infection or culture procedures due to mechanisms such as phase variation or single base mutations or deletions. Strain GB1 did not express ganglioside mimics due to a frame-shift mutation in the *cst-III* gene. It is possible that GBS was induced by a ganglioside-mimicking GB1 strain and that this mutation occurred later in the course of the infection or during laboratory processing. This hypothesis is concordant with the presence of antibodies against both GM1 and asialo-GM1 in the patient serum (5). The same scenario may also apply to strains GB26 and GB27, which had been isolated from two family members of a GBS patient who did not develop neurological symptoms. Both isolates are genetically highly related, indicating that all family members had probably been infected with the same strain. Interestingly, we found that variation in the poly-G tract of the *cst-II* gene was responsible for the lack of ganglioside mimics in the LOS of GB27. In this case, GBS may have been triggered by the ganglioside-mimicking variant of the strain (GB26) and not by the variant without ganglioside mimics (GB27). Strain GB26 expresses an LOS outer core that mimics GM1b which, unfortunately, is not commercially available. Consequently we could not determine if the family member who developped GBS had any anti-GM1b antibodies.

On the other hand, our findings indicate that sometimes, molecular mimicry with non-sialylated LOS may be involved in the pathogenesis of GBS. We demonstrated previously that the GB5 patient serum contains anti-asialo-GM2 antibodies cross-reactive with GB5 LOS, which suggests that GBS was induced by molecular mimicry with *C. jejuni* LOS without ganglioside mimics (25). Other mechanisms, including mimicry with *C. jejuni* structures other than LOS, either sialylated or non-sialylated, or with other microorganisms should also be considered in some cases. Strains GB4, GB15 and GB24 do not express ganglioside mimics because they do not have the genes that are required for sialylation of the LOS. The acute phase patient sera of GB4, GB15 and GB24 did not contain anti-ganglioside antibodies ((5) and M. Kuijf, unpublished data), suggesting a pathogenic mechanism other than ganglioside mimicry. Further investigations are needed to elucidate the pathogenesis of GBS and the role of *C. jejuni* in these cases.

Genetic polymorphism of *C. jejuni* determines the LOS structure and thereby also the specificity of the anti-ganglioside antibody response and clinical features of GBS (5,15,16). Presence of and polymorphism within the *cst-II* gene has been associated with the expression of ganglioside mimics and with clinical features of GBS (31,32). We found that the Cst-II Asn51 variant was associated with the expression of disialylated LOS and seemed to occur more frequently in class B strains and strains related with clinical symptoms of

MFS or GBS with ophthalmoplegia. The Thr51 variant was associated with monosialylated LOS and seemed to occur more frequently in class A strains and in GBS-related strains. These observations suggest that the previously described associations between a class A LOS locus and GBS and class B LOS locus and MFS may be based on the high prevalence of the Thr51 variant in the class A LOS locus and the Asn51 variant in the class B LOS locus (16). Our findings are concordant with the recent reports of Koga *et al.* (27,32).

We conclude that the majority of *C. jejuni* strains isolated from GBS or MFS patients express single or multiple ganglioside mimics in their LOS. However, a substantial portion of the strains is apparently lacking the antigen that is supposed to give rise to the potentially pathogenic anti-ganglioside antibodies. Further examination of the disease etiology in these patients is mandatory.

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SUPPLEMENTARY TABLES I TO XXVII

Supplementary Table I. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB1 using a basic structure containing Kdo_2 Hep_2 Hex_5.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3060.0	3059.89
GlcN3N-GlcN-2*PP*Etn	3183.0	3182.94
GlcN3N-GlcN3N-*PP*Etn	3285.0	3285.27
GlcN3N-GlcN3N-2*PP*Etn	3408.0	3408.32

Assignments are from a comparison of the observed and calculated mass (Da) based on the proposed structures (Table 2). Average mass units were used for calculation of molecular mass values based on proposed compositions as follows: Glc/Gal, 162.14; HexNAc, 203.19; Hep, 192.17; Kdo, 220.18; GlcN, 161.16; GlcN3N 160.18; NeuAc, 291.26; phosphate, 79.98; *P*Etn, 123.05; phosphoramidate, 78.98; 3-OH C14:0 fatty acid, 226.36.

Supplementary Table II. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB2 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN	-	3108.0	3107.01
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3522.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table III. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB3 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3744.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table IV. Assignment of the variants for the lipid A backbone and variable hexose (Hex) of the *O*-deacylated LOS from *C. jejuni* GB4 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_3$ $QuiNAc_1$ $phosphoramidate_1$.

Lipid A variant	Hex	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3327.6	3326.16
GlcN3N-GlcN-2*PP*Etn	-	3450.9	3449.21
GlcN3N-GlcN-*PP*Etn	+	3489.7	3488.30
GlcN3N-GlcN3N-*PP*Etn	-	3553.3	3551.54
GlcN3N-GlcN-2*PP*Etn	+	3613.3	3611.35
GlcN3N-GlcN3N-2*PP*Etn	-	3676.2	3674.59
GlcN3N-GlcN3N-*PP*Etn	+	3715.1	3713.68
GlcN3N-GlcN3N-2*PP*Etn	+	3838.3	3836.73

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table V. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB5 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN-GlcN-*PP*Etn	2713.5	2713.42
GlcN3N-GlcN	2815.5	2815.75
GlcN3N-GlcN-*PP*Etn	2938.3	2938.80
GlcN3N-GlcN-2*PP*Etn	3061.8	3061.85
GlcN3N-GlcN3N-*PP*Etn	3166.3	3164.18
GlcN3N-GlcN3N-2*PP*Etn	3286.3	3287.23

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table VI. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB11 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3230.4	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3354.1	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.8	3455.44
GlcN3N-GlcN-*PP*Etn	+	3522.7	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3580.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.9	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3748.2	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3871.1	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table VII. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB13 using a basic structure containing Kdo_2 Hep_2 Hex_6 $HexNAc_1$ $NeuAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3717.0	3716.48
GlcN3N-GlcN-2*PP*Etn	3840.0	3839.53
GlcN3N-GlcN3N-*PP*Etn	3942.0	3941.86
GlcN3N-GlcN3N-2*PP*Etn	4065.0	4064.91

See Supplemetary Table I for the average mass units used for calculation.

Supplementary Table VIII. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB14 using a basic structure containing Kdo_2 Hep_2 Hex_6 $HexNAc_1$ $NeuAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3717.0	3716.48
GlcN3N-GlcN-2*PP*Etn	3840.0	3839.53
GlcN3N-GlcN3N-*PP*Etn	3942.0	3941.86
GlcN3N-GlcN3N-2*PP*Etn	4065.0	4064.91

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table IX. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB15 using a basic structure containing Kdo_2 Hep_2 Hex_5 $HexNAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3141.0	3140.03
GlcN3N-GlcN-2*PP*Etn	3261.0	3263.08
GlcN3N-GlcN3N-*PP*Etn	3365.0	3365.41
GlcN3N-GlcN3N-2*PP*Etn	3488.5	3488.46

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table X. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB16 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$ $NeuAc_2$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	3805.5	3806.51
GlcN3N-GlcN3N-*PP*Etn	3908.5	3908.84
GlcN3N-GlcN3N-2*PP*Etn	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XI. Assignment of the variants for the lipid A backbone and variable terminal sialic acids (NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB17 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage while terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

Lipid A variant	t-2,3 NeuAc	t-2,8 NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN3N-*PP*Etn	-	-	3327.0	3326.32
GlcN3N-GlcN-*PP*Etn	+	-	3393.0	3392.20
GlcN3N-GlcN-2*PP*Etn	+	-	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	-	3618.0	3617.58
GlcN3N-GlcN-*PP*Etn	+	+	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	+	+	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	+	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	+	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XII. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB18 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XIII. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB19 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$ $NeuAc_2$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XIV. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB21 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3 NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3744.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XV. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB22 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XVI. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB23 using a basic structure containing Kdo_2 Hep_2 Hex_2 $HexNAc_1$ $NeuAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3066.0	3067.92
GlcN3N-GlcN-2*PP*Etn	3189.0	3190.97
GlcN3N-GlcN3N-*PP*Etn	3291.0	3293.30
GlcN3N-GlcN3N-2*PP*Etn	3414.0	3416.35

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XVII. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB24 using a basic structure containing Kdo_2 Hep_2 Hex_6 $HexNAc_1$ P_2.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3582.8	3585.18
GlcN3N-GlcN-2*PP*Etn	3705.8	3708.23
GlcN3N-GlcN3N-*PP*Etn	3808.2	3810.56
GlcN3N-GlcN3N-2*PP*Etn	3931.6	3933.61

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XVIII. Assignment of the variants for the lipid A backbone and variable terminal sialic acids (NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB25 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage while the terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8- linkage.

Lipid A variant	t-2,3-NeuAc	t-2,8-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-2*PP*Etn	-	-	3225.0	3223.99
GlcN3N-GlcN3N-*PP*Etn	-	-	3327.0	3326.32
GlcN3N-GlcN-*PP*Etn	+	-	3393.0	3392.20
GlcN3N-GlcN3N-2*PP*Etn	-	-	3447.0	3449.37
GlcN3N-GlcN-2*PP*Etn	+	-	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	-	3618.0	3617.58
GlcN3N-GlcN-*PP*Etn	+	+	3681.0	3683.46
GlcN3N-GlcN3N-2*PP*Etn	+	-	3741.0	3740.63
GlcN3N-GlcN-2*PP*Etn	+	+	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	+	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	+	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XIX. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB26 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3099.0	3100.94
GlcN3N-GlcN-2*PP*Etn	-	3222.0	3223.99
GlcN3N-GlcN3N-*PP*Etn	-	3327.0	3326.32
GlcN3N-GlcN-*PP*Etn	+	3393.0	3392.20
GlcN3N-GlcN3N-2*PP*Etn	-	3447.0	3449.37
GlcN3N-GlcN-2*PP*Etn	+	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	3618.0	3617.58
GlcN3N-GlcN3N-2*PP*Etn	+	3741.0	3740.63

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XX. Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB27 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3099.0	3100.94
GlcN3N-GlcN-2*PP*Etn	3222.0	3223.99
GlcN3N-GlcN3N-*PP*Etn	3324.0	3326.32
GlcN3N-GlcN3N-2*PP*Etn	3447.0	3449.37

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXI. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB28 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3453.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXII. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB31 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3228.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3522.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Table I for the average mass units used for calculation.

Supplementary Table XXIII. Assignment of the variants for the lipid A backbone and variable terminal sialic acid (NeuAc) of the *O*-deacylated LOS from *C. jejuni* MF6 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$ $NeuAc_1$. The terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8- linkage.

Lipid A variant	t-2,8-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3390.0	3392.20
GlcN3N-GlcN-2*PP*Etn	-	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	-	3618.0	3617.58
GlcN3N-GlcN-*PP*Etn	+	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	+	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXIV. Assignment of the variants for the lipid A backbone, variable terminal sialic acids (NeuAc) and HexNAc of the *O*-deacylated LOS from *C. jejuni* MF7 using a basic structure containing Kdo_2 Hep_2 Hex_3 $NeuAc_1$. The terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

Lipid A variant	t-2,8-NeuAc	HexNAc	Observed mass	Calculated mass
GlcN3N-GlcN-2*PP*Etn	-	+	3354.0	3353.11
GlcN3N-GlcN-2*PP*Etn	+	-	3441.0	3441.18
GlcN3N-GlcN3N-*PP*Etn	+	-	3543.0	3543.51
GlcN3N-GlcN3N-2*PP*Etn	-	+	3576.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	+	3747.0	3746.70
GlcN3N-GlcN3N-*PP*Etn	3	-	3834.0	3834.77
GlcN3N-GlcN3N-2*PP*Etn	+	+	3870.0	3869.75
GlcN3N-GlcN3N-2*PP*Etn	3	-	3957.0	3957.82
GlcN3N-GlcN3N-*PP*Etn	3	+	4038.0	4037.96

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXV. Assignment of the variants for the lipid A backbone, variable terminal sialic acids (NeuAc) and HexNAc of the *O*-deacylated LOS from *C. jejuni* MF8 using a basic structure containing Kdo_2 Hep_2 Hex_3 $NeuAc_1$. The terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

Lipid A variant	t-2,8-NeuAc	HexNAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	-	3027.0	3026.87
GlcN3N-GlcN-2*PP*Etn	-	-	3150.0	3149.92
GlcN3N-GlcN-2*PP*Etn	-	+	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	+	-	3543.0	3543.51
GlcN3N-GlcN3N-2*PP*Etn	-	+	3579.0	3578.49
GlcN3N-GlcN3N-2*PP*Etn	+	-	3666.0	3666.56

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXVI. Assignment of the variants for the lipid A backbone and variable terminal sialic acids (NeuAc) of the *O*-deacylated LOS from *C. jejuni* MF20 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage while terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

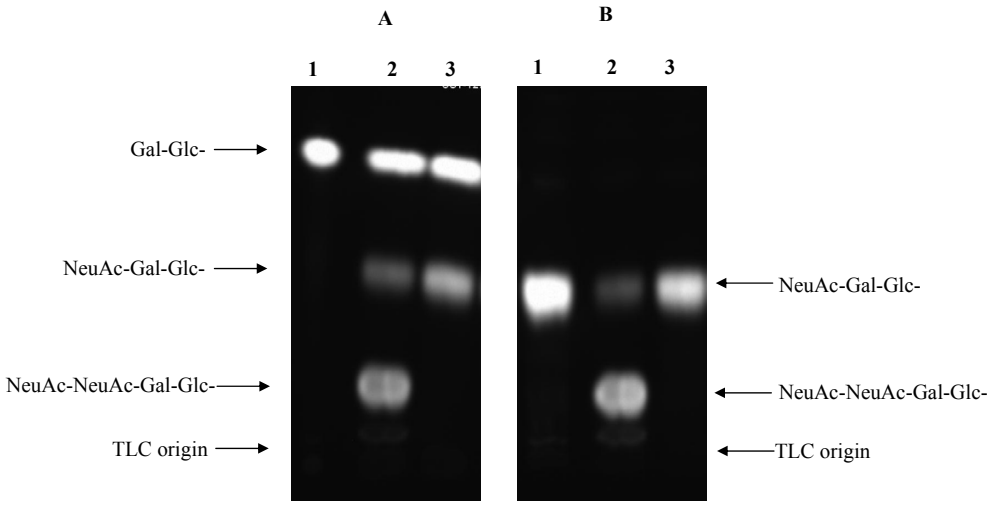
Lipid A variant	t-2,3-NeuAc	t-2,8-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	+	-	3392.2	3392.20
GlcN3N-GlcN-2*PP*Etn	+	-	3516.3	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	-	3618.9	3617.58
GlcN3N-GlcN-*PP*Etn	+	+	3684.3	3683.46
GlcN3N-GlcN3N-2*PP*Etn	+	-	3742.1	3740.63
GlcN3N-GlcN-2*PP*Etn	+	+	3807.1	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	+	3910.3	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	+	4033.1	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXVII. Information about the DNA sequencing of the lipooligosaccharide loci from GBS- and MFS-associated strains.

Strain	GenBank Accession number	Contig size (bp)	Double-Strand coverage (%)
GB1	EF066651	1,906	98.3
GB2	DQ813306	6,047	86.1
GB3	DQ906040	6,047	99.4
GB4	AY943308	15,092	96.9
GB5	AY854153	12,403	93.0
MF6	AY422196	12,370	85.8
MF7	DQ140270	12,354	89.3
MF8	DQ102714	12,359	92.3
GB11	AY422197	24,425	98.0
GB13	EF101695	1,906	99.0
GB14	EF101696	1,906	96.3
GB15	AY423554	7,633	87.5
GB16	EF076703	6,036	93.7
GB17	EF094857	7,930	86.0
GB18	DQ868320	6,047	99.4
GB19	DQ357237	11,413	73.1
MF20	EF064287	9,357	96.3
GB21	EF076704	6,047	98.0
GB22	EF091821	6,047	97.8
GB23	EF107518	6,045	83.3
GB24	AY573819	9,295	98.3
GB25	EF064288	9,357	91.5
GB26	DQ351737	11,427	85.3
GB27	EF095404	880	1.2 [a]
GB28	DQ906041	6,047	99.1
GB31	DQ518908	11,419	96.9

[a] Only the phase-variable *cstII* gene was sequenced in GB27. The heterogeneous G-tract prevented sequencing on both strands but the sequence was confirmed by multiple same strand sequencing reactions.



Supplementary Figure 1. Demonstration that Cst-II from *C. jejuni* GB26 is mono-functional.
The α-2,3-sialyltransferase activity was assayed using Galβ-1,4-Glc-FCHASE as acceptor (Panel A). The α-2,8-sialyltransferase activity was assayed using Neu5Acα-2-3-Galβ-1,4-Glc-FCHASE as acceptor (Panel B). Purified recombinant MalE-Cst-II from GB26 was incubated with either Galβ-1,4-Glc-FCHASE (Panel A, lane 3) or Neu5Acα-2-3-Galβ-1,4-Glc-FCHASE (Panel B, lane 3) and a 2-fold excess of CMP-NeuAc. Product is observed only with Galβ-1,4-Glc-FCHASE (lane 3) as acceptor. Lane 1 from panel A shows where Gal-Glc-FCHASE migrates and lane 1 from panel B shows where Neu5Acα-2-3-Galβ-1,4-Glc-FCHASE migrates. Lanes 2 show an example of a Cst-II that is bifunctional (Cst-II(Ile53Ser) from *C. jejuni* OH4384).

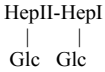
Appendix A: Key to LOS outer core structures from Class A:

The glycosyltransferase variants for each *C. jejuni* strain are indicated in Table 3

Step 1: Glucosyltransferase variant

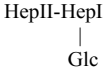
Two-domain Cj1135

Basic inner core:



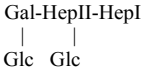
One-domain Cj1135

Basic inner core

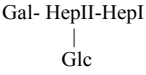


Step 2: Cj1136 variant is always observed as complete product in class A strains studied in this work

Case 1: Gal extension from HepII with two glucose units on inner core

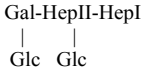


Case 2: Gal extension from HepII with one glucose unit on inner core

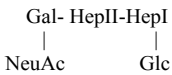


Step 3: Addition of NeuAc to the inner Gal by Cst-II when there is no Glc on HepII.

Case 1: No NeuAc on inner Gal

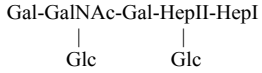


Case 2: NeuAc on inner Gal

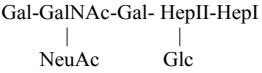


Step 4: Specificity of CgtA and CgtB

Case1: CgtA and CgtB use non-sialylated acceptors

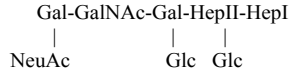


Case 2: CgtA and CgtB use sialylated acceptors

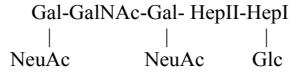


Step 5: Mono- or bi-functional Cst-II

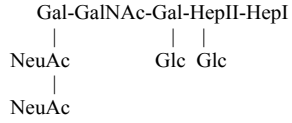
Case1.1: Mono-functional Cst-II



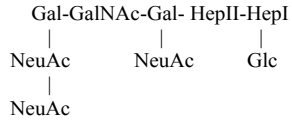
Case 1.2: Mono-functional Cst-II



Case 2.1: Bi-functional Cst-II



Case 1.2: Bi-functional Cst-II



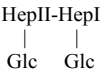
Appendix B: Key to LOS outer core structures from Class B:

The glycosyltransferase variants for each *C. jejuni* strain are indicated in Table 3

Step 1: Glucosyltransferase variant

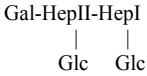
All class B strains have a two-domain Cj1135:

Basic inner core:

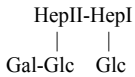


Step 2:

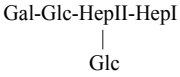
Case 1: Cj1136 encodes a full length galactosyltransferase which results in a Gal extension from HepII with two glucose units on inner core:



Case 2: Cj1136 encodes a truncated galactosyltransferase which prevent extension from HepII. An un-identified gene encodes a galactosyltransferase that uses the Glc on HepII as acceptor:

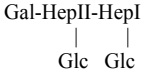


Note: equivalent to:

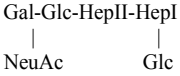


Step 3: Addition (or not) of NeuAc to the inner Gal by Cst-II:

Case 1: No NeuAc added to the Gal extension from HepII because of the presence of a Glc on HepII.

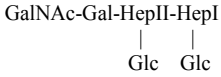


Case 2: Addition of NeuAc on Gal attached to Glc

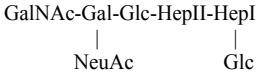


Step 4: Addition of GalNAc

Case1: CgtA uses a non-sialylated acceptor

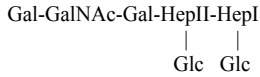


Case 2: CgtA uses a sialylated acceptor



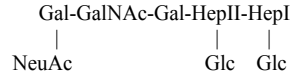
Step 5: Addition of a terminal Gal is observed only in the non-sialylated extension

Case 1:

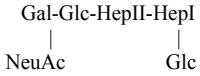


Step 6: Mono- or bi-functional Cst-II

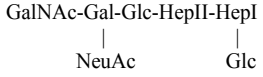
Case1.1: Mono-functional Cst-II



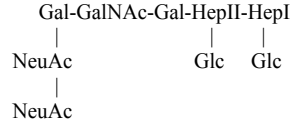
Case 1.2: Mono-functional Cst-II



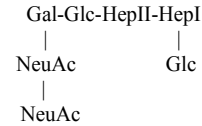
Case 1.3: Mono-functional Cst-II



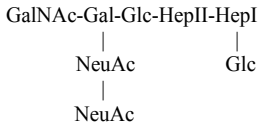
Case 2.1: Bi-functional Cst-II



Case 2.2: Bi-functional Cst-II



Case 2.3: Bi-functional Cst-II



3.2

Origin of ganglioside complex antibodies in Guillain-Barré syndrome

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ABSTRACT

The origin of antibodies to ganglioside complexes, as new immunotargets for Guillain-Barré syndrome (GBS), is unknown. This was investigated in 21 GBS patients from which *Campylobacter jejuni* was isolated. Two of these patients had serum IgG to the GM1/GD1a complex and two other patients had IgG to the GQ1b/GD1a complex. These pairs of patients were clinically distinct. These antibodies all cross-reacted to lipo-oligosaccharides (LOS) from the autologous *C. jejuni* strain. Previous mass spectrometry studies on these LOS showed the presence of oligo-saccharides with a similar structure, further supporting the hypothesis that in these patients LOS induced the ganglioside complex antibodies.

INTRODUCTION

Molecular mimicry between *Campylobacter jejuni* lipo-oligosaccharides (LOS) and peripheral nerve gangliosides plays a crucial role in the pathogenesis of the Guillain-Barré syndrome (GBS) (1). *C. jejuni* is the predominant cause of infection preceding GBS and highly associated with the presence of antibodies to a variety of gangliosides (2,3). The *C. jejuni* LOS contains ganglioside-like moieties that dictate the specificity of the cross-reactive anti-ganglioside antibodies (4). These antibodies can also be induced by immunization with *C. jejuni* LOS in animal models that develop a neuropathy resembling GBS (5). Interestingly, the specificity of the anti-ganglioside antibodies is related to distinct clinical subgroups of GBS (1). However, not all GBS patients associated with *C. jejuni* infections have antibodies to single gangliosides (3,6).

Recent studies showed that some GBS patients produce antibodies to mixtures or complexes of gangliosides, such as GM1/GD1a and GQ1b/GD1a, rather than to single gangliosides (7,8). Conformational epitopes recognized by the antibodies may be present in the Schwann cell and neuronal plasma membranes where various gangliosides reside in clusters in functional domains (9). Interestingly, *C. jejuni* isolates from GBS patients also frequently express various forms of (truncated) LOS each mimicking a different ganglioside (10). At present however, it is unknown whether the conformational epitopes formed by ganglioside complexes are also present in the LOS of *C. jejuni*.

In this study the origin of the antibodies to ganglioside complex was determined in patients with GBS from which *C. jejuni* was isolated. First, acute phase serum samples from 21 of these patients were screened for the presence of IgG antibodies to various ganglioside complexes in relation to the neurological deficits. Secondly, we studied the cross-reactivity of these antibodies to LOS from autologous *C. jejuni* isolates in relation to the previously determined molecular structure of these LOS. Our study provides strong evidence that conformational molecular mimicry is also involved in the induction of ganglioside complex antibodies in these patients with *C. jejuni*-related GBS.

MATERIALS AND METHODS

Patients and bacterial strains

Twenty-six *C. jejuni* strains were isolated from 24 GBS patients or infected family members with enteritis from The Netherlands, Belgium and the Netherlands Antilles between 1991 and 2000. The family members were excluded from the serological study. From 21 of the remaining patients, pre-treatment serum obtained within two weeks of onset of weakness was available for the present study. Fourteen of these patients had GBS without ophthalmoplegia, three had GBS with ophthalmoplegia and four had the Miller Fisher syndrome (MFS) variant, characterized by the presence of ophthalmoplegia, ataxia and areflexia without evident limb paresis. All *C. jejuni* strains were isolated during the acute phase of disease. From all patients informed consent was obtained.

Determination of ganglioside complex serum reactivity
Serum samples obtained from the 21 *C. jejuni*-related GBS or MFS patients were diluted 1:100 and tested in duplicate for IgG reactivity to the gangliosides GM1, GM2, GD1a, GD1b, GD3 and GQ1b and to all combinations of two of these gangliosides. The antibody activity was determined by enzyme-linked immunosorbent assay (ELISA) according to previously described methods (11) and by using 150 pmol/well for each ganglioside. Serum reactivity for ganglioside complexes was considered positive when the corrected optical densities (OD) (extinctions of ganglioside complex coated wells minus OD of non-coated wells) were 0.2 higher than the corrected OD for a single ganglioside, in accordance with previously defined criteria (7). Positive serum samples were titrated using two-fold serial dilution series starting at 1:100. The reciprocal of the highest dilution that resulted in an OD higher than the cut-off value (OD 0.2) was taken to be the titer (11). Residual antibody activity to ganglioside complexes was determined in convalescent serum samples obtained at three to six months after onset of disease.

LOS isolation
The LOS fractions from all *C. jejuni* strains were isolated by hot phenol-water extraction and processed as described before (2). The molecular structure of the *C. jejuni* LOS core antigen was previously determined by electrospray ionization mass spectrometry and summarized in Table 1 (10).

Determination of cross-reactivity
Cross-reactivity of anti-ganglioside complex antibodies to *C. jejuni* LOS was determined by pre-incubation of serum with LOS from *C. jejuni* isolated from the autologous patients and with LOS from the *C. jejuni* HS:3 Penner serostrain as a control, according to methods previously described (2,12). Cross-reactivity of anti-GM1/GD1a complex antibodies was also determined to LOS from three non-autologous *C. jejuni* strains (GB2, GB21 and GB22) containing both GM1 and GD1a mimics and to LOS from NTC 11168 reference strain containing a GM1 mimic (10,13). LOS concentrations of 50, 13, 3.0, 0.8 and 0.2 μg/ml were incubated with serum (diluted 1:100) for 3 hours at 4ºC. The supernatants were centrifuged and tested for residual anti-ganglioside activity. Percentage of inhibition was defined as:

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

RESULTS

Antibodies to ganglioside complexes
Sera from four of 21 (19%) patients with *C. jejuni*-related GBS had IgG antibodies to one or more of the gangliosides complexes. Two patients (GB11 and GB17) had IgG antibodies to the GM1/GD1a complex and two other patients (GB16 and GB19) had IgG antibodies to the GQ1b/GD1a complex (Table 2). The patients GB17 and GB16 also had IgG antibodies to the GD1a/GD1b and GD3/GQ1b complexes respectively (Table 2). Convalescent serum samples available from the patients GB11, GB16 and GB19 contained no residual antibody activity to ganglioside complexes. None of the patients had antibodies to the ganglioside complexes

Table 1. Molecular structures and ganglioside mimics of LOS from *C. jejuni* isolates from GBS patients used in the study[a].

Strain(s)	LOS structure	Ganglioside mimics
GB2, GB11, GB21, GB22	Gal-GalNAc-Gal-Hep-Hep | NeuAc Glc	GM1
	Gal-GalNAc-Gal-Hep-Hep | | | NeuAc NeuAc Glc	GD1a
GB17	Gal-GalNAc-Gal-Hep-Hep | | Glc Glc	none (GA1)
	Gal-GalNAc-Gal-Hep-Hep | | | NeuAc Glc Glc	GM1b
	Gal-GalNAc-Gal-Hep-Hep | | | NeuAc Glc Glc | NeuAc	GD1c[b]
GB16, GB19	Gal-GalNAc-Gal-Hep-Hep | | | NeuAc Glc Glc | NeuAc	GD1c[b]

[a] Structures were previously published (10).
[b] GD1c structurally similar to GD3.

composed of GM1/GM2, GM1/GD1b, GM1/GD3, GM1/GQ1b, GM2/GD1a, GM2/GD1b, GM2/GD3, GM2/GQ1b, GD1a/GD3, GD1b/GD3, GD1b/GQ1b. Some of the patients without anti-ganglioside complex antibodies have antibodies to single gangliosides as was previously published (4).

Characteristics of patients with ganglioside complex antibodies

The patients with ganglioside complex antibodies all had preceding symptoms of an infectious disease, serological evidence for a recent *C. jejuni* infection and serum IgM and IgG antibodies to the LOS from the autologous *C. jejuni* isolate. All patients had a rapidly progressive form of GBS without ataxia. Patient GB11 and GB17, with antibodies to the GM1/GD1a complex, had a pure motor variant of GBS without cranial nerve involvement (except for a mild facial palsy in patient GB11) (Table 2). Patients GB16 and GB19, with antibodies to the GQ1b/GD1a complex had a severe variant of GBS with ophthalmoplegia (Table 2). Patient GB16 had to be ventilated and GB19 had an additional bulbar palsy.

Table 2. Serology and clinical features of GBS patients with ganglioside complex antibodies.

Patients	GB11	GB17	GB16	GB19
Clinical characteristics				
Age, years	50	64	53	29
Sex	Male	Male	Male	Male
Days to nadir[a]	13	14	2	2
Ophthalmoplegia	-	-	+	+
Sensory involvement	-	-	-	+
Motor involvement[b]	46	39	6	34
GBS disability score[c]				
At nadir	4	3	5	4
At 26 weeks	1	3	0	NA
Serum IgG antibody titers				
GM1	800	100	0	0
GD1a	0	0	12800	100
GD1b	3200	0	0	0
GD3	0	100	3200	0
GQ1b	0	0	102400	12800
GM1/GD1a	12800	12800	0	0
GD1a/GD1b	0	12800	0	0
GD1a/GQ1b	0	100	409600	25600
GD3/GQ1b	0	0	25600	0

[a] Number of days between onset and most severe weakness (nadir).
[b] Severity of weakness expressed as MRC-sum scores: sum of scores according to the Medical Research Council grading system of 6 bilateral muscle groups (deltoid, biceps, triceps, iliopsoas, quadriceps and tibialis anterior muscles) ranging from 60 (normal strength) to 0 (tetraparalytic).
[c] GBS disability score: 0 = no symptoms, 1 = minor signs or symptoms, 2 = able to 10 m without support but incapable of manual work, 3 = able to walk 10 m with walking aid, appliance or support, 4 = bed- or chair-bound, 5 = requiring assisted ventilation, 6 = dead.
NA = not available.

Cross-reactivity of ganglioside complex antibodies

The cross-reactivity of the ganglioside complex antibodies to *C. jejuni* LOS was studied by inhibition ELISA. Up to 90-100% of serum reactivity to the ganglioside complexes in the four patients was inhibited by 50 μg/ml LOS from the autologous *C. jejuni* isolate (Figure 1). This inhibition was dose dependent whereas no inhibition of serum reactivity was found using LOS from the control *C. jejuni* HS:3 Penner serostrain which lacks ganglioside mimics (Figure 1).

Anti-GM1/GD1a complex antibodies from patient GB11 were also inhibited by pre-incubation with LOS containing GM1 and GD1a mimics from three non-autologous *C. jejuni* strains isolated from patients without these antibodies (Figure 2). This activity was also inhibited by 50 μg/ml of LOS from the GB17 *C. jejuni* strain, but not by LOS from the *C. jejuni* NTC 11168 reference strain containing a GM1 mimic but not the GD1a mimic (data not shown).

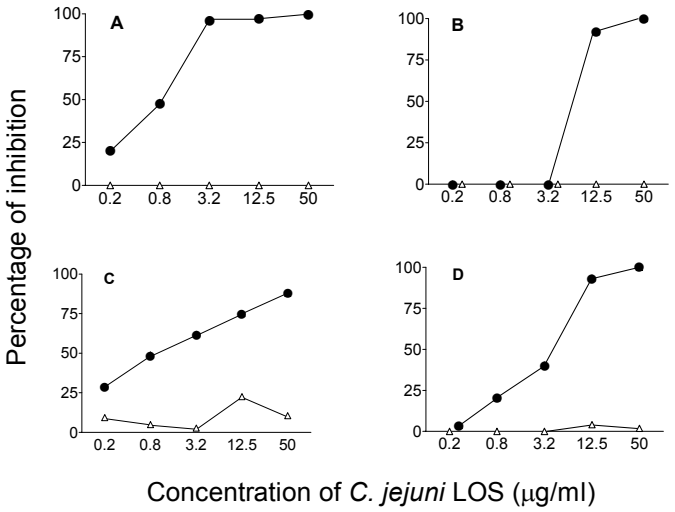

Figure 1. Cross-reactivity of ganglioside complex antibodies.
Antibodies to ganglioside complexes cross-react with LOS from the autologous *C. jejuni* strains (filled circles) but not with LOS from the control HS:3 Penner serostrain lacking ganglioside mimicry (open triangles). **(A)** Inhibition of IgG anti-GM1/GD1a complex reactivity in serum from patient GB11 by pre-incubation with LOS from the autologous *C. jejuni* GB11 strain (GM1/GD1a mimic, Table 1) and from Penner serostrain HS:3. **(B)** Inhibition of anti-GM1/GD1a complex reactivity in serum from patient GB17 by pre-incubation with LOS from the autologous GB17 strain (GM1b/GD1c mimic) and from Penner serostrain HS:3. **(C)** Inhibition of anti-GD1a/GQ1b complex reactivity in serum from patient GB16 by pre-incubation with LOS from the autologous GB16 strain (GD1c mimic) and from Penner serostrain HS:3. **(D)** Inhibition of anti-GD1a/GQ1b complex reactivity in serum from patient GB19 by pre-incubation with LOS from the autologous GB19 strain (GD1c mimic) and from Penner serostrain HS:3.

DISCUSSION

In this study we demonstrated that serum ganglioside complex antibodies cross-reacted with LOS from autologous *C. jejuni* isolates, strongly indicating that these antibodies were induced by *C. jejuni* LOS. Structural analysis had previously identified ganglioside-like moieties in the LOS from these autologous strains (10). The *C. jejuni* strain from one patient with anti-GM1/GD1a antibodies (GB11) expresses an heterogeneous LOS mimicking the expected GM1 and GD1a. The *C. jejuni* strain from the other patient with anti-GM1/GD1a antibodies (GB17) expresses a slightly different but also heterogeneous LOS that mimics asialo-GM1, GM1b and GD1c. These mimics share the terminal Gal-GalNAc- and NeuAc-Gal-GalNAc-moieties with GM1 and GD1a respectively. The serum anti-GM1/GD1a complex antibodies from patient GB11 also cross-reacted to this LOS suggesting the recognition of a similar epitope. The anti-GM1/GD1a complex antibodies from patient GB11 did not cross-react to LOS from HS:3 Penner serostrain lacking ganglioside-mimics and the NTC 11168 reference strain expressing a GM1 mimic only, illustrating the specificity of this recognition. Surprisingly, the two strains isolated from the patients with anti-GQ1b/GD1a antibodies (GB16 and GB19) both express

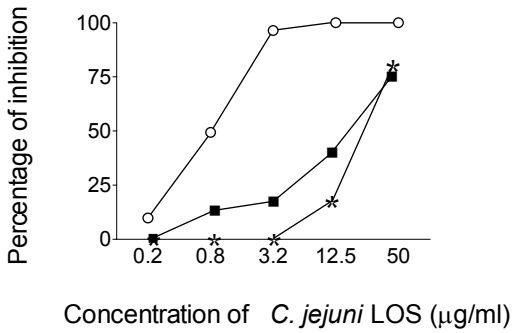

Figure 2. Cross-reactivity of IgG GM1/GD1a complex antibodies to non-autologous *C. jejuni* strains containing GM1/GD1a mimics.
Inhibition of IgG anti-GM1/GD1a complex reactivity in serum from patient GB11 by pre-incubation with LOS from *C. jejuni* strains GB2 (open circles), GB21 (asterisks) and GB22 (filled squares) isolated from GBS patients without ganglioside complex antibodies.

a homogeneous LOS with only a GD1c mimic. This mimic however, shares the terminal NeuAc-NeuAc-Gal-GalNAc epitope with GQ1b and the NeuAc-Gal-GalNAc with GD1a. Both patients had additional high titers of anti-GQ1b antibodies, although the titer was lower than the anti-GQ1b/GD1a antibodies. We can not exclude the possibility that the detected antibody activity to GQ1b is caused by antibodies to GQ1b/GD1a complexes, since the GQ1b used in these studies may be contaminated with other gangliosides.

Previous results showed that infection with *C. jejuni* expressing ganglioside-like LOS does not always lead to production of anti-ganglioside antibodies and GBS (10). In the present study we demonstrated that this restriction also applies for ganglioside complex mimics in LOS. Ganglioside complex antibodies were absent in some GBS patients that were infected with *C. jejuni* strains containing both GM1 and GD1a mimics in LOS. Interestingly, the GM1/GD1a complex antibodies from patient GB11 cross-reacted to at least three of these isolates (Figure 2). This illustrates the important role of host-factors in addition to molecular mimicry in the production of cross-reactive antibodies.

Several findings in the current study support the hypothesis that the cross-reactive antibodies to ganglioside complexes in these patients contributed to the development of GBS. First, the specificity of the antibodies to ganglioside complexes in these patients was associated with a specific clinical phenotype: the two patients with anti-GM1/GD1a antibodies both had a pure motor variant without severe cranial nerve involvement, while the two patients with anti-GQ1b/GD1a antibodies both had a severe GBS with ophthalmoplegia. Serum reactivity to the NeuAc-Gal-GalNAc-moiety of the GQ1b/GD1a complex antibodies may have contributed to the severe limp paresis and may explain why these patients did not develop ophthalmoplegia only. Secondly, this dichotomy in antibody specificity and clinical symptoms corresponded to the molecular structure of the LOS from the *C. jejuni* isolates from these patients. Thirdly, the titer of the serum antibodies to ganglioside complexes declined with clinical recovery of the patients. Studies in bioassays are required to further address the presumed neurotoxic effects of these antibodies.

Our study illustrates both the relevance and the intricacy of molecular mimicry in the induction of antibodies to ganglioside complexes in patients with *C. jejuni*-related GBS. In three cases the carbohydrate structures in the bacterial LOS were similar, but not identical to those in the associated ganglioside complexes. Furthermore, we found that in 11 of 17 ganglioside complex seronegative patients a heterogeneous *C. jejuni* LOS, as determined previously by mass spectrometry, is present as well (10). The configuration of the oligosaccharide moieties is highly influenced by the micro-environment, and depends on the epitope density and proximity of other oligosaccharides and possibly on the nature of the lipid carrier (9,14). Apparently, these different oligosaccharide moieties in the LOS and the ganglioside complexes form similar conformational epitopes, since both structures were recognized by the same serum antibodies. Immunizations with protein 'mimotopes' to induce antibodies to *Haemophilus influenzae* LOS also show that structural identity is not required to produce cross-reactive antibodies (15). Alternatively, the carbohydrate composition of LOS may also have changed due to genetic alterations in the LOS biosynthesis locus after the initial infection. However, the observed cross-reactivity of ganglioside complex antibodies and the similarity between the ganglioside mimics present in LOS clearly indicates that conformational epitopes can be formed by ganglioside-like complexes in *C. jejuni* LOS.

Our findings illustrate the importance of demonstrating antibody cross-reactivity in addition to defining molecular structures. The conformation of the oligosaccharide moieties in *C. jejuni* LOS and the recognition of these complex moieties by the adaptive immune system can not be predicted by the biochemical structure only. This may partly explain why *C. jejuni* strains expressing ganglioside-like structures have also been isolated in patients with uncomplicated enteritis, without the production of anti-ganglioside antibodies and subsequent development of GBS.

ACKNOWLEDGMENTS

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4

Immune response to *Campylobacter jejuni* and antigen presentation in GBS

4.1

Susceptibility to Guillain-Barré syndrome is not associated with CD1A and CD1E gene polymorphisms

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ABSTRACT

Immune responses to microbial glycolipids that cross-react to neural epitopes may trigger the Guillain-Barré syndrome (GBS). CD1 molecules are involved in antigen presentation of glycolipids and variation in CD1 genes was recently reported to confer susceptibility to develop GBS. This hypothesis was tested by comparing single nucleotide polymorphisms (SNPs) of CD1A and CD1E in 312 well defined GBS patients and 212 healthy controls. SNPs in CD1A and CD1E were not associated with GBS susceptibility, specific clinical subgroups, anti-ganglioside antibodies, antecedent infections and prognosis. Based on this study, CD1 polymorphisms are not a susceptibility or disease modifying factor in GBS.

INTRODUCTION

Guillain-Barré syndrome (GBS) is an immune mediated polyneuropathy that rarely follows common types of infections. *Campylobacter jejuni* is the predominant antecedent infection in GBS but only one in an estimated 1,000 to 5,000 persons with this type of infection will develop GBS (1). A crucial step in the pathogenesis of *C. jejuni*-related GBS is the production of cross-reactive antibodies to nerve gangliosides. These antibodies are induced by ganglioside mimicking *C. jejuni* lipo-oligosaccharide (LOS) and have shown to damage peripheral nerves (2). Antigen presentation of these bacterial glycolipids by dendritic cells or other antigen presenting cells may determine whether this aberrant immune response will occur or not.

Single nucleotide polymorphisms (SNPs) in the genes coding for antigen presenting molecules may influence the susceptibility to develop GBS. Extensive studies showed no general or consistent association between the human leucocyte antigen (HLA) class II haplotypes and GBS (3). HLA class II molecules yet present peptides whereas CD1 molecules are involved in antigen presentation and processing of glycolipids. In humans, five types of CD1 molecules exist, of which CD1a, CD1b, CD1c and CD1d are expressed at the cellular surface. The CD1e is an intermediate form that accumulates intracellular in late endosomal compartments and co-localizes with CD1b that in turn is transported to the cell membrane (4). CD1b is expressed on dendritic cells and is involved in activation of GM1-specific T-cells upon infection with *C. jejuni* LOS (5). Variation occurs in exon 2 of all CD1 genes, but only result in amino acid substitutions in CD1a and CD1e (6). In CD1A, the two alleles result in the replacement of threonine by isoleucine (1) and the replacement of cysteine by tryptophan (2). Both variants are expressed at the cell surface (7). In CD1E, the replacement of glutamine by arginine occurs in the region flanking the lipid binding groove. The effects of these SNPs on antigen presentation of lipids are however unknown. Interestingly, a recent report indicated that genotype variants in exon 2 of CD1A and CD1E genes are susceptibility factors for developing GBS, although this study was based on a relatively small cohort of 65 patients (8).

In the present study, a detailed described cohort of 312 GBS patients and 212 healthy controls was used to determine if these CD1 SNPs are related to the development of GBS. Furthermore, we assessed the relationship between these SNPs and subgroups of GBS patients defined by preceding infections, serum antibodies and clinical characteristics.

PATIENTS AND METHODS

Patients and controls

DNA was obtained from 312 Dutch patients (median age 47 years, range 7-82 years, male-female ratio =1.1) fulfilling the diagnostic criteria for GBS (9). Detailed clinical and serological data were available in a subgroup. This group consisted of 213 patients who participated in one of the Dutch GBS trials or survey studies (10-13). Disease severity was defined by the GBS disability score, ranging from 0 (no symptoms) to 6 (dead), and the MRC sumscore, ranging from 0 (tetraplegia) to 60 (normal) at nadir (3). Clinical outcome was evaluated as the number of days required for patients to be able to walk independently (i.e. reaching GBS

disability score of 2) with a follow-up time of 6 months. Time to nadir was expressed as the number of days before reaching the highest disability score or lowest MRC sumscore. Serological screening for most common antecedent infections and antiganglioside antibodies was performed using standard procedures (3). Dutch healthy blood bank donors were used as controls (*N*=212, median age 35 years, range 19-60 years, male/female ratio=0.66). The study was approved by the Medical Ethical Committee of Erasmus MC. All patients and controls gave their written informed consent (14).

Genotyping

Genotyping was performed using the LightCycler® technique (Roche Diagnostics, Mannheim, Germany) (3). The set of primers and hybridization probes were designed by TibMolBiol and are listed in table 1 (Berlin, Germany). Sequence-verified control donors were used in every experiment as internal control. The PCR-reaction and the final melting curve were performed in LightCycler capillaries (Roche Diagnostics) with a final volume of 20 µl, containing 10 ng genomic DNA, 0.5 µM of each primer, 0.15 µM of each hybridization probe, 1x LightCycler DNA Master Hybridization Probes (Roche Molecular Biochemicals, Mannheim, Germany) and 2 mM MgCl2. Sealed capillaries were centrifuged and placed in the rotor of the LightCycler instrument (Roche Diagnostics). The PCR thermocycling profile included 10 min at 95°C, followed by 45 cycles of 95°C for 0.1 sec, 60°C for 15 sec and 72°C for 20 sec. Next, the melting curve profile was performed, which included 1 cycle of 95°C for 2 min, 55°C for 1 min, 45°C for 30 sec, 40°C for 3 min after which the temperature was slowly increased (0.1°C/sec) to 85°C under continuous detection of the emitted light. Finally, the cooling down was performed at 40°C for 30 sec. Data were analysed using the melting curve program. In each experiment we used sequenced DNA from confirmed control donors for each genotype.

Table 1. Used primer sets and hybridazation probes.

Gene	Primers (5' → 3')	Hybridization probes (5' → 3')
CD1A	CCGCACTCTGGCACCTTTCT (forward)	CTCATACCTGGGACAGCAATTCCAGCACC-fluorescein
	TTGCTGAAGTTTCCACTGGACC (reverse)	LC Red705-TCGTTTTCCTGTGCCCCTGG
CD1E	TGGGCACCATCCGCTTTC (forward)	TCCATAGTTTTATCCAGATAGTGCAAGCT-fluorescein
	CATGCAGGCCCTTTTGTGT (reverse)	LC Red640-CTGCTGGTCAATTTCAGCTTGA ATGTAAGTTC

Statistical analyses

Verification of Hardy-Weinberg equilibrium, genotype and allele frequencies was compared using two-sided Chi-square or Fisher's exact test. An expectation-maximization algorithm was used to test for linkage disequilibrium and to compare estimated haplotype distributions. SNP associations for clinical outcome were studied by Kaplan-Meier analysis using logrank tests. Correction for type I errors was performed by Bonferroni adjustment. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

All SNPs tested were in Hardy-Weinberg equilibrium and in linkage disequilibrium. No differences were found between the frequencies of the various alleles, genotypes and haplotypes in GBS patients and controls (Table 2). In GBS patients there was no significant association between the SNPs and sex, age, symptoms of preceding infection (diarrhea or upper-respiratory tract infection), serology (for recent infections with *C. jejuni*, *Mycoplasma pneumoniae,* CMV and EBV), disease severity at nadir, presence of sensory or cranial nerve deficits, mechanical ventilation and serum IgM/IgG antibodies to GM1 or GD1a. Clinical outcome of GBS patients, after 6 months of onset and during follow-up, was not associated with specific CD1A and CD1E SNPs in Kaplan-Meier analysis.

A subgroup analysis was performed in patients with anti-GM1 antibodies (n=46). There was an association with the CG variant of the CD1A genotype and the GG variant of the CD1E genotype and good clinical outcome (logrank respectively p=0.04 and p=0.03) but after correction for multiple testing this was not significant. No associations were found for clinical deficits and disease severity in relation to CD1A and CD1E SNPs within patients having antiganglioside antibodies and/or *C. jejuni* infections.

Table 2. CD1A and CD1E polymorphisms in GBS patients and controls.

SNP	GBS patients (n=312)	Healthy controls (n=212)
	%	%
CD1A		
G-allele	6.3	6.4
C-allele	93.7	93.6
CD1E		
A-allele	64.6	66.5
G-allele	35.4	33.5
CD1A (genotype)		
GG	0	0
GC	12.5	12.7
CC	87.5	87.3
CD1E (genotype)		
AA	41.2	44.3
AG	46.9	44.3
GG	11.9	11.3
CD1 haplotype (CD1E / CD1A)		
AC	64.5	66.5
AG	0	0
GC	29.2	27.1
GG	6.3	6.4

Allele-, genotype and haplotype distributions were compared between GBS patients and healthy controls using two-sided Pearson chi-square tests. No significant associations were found for SNPs in exon 2 of CD1A and CD1E.

DISCUSSION

In our study, no difference was found in the frequency of CD1A and CD1E SNPs between the group of GBS patients and the group of healthy controls, indicating that these gene polymorphisms do not influence the general susceptibility to develop GBS. In addition, there was no genetic association with clinical outcome, clinical characteristics or serological subgroups of GBS, including antecedent *C. jejuni* infections and anti-ganglioside antibodies. These results give no support to the hypothesis that CD1A and CD1E influence the risk of getting GBS as raised in a previous study that was based on results in a much smaller cohort of GBS patients (8). It is unlikely that this discrepancy is caused by differences in patient populations, since the subgroup analysis in the current study showed no association with specific subgroups of patients. Population stratification can not be excluded although both groups had a similar ethnic background and showed a similar distribution of CD1 gene polymorphism frequencies. Differences may partly be explained by the methods of statistical analysis (15,16). In the current study, two-sided tests were used because we were unable to predict *a priori* whether a certain CD1 gene polymorphism was related to an increased or decreased susceptibility to develop GBS. Most likely, the sample size of the studies has influenced the findings. Studies based on smaller number of patients may have more frequent chance associations. Although the current study population was almost five-times larger, even 524 patients and controls is a relatively small number for candidate gene association studies. Confirmation in more extensive studies is required that fulfill the criteria put forward by the NCI-NHGRI working group (17). Given the relative rarity of GBS, this can only be accomplished by substantial international collaboration.

CD1 molecules are key players in immune responses to glycolipids and may be involved in the pathogenesis of GBS, especially in patients with antecedent *C. jejuni* infections and crossreactive anti-ganglioside antibodies. CD1b binds to GM1 on antigen presenting cells and may induce GM1-specific T-cell activation independent from CD1e (5,8). SNPs in the CD1B, CD1C and CD1D genes were not determined in the current study since these are either very rare and/or silent (6). Sequencing of the nucleotides that encode amino-acids directly involved in binding antigens in the CD1 groove may possibly reveal unknown mutations. Recently, a naturally occurring mutation in CD1E has been reported that influences antigen presentation via CD1b (18). The absence of association with CD1 gene polymorphisms does not exclude the possibility that CD1 molecules play an important role in the pathogenesis of GBS. Further research is needed to determine whether CD1 molecules or pathways downstream of CD1 are involved in the process of antigen presentation of glycolipids in GBS.

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4.2

Sialylation of lipo-oligosaccharide from Guillain-Barré syndrome-related *Campylobacter jejuni* modulates DC and B-cell responses

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ABSTRACT

In Guillain-Barré syndrome (GBS), ganglioside mimicry of *Campylobacter jejuni* lipo-oligosaccharide (LOS) drives the production of cross-reactive antibodies to peripheral nerve gangliosides. The mechanism for this aberrant humoral immune response to *C. jejuni* in GBS is unknown. Here we address whether human dendritic cell (DC) activation and subsequent B-cell proliferation is modulated by sialic acid residues in GBS associated *C. jejuni* LOS. Highly purified sialylated LOS of *C. jejuni* isolates from GBS patients induced human DC maturation and secretion of inflammatory cytokines that was mediated by TLR4. The extent of TLR4 signaling and DC activation was higher with LOS from wild type isolates than with non-sialylated LOS of the corresponding *sialyltransferase* gene knockout (*cst-II* mutant) strains, indicating that sialylation boosts the DC response to *C. jejuni* LOS. Supernatants of LOS-activated DCs induced B-cell proliferation after cross-linking of surface immunoglobulins in absence of T-cells. Lower B-cell proliferation indices were found with DC supernatants after stimulation with *cst-II* mutant or neuraminidase desialylated LOS. In serum from *C. jejuni*-related GBS patients (n=27), antibody activity to sialylated LOS was significantly higher compared to *C. jejuni* enteritis patients without GBS (n=20) or healthy controls (n=30). This study shows that sialylation of *C. jejuni* LOS enhances human DC activation and subsequent B-cell proliferation, which may explain the development of cross-reactive anti-ganglioside antibodies found in GBS patients following *C. jejuni* infection.

INTRODUCTION

The Guillain-Barré syndrome (GBS) is a post-infectious neuropathy characterized by rapidly progressive muscle weakness. In its most severe form, patients have a paralysis of all cranial, limb and respiratory muscles for which they need mechanical ventilation for months. Various types of infection may precede GBS, but *Campylobacter jejuni* is the predominant cause, especially in patients with extensive weakness (1,2). Patients with *C. jejuni*-related GBS frequently have serum antibodies to human peripheral nerve gangliosides, which induce multiple neurotoxic effects after passive transfer to mice (3,4). *C. jejuni* triggers the production of anti-ganglioside antibodies in these patients by molecular mimicry. More specifically, some *C. jejuni* strains express lipo-oligosaccharides (LOS) with similar sialic acid (*N*-acetyl-neuraminic acid) carbohydrate moieties as present in gangliosides (5,6), resulting in the production of cross-reactive antibodies (7). Rabbits sensitized with GM1-like *C. jejuni* LOS produce cross-reactive anti-ganglioside antibodies and develop a neuropathy and flaccid paresis similar to patients (8). The variation in ganglioside mimicry is controlled by the sialyltransferase *cst-II* gene, which is associated with *C. jejuni* from GBS patients (9, 10). LOS sialylation is therefore a key factor in the development of GBS after *C. jejuni* infection.

In cases with uncomplicated *C. jejuni* gastroenteritis not leading to GBS, however, the antibody response to LOS is usually very low or even undetectable (6). The mechanism responsible for the induction of a high antibody response to LOS in GBS is unknown. We hypothesize that the immunogenicity of LOS during *C. jejuni* infection, which is required to induce the high titer cross-reactive antibodies to gangliosides in GBS, also depends on the sialylation of the LOS. Sialylated LOS is more frequently found in *C. jejuni* isolates from GBS patients than in isolates from enteritis controls, and sialic acids are known to modulate the immune response in other types of infection (6,11). This process may be controlled by dendritic cells (DCs), which constitute one of the first lines of mucosal immune defense and are pivotal in bridging innate and adaptive immunity (12). In the present study, the maturation and cytokine production of human DCs in response to sialylated LOS from three GBS-related *C. jejuni* isolates was compared to the DC response to non-sialylated LOS from the corresponding *cst-II* mutant strains. In addition, the proliferation of human tonsillar B-cells in response to soluble factors from these activated DCs was determined. Our study shows that the sialylation of *C. jejuni* LOS modulates the DC activation which subsequently contributes to stronger proliferation of naïve mucosal B-cells.

RESULTS

High antibody titers to sialylated *C. jejuni* LOS in serum from GBS patients

To demonstrate that the production of antibodies to sialylated LOS is related to the development of GBS, acute phase serum samples were tested from GBS patients with a preceding *C. jejuni* infection (n=27), enteritis controls with a *C. jejuni* infection but no GBS (n=20) and healthy controls without a recent *C. jejuni* infection (n=30). The LOS for this serological study was purified from the GB11 wild-type (WT) strain, a *C. jejuni* isolated from a patient with GBS,

which is highly sialylated and mimics the gangliosides GM1 and GD1a (Table 1). High titers of IgM and IgG antibodies to the LOS from the GB11 WT were found exclusively in the serum from patients with GBS (Figure 1A). Based on the extinctions for anti-LOS antibodies found in the healthy control group, cut-off values for positivity were defined (Figure 1A). In the group of GBS patients, 18 (67%) were positive for anti-LOS IgG and 13 (48%) for anti-LOS IgM. These results show that only in GBS patients the *C. jejuni* infections had resulted in significant antibody responses to sialylated LOS.

Next, we determined whether these antibodies in the serum from GBS patients are directed to the sialic acid residues in *C. jejuni* LOS by performing adsorption studies with the non-sialylated LOS from the corresponding *cst-II* knockout mutant strain. *Cst-II* is a sialyltransferase that is essential for the synthesis of α(2-3)- and α(2-8)-linked sialic acids to galactose in the outer core of LOS from *C. jejuni* (10). The GB11 *cst-II* knockout mutant strain expresses a truncated LOS outer core without sialic acids and lacks ganglioside mimicry (Table 1) (5,10). IgG antibody activity to GB11 WT LOS in serum from GBS patients was inhibited much more efficiently when pre-incubated with LOS from the same strain (median 96% inhibition), than with LOS from the GB11 *cst-II* mutant strain (median 37% inhibition) (p=0.002) (Figure 1B). These results confirm that the high antibody activity to *C. jejuni* LOS in patients with GBS is caused by an immune response mainly directed to the sialylated moieties in LOS.

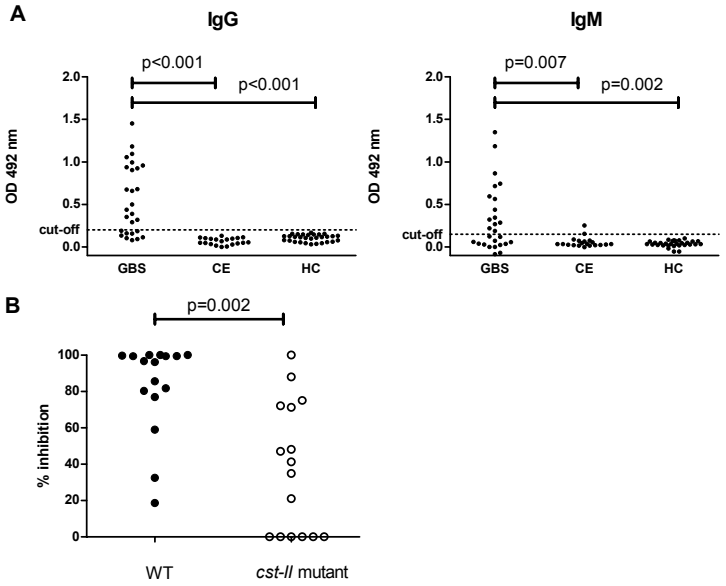

Figure 1. High serum reactivity to *C. jejuni* LOS in GBS patients due to sialylation of LOS.
(A) Serum antibody activity to sialylated LOS from GB11 WT, a *C. jejuni* isolate from a GBS patient, was significantly higher in acute phase serum from *C. jejuni* related GBS patients than in acute phase serum from *C. jejuni* enteritis patients without GBS or healthy controls. **(B)** Absorption of IgG antibody activity, expressed as percentage of inhibition, to LOS from GB11 WT in serum samples from GBS patients by pre-incubation with 100 µg/ml LOS was significantly lower with LOS from the GB11 *cst-II* mutant strain, indicating that the antibodies recognize the sialic acids in LOS from GB11 WT.

Table 1. Carbohydrate outer core structure of LOS from *C. jejuni* strains isolated from GBS patients used in the current study[a].

Strain(s)	LOS structure	Ganglioside mimic
GB2-, GB11 WT	Gal-GalNAc-Gal-Hep-Hep Neu5Ac Glc	GM1
	Gal-GalNAc-Gal-Hep-Hep Neu5Ac Neu5Ac Glc	GD1a
GB19 WT	Gal-GalNAc-Gal-Hep-Hep Neu5Ac Glc Glc Neu5Ac	GD1c[b]
GB2-, GB11-*cst-II* mutant	Gal-GalNAc-Gal-Hep-Hep Glc	none
	GalNAc-Gal-Hep-Hep Glc	none
	Gal-Hep-Hep Glc	none
GB19-*cst-II* mutant	Gal-GalNAc-Gal-Hep-Hep Glc Glc	none

[a] Molecular structures were determined by mass spectrometry and previously published (7).
[b] GD1c is structurally similar to GD3. Neu5Ac: *N*-acetylneuraminic acid, Gal: galactose, GalNAc: *N*-acetyl galactosamine, Hep: heptose, WT: wildtype, *cst-II: sialyltransferase* knockout mutant.

C. jejuni LOS induces DC activation through TLR4

To further explore the human immune response to *C. jejuni* LOS, DC derived from peripheral blood monocytes from healthy blood donors were cultured in the presence of heat-inactivated *C. jejuni* whole bacteria. The three *C. jejuni* WT strains isolated from GBS patients were cultured with DC in multiplicities of infection (MOI) ranging from 1:2 to 1:100. MOIs of 1:2 already induced upregulation of the DC surface-expressed co-stimulatory molecules CD80, CD86, CD40 and HLA-DR, indicating cellular activation (Figure 2A). In agreement with these observations, highly purified LOS from these *C. jejuni* WT strains also induced a dose-dependent up-regulation of CD80 on these DCs from a starting concentration of 0.1 ng/ml (Figure 2B). Activation of DCs was also reflected by secretion of inflammatory cytokines in the supernatant (Figure 3A). After stimulation of DCs with *C. jejuni* LOS there was a dose-dependent increase in supernatant levels of IL-6, IL-8, IL-10, IL12p40 and TNF-α (Figure 4B).

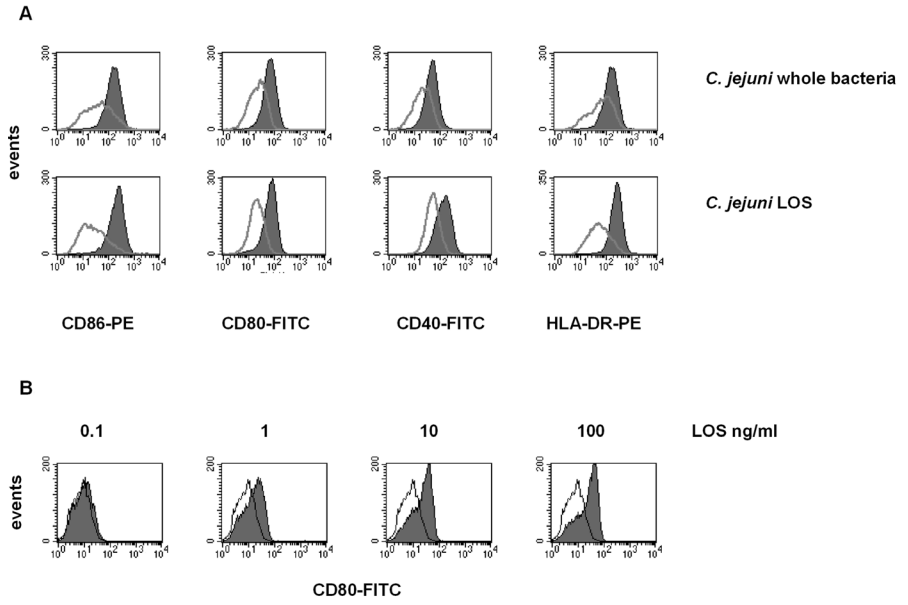

Figure 2. Human DCs are dose-dependent activated by *C. jejuni* whole bacteria and *C. jejuni* LOS.
(A) Immature DCs (10^5 cells/well) were incubated for 18 h with either heat-inactivated *C. jejuni* GB11 WT whole bacteria at a MOI of 1:10 (top panel) or 100 ng/ml of purified LOS from the same strain (lower panel). Cells were stained with mAbs to the co-stimulatory molecules CD86, CD80, CD40 as well as to HLA-DR to determine DC activation. Histogram represents unstimulated DCs (void histogram) and DCs stimulated with *C. jejuni* whole bacteria or LOS (filled histogram). Representative results of one of 15 experiments. **(B)** DCs were stimulated with increasing concentrations of LOS from *C. jejuni* GB19 WT. Upregulation of CD80-FITC indicating DC activation was observed at 0.1 ng/ml and increased further at higher concentrations of LOS. Void histogram represents unstimulated cells and grey histograms LOS stimulated cells. Representative results of one of 15 experiments.

DC activation by microbial patterns is mediated through toll-like receptors (TLRs) expressed at the cell-surface or intra-cellular (13). Interactions between microbial antigens and TLR4 lead to downstream activation of NF-κB that regulates the transcription of pro-inflammatory cytokines such as TNF-α, IL-8 and IL-12p40 (14). TLR4 is a member of the pattern-recognition receptor family (PRR) and binds to endotoxin or lipid A present in LPS from gram-negative bacteria. The *C. jejuni* LOS is devoid of the repetitive oligosaccharides present in the O-specific chain of LPS but it does contain the evolutionary conserved lipid A structure. Therefore, it is presumed that LOS may also induce DC activation through TLR4. To test this, DC were cultured with a neutralizing mouse anti-human TLR4 mAb during LOS stimulation. Neutralization of TLR4 inhibited *C. jejuni* LOS induced secretion of IL-12p40 by DCs (Figure 3B). In addition, DC activation and secretion of IL-6, IL-8, IL-10, and TNF-α were also attenuated by the anti-TLR4 mAb. In sum, human DC activation occurs at low concentrations of *C. jejuni* LOS and is partly mediated through TLR4 leading to induction of cytokine secretion.

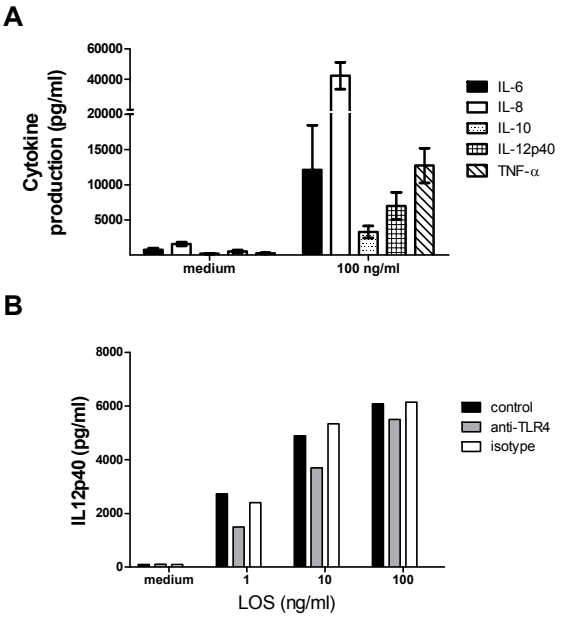

Figure 3. DCs secrete inflammatory cytokines partially through TLR4 upon stimulation with *C. jejuni* LOS. **(A)** DCs stimulated with 100 ng/ml LOS from *C. jejuni* GB11 WT or medium as control were harvested after overnight incubation. Mean cytokine levels (10 experiments) in DC supernatants ± standard errors. **(B)** DCs were stimulated with LOS from *C. jejuni* GB11 WT (control) and incubated concomitantly with a neutralizing mouse anti-humanTLR4 mAb or an isotype control mAb.

C. jejuni LOS carbohydrate moiety modulates DC activation

To assess whether the carbohydrate moiety of *C. jejuni* LOS influences the DC response, LOS from the three WT strains and corresponding *cst-II* mutant strains were compared for their ability to induce DC activation and secretion of cytokines. The expression of surface co-stimulatory molecules on DCs was higher after incubation with sialylated LOS from the GB11 WT strain as compared to non-sialylated LOS from the GB11 *cst-II* mutant strain (Figure 4A). A similar higher expression of these markers on DCs was found after stimulation with LOS from GB2 and GB19 WT compared to LOS from the GB2 and GB19 mutant strains. In addition, the secretion of pro- and anti-inflammatory cytokines was significantly higher when DCs were stimulated with LOS from WT strains compared to their *cst-II* mutant strains (Figure 4B). An approximately 10-fold higher concentration of LOS from the GB11 *cst-II* mutant compared to the GB11 WT was needed to obtain similar levels of induced cytokine secretion. The *cst-II* mutants of two other GBS-associated tested *C. jejuni* isolates showed similar results (Figure 4B, last panel). These data indicate that the carbohydrate moiety of *C. jejuni* LOS indeed modulates the human DC activation and cytokine secretion.

Sialylation of *C. jejuni* LOS modulates DC response

The observations presented above showed that the carbohydrate structure of LOS modulates the DC response and suggest that the sialylation of LOS mediated this effect.

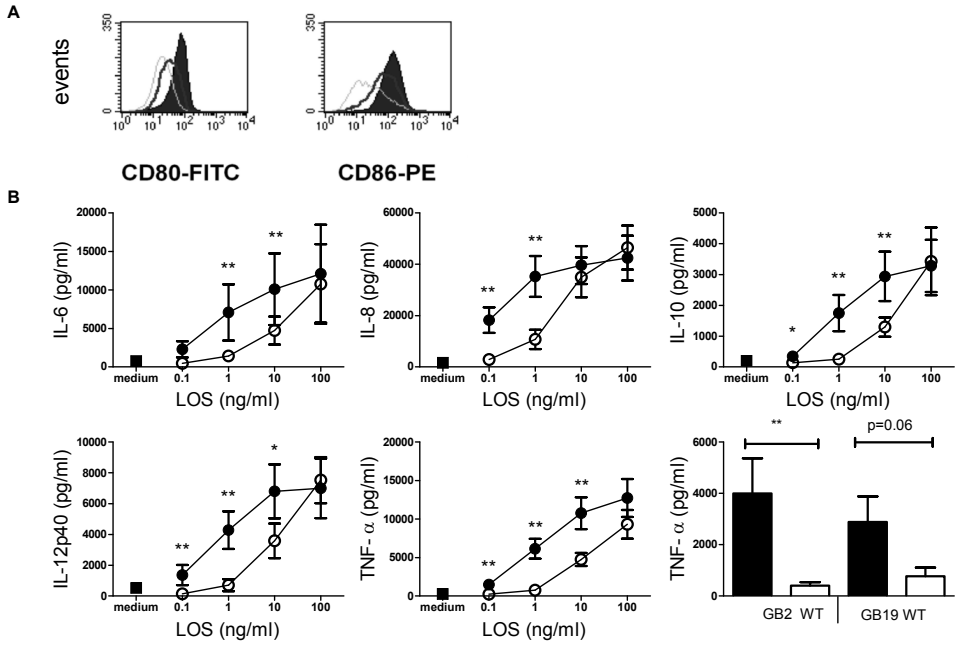

Figure 4. Sialylation of *C. jejuni* LOS increases DC activation and cytokine secretion.
(A) DCs were stimulated with 1 ng/ml LOS from *C. jejuni* GB11 WT (filled purple histogram), GB11 *cst-II* mutant strain (dark gray overlay histogram) or not stimulated (light gray overlay histogram). After overnight incubation DCs were harvested and surface expression of CD80 and CD86 was determined by flowcytometry. **(B)** DCs were incubated overnight with a concentration range of 0.1 to 100 ng/ml of LOS from GB11 WT (filled data points) and GB11 *cst-II* mutant strains (void data points). Cytokine levels in supernatant were determined by ELISA. Filled data points represent GB11. In the last panel, 1 ng/ml of LOS from GB2 WT and GB19 WT (black bars) were also compared with their corresponding *cst-II* mutant strains (void bars). Wilcoxon-signed rank tests were used for statistical comparisons, * $P<0.05$; ** $P<0.005$.

To further determine the modulating effects of sialylation, LOS from the *C. jejuni* GB19 WT was incubated with neuraminidase from *Arthrobacter ureafaciens*, cleaving off the α(2-8)-linked- and terminal α(2-3)-linked sialic acid residues. The desialylation of LOS from the GB19 WT by this neuraminidase treatment (GB19 WT NA) was confirmed by showing that the binding of a mAb to GD3 was lost, while instead the LOS gained reactivity to a serum with antibodies to asialo-GM1 (Supplemental figure S1). This LOS was subsequently incubated overnight with DCs and compared with LOS from GB19 WT and *cst-II* mutant strains. The DC activation and TNF-α secretion after incubation with LOS from the GB19 WT NA was lower than after incubation with LOS from the GB19 WT, and just as low as after incubation with LOS from the GB19 *cst-II* mutant (Figure 5A-B). The secreted levels of IL-6, IL-8, IL-10 and IL-12p40 were also lower, similar to the levels seen after stimulation with LOS from the *cst-II* mutant. These data demonstrate that the DC activation is enhanced by sialylation of the LOS from *C. jejuni.*

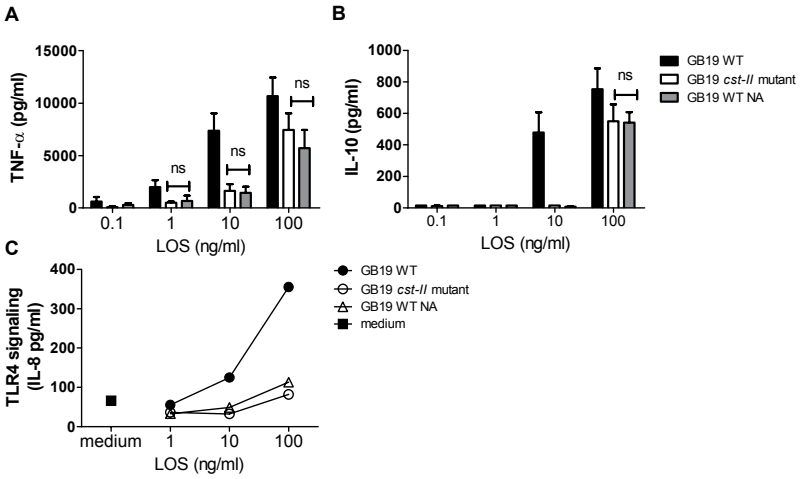

Figure 5. Neuraminidase treatment of *C. jejuni* LOS attenuates DC activation.
LOS from *C. jejuni* GB19 WT was incubated at 37 ºC for 3 h with 0.8 U/ml of neuraminidase (NA). After inactivation of neuraminidase, this desialylated LOS (GB19 WT NA) was incubated overnight with immature DCs or HEK/TLR4 cells (10^5 cells/ml). The effects were compared with those observed after incubation with LOS from GB19 WT and GB19 WT *cst-II* mutant strains. TNF-α and IL-10 levels in DC cell media **(A and B)** and IL-8 levels (pg/ml) in HEK/TLR4 cell media **(C)**, as a measure for TLR4 signaling, were determined by ELISA. TNF-α and IL-10 levels represent pooled data from 5 separate experiments, error bars represent standard errors. TLR4 signaling are mean results from 2 experiments. Ns: not significantly different.

To assess whether the enhanced cell activation by sialylated LOS was specifically due to differences in TLR4 signaling, a TLR4 transfected cell line (HEK293/TLR4) was used that secretes IL-8 in response to TLR-4 ligation (14). Non-sialylated LOS from both the GB19 *cst-II* mutant and the GB19 WT NA induced significant lower IL-8 levels compared to LOS from the GB19 WT (Figure 5C). These data suggest that the sialylation of *C. jejuni* LOS modulates activation of DCs through differences in LOS/TLR4 signaling.

Sialylation of *C. jejuni* LOS influences proliferation of naïve B-cells through DC-derived soluble factors

Supernatants from DCs stimulated with microbial products are known to enhance B-cell responses (15). In light of the previous observations, we hypothesized that the DC activation by *C. jejuni* may also affect B-cell responses. A proliferation assay was designed in which human tonsillar B-cells were cultured in the presence of DC-derived supernatant. These mucosal B-cells were stimulated with low concentrations of goat-anti-human-IgM to cross-link B-cell receptors (BcRs), mimicking antigen-recognition as a first signal for B-cell activation. Stimulation of B-cells with *C. jejuni* LOS alone up to concentrations of 10 μg/ml had no effect, consistent with the absence or low TLR4 expression in human non-activated B-cells (16). Next, B-cells were cultured in the presence of supernatant from DCs previously stimulated with *C. jejuni* LOS, which resulted in an enhancement of B-cell proliferation (Figure 6A). Because centroblasts previously activated *in vivo* may proliferate without

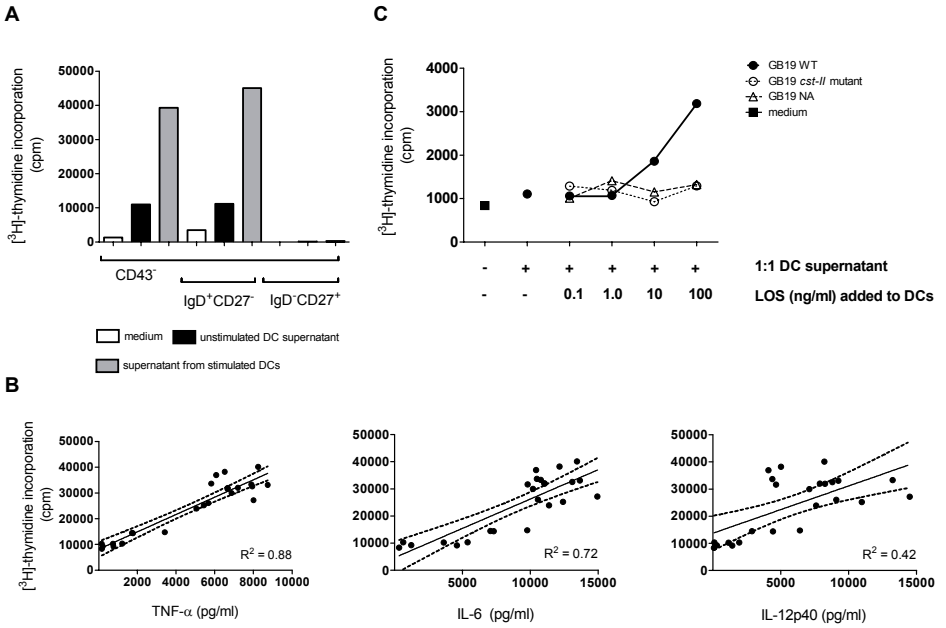

Figure 6. B-cell proliferation is modulated by LOS sialylation, DC-derived solubles and correlates with cytokine levels.

(A) Human tonsils were depleted of $CD43^+$ cells by magnetic labeling. The cells were further sorted by flowcytometry into IgD^+CD27^- and IgD^-CD27^+ fractions. B-cells ($4*10^6$ cells/ml) were cultured on plates coated with polyclonal anti-IgM in the presence or absence of 1:1 supernatant from DCs. Supernatant used to stimulate B-cells was used from various experiments in which DCs were either unstimulated or stimulated with *C. jejuni* LOS in different concentrations. After 48 h co-culture, proliferation was determined by (^{3}H)-thymidine incorporation. **(B)** DCs were stimulated with LOS from different *C. jejuni* strains (GB2, GB11, GB19 WT and *cst-II* mutants) at concentrations ranging from 0.1 to 100 ng/ml. DC supernatant was used to stimulate B-cells. B-cell proliferation was assessed by ^{3}H-thymdine incorporation. Linear regression lines with 95% confidence intervals (dotted) are shown. Spearmann correlations were significant at $p<0.0001$ for TNF-α and IL-6 and at $p=0.002$ for IL-12p40. Results are from one out of three representative experiments. **(C)** DCs were stimulated with sialylated LOS from GB19 WT or with non-sialylated LOS from GB19 *cst-II* mutant strains or from GB19 WT treated with neuraminidase (GB19 WT NA). A range of LOS concentrations was used to demonstrate a dose dependent effect. Supernatant from DCs stimulated with GB19 WT LOS induced higher B-cell proliferation compared to GB19 *cst-II* mutant strain and GB19 WT NA LOS. Representative results from one of two experiments.

further exogenous stimulation, flowcytometric sorting of naïve IgD^+CD27^- and IgD^-CD27^+ centroblasts cells was performed. Comparison of the two subsets derived from $CD43^-$ cells showed that the naïve B-cell population ($CD43^-IgD^+CD27^-$) proliferated in the presence of DC supernatant (Figure 6A). The extent of B-cell proliferation correlated with the cytokine response and activation of DCs by LOS (Figure 6B), and increased dose-dependently with the LOS concentrations (Figure 6C). Supernatants from DC cultures stimulated with desialylated LOS from GB19 WT NA and GB19 *cst-II* mutant were less effective in enhancing B-cell proliferation (Figure 6C). These results demonstrate that *C. jejuni* LOS influence mucosal B-cells proliferation through DC activation, even without cell-cell interactions between DCs

and B-cells. Sialylated LOS from *C. jejuni* enhances this proliferation of B-cells through activation of DCs.

Contributing factors for DC-induced B-cell proliferation

In order to determine which DC-derived soluble factor(s) enhances B-cell proliferation, inhibition studies were performed for candidate molecules. Heating the supernatant up to 80 °C abrogated its stimulatory effect. This confirmed our previous observation that the thermo stable LOS does not interact directly with B-cells. The observed thermo-instability suggests that temperature sensitive molecules such as proteins may be involved. In addition, freeze thawing had mild attenuating effects. Next, the role of candidate cytokines known to influence B-cells was investigated. Supplementing culture media with neutralizing antibodies to IL-6 and IL-12 did not inhibit or abrogate the effect (Supplemental figure S2). These results imply that the enhanced B-cell proliferation orchestrated by *C. jejuni* stimulated DCs is likely induced by heat instable factors and do not seem to be the result of common B-cell stimuli, such as IL-6 and IL-12 (15,17).

DISCUSSION

The current study demonstrates that the sialylation of *C. jejuni* LOS enhances human DC activation via TLR4 signaling and that supernatants from activated DCs induce proliferation of mucosal B-cells. *C. jejuni* strains isolated from GBS patients more frequently express sialylated LOS than *C. jejuni* strains from patients with uncomplicated gastro-enteritis (6). The sialylated outer core of *C. jejuni* LOS determines the specificity of the cross-reactive antibodies to gangliosides and thereby the site of nerve damage and clinical phenotype in GBS. The current study shows complementary that sialic acids in *C. jejuni* LOS boost DC activation and stimulate the subsequent B-cell response. This enhancement may explain the high antibody activity to sialylated LOS in serum from patients who develop GBS after a *C. jejuni* infection. Breaking of the natural tolerance to host gangliosides may result in a cross-reactive antibody response to peripheral nerves and subsequent neuropathy.

C. jejuni infections are usually resolved before an adaptive immune response is mounted (18), indicating a key role of the innate immune response in clearing the infection. *C. jejuni* strains expressing sialylated LOS have been found to invade intestinal epithelial cells significantly better as compared to strains with non-sialylated LOS (19). DCs reside directly under the epithelial cell layer are therefore well positioned to interact with both invasive but also with non-invasive pathogens (20). Previous studies showed that *C. jejuni* are readily internalized by DCs and induce cell maturation and cytokine production (21). In agreement with these findings in DCs, the current study demonstrates that LOS from GBS-related *C. jejuni* strains induced an up-regulation of co-stimulatory cell surface markers, including CD40, CD80, CD86 and HLA-DR, and secretion of inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-10, IL-12p40, and TNF-α. Interestingly, high levels of these cytokines are also found in serum from patients in the acute stage of GBS (22,23).

For most bacteria, surface sialic acids provide protection from host innate defenses by mimicry of host glycoconjugates found on cell surfaces and in mucus. In *Neisseria meningitidis,*

sialylation of LOS results in a reduced phagocytosis by DCs without influencing cytokine secretion (24). In contrast, sialylation of *N. meningitidis* LOS resulted in enhanced siglec-dependent macrophage-mediated phagocytosis (25). In addition, *Haemophilus influenzae* mutant strains expressing non-sialylated LOS induce an attenuated response in an *in vivo* model of otitis media in chinchillas (11). Sialic acids, therefore, do not always function as anti-recognition molecules and may in fact also sterically impede microbial-host interactions (26). The present study clearly shows that removal of sialic acids in LOS of *C. jejuni* by neuraminidase treatment or site-directed mutagenesis of the sialyltransferase *cst-II* results in attenuated DC activation and subsequent B-cell proliferation. This could explain the usually low antibody response to non-sialylated LOS after uncomplicated *C. jejuni* gastro-enteritis.

TLR4 engages LOS via lipid A, which leads to secretion of several inflammatory cytokines through activation of NF-κB transcription (14). Previous studies demonstrated NF-κB transcription in human DCs upon stimulation with *C. jejuni* LOS (21). Accordingly, the observed secretion of IL-12p40 and other cytokines induced by GBS-associated *C. jejuni* LOS in the current study was reduced when binding to TLR4 was prevented. HEK/TLR4 cells stimulated with *C. jejuni* LOS secreted high levels of IL-8, further indicating that cellular activation is mediated by direct interaction of *C. jejuni* LOS with TLR4. Interestingly, IL-8 production by HEK/TLR4 cells was significantly reduced after stimulation with non-sialylated LOS from GB19 WT after neuraminidase treatment or the corresponding *cst-II* mutant strain. This finding suggests that sialic acids in *C. jejuni* LOS enhance the TLR4 signaling. Besides, gangliosides alter the expression of TLR4 in microglia and astrocytes and transcription of inflammatory cytokines after exposure to gangliosides (27). Sialic acids in glycoconjugates may directly interact with TLR4 or other adjacent receptors forming functional units or influence the conformation of LOS in such way that lipid A more efficiently binds to TLR4. Human DCs also express sialic acid binding Ig-like lectins (siglecs) that bind to *C. jejuni (*28*)*, which may play a critical role in the efficiency of TLR4 signaling after stimulation with *C. jejuni* with sialylated LOS.

Activation of B-cells is required to produce a cross-reactive antibody response to gangliosides and is an essential step in the pathogenesis of *C. jejuni* -related GBS. The relatively low affinity of the anti-ganglioside antibodies and the absence of sustained serum titers in GBS (3), may be compatible with a T-cell independent immune response. Recently, a mechanism was described in which activated DCs provide direct B-cell help in absence of T-cells (15, 17,29-31). Our observation that *C. jejuni* LOS-activated DCs induce proliferation of human tonsillary B-cells via soluble factors, in which sialylation of LOS further enhances this effect, suggests a comparable mechanism for the antibody response to infections with *C. jejuni*. Co-incubation with blocking monoclonal antibodies to IL-6 or IL-12, or a combination of both, did not inhibit this B-cell proliferation. In a previous study in which DCs were stimulated with *E. coli* LPS, neutralizing monoclonal antibodies to IL-6 and IL-12 abolished a similar effect on B-cells (15). However, in this study T-cell help was also added to DC supernatant. DCs can directly induce T-cell independent proliferation and class-switching in B-cells via expression of B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) and skew adaptive immune responses by TLR-dependent mechanisms (30,31). Further studies are required to demonstrate which soluble factors are responsible for the proliferation of B-cells by supernatants from *C. jejuni* -activated DCs and whether B-cells show gene rearrangements

and maturate into antibody producing plasma cells in this particular context.

In conclusion, our study shows that sialylation of LOS in *C. jejuni* is a key determinant in the extent of human DC activation and subsequent B-cell proliferation. This feature of *C. jejuni* LOS may explain the high titers of serum antibodies to LOS found in patients with GBS that cross-react with peripheral nerve gangliosides.

MATERIALS AND METHODS

Patients and controls

Pre-treatment, acute phase serum samples were obtained from 27 GBS patients who participated in a previous clinical trial (32). All these patients fulfilled the diagnostic criteria for GBS (33) and had preceding diarrhoea and a positive serological for a recent *C. jejuni* infection (34). As controls, acute phase serum samples from 20 patients with culture proven *C. jejuni* gastro-enteritis without GBS were used, as well as serum samples from 30 healthy blood donors. All experiments were performed according to the guidelines of the medical ethical committee of the Erasmus MC and all patients had signed informed consent.

Cell cultures

Peripheral blood mononuclear cells (PBMC) from healthy volunteers were isolated by density gradient (Lymphoprep) centrifugation. Monocytes were isolated by positive selection using CD14-microbeads (Miltenyi Biotec) according to the manufacturer's instructions. To obtain monocyte-derived DCs, monocytes were cultured in 6-well plates for 6 days in the presence of GM-CSF (800 U/ml, Novartis) and IL-4 (400 U/ml, R&D systems). Immature monocyte-derived DCs were washed, seeded at 1 x 10^6 cells/ml in 96-wells plates and stimulated with either heat-inactivated *C. jejuni* whole bacteria or purified *C. jejuni* LOS fractions for 18 h. Neutralizing mAb to TLR4 (clone HTA125) and isotype control mIgG2a (both used at 25 μg/ml) were purchased from eBioscience. Human tonsils were obtained from children that underwent routine tonsillectomy at the local Sophia Children's Hospital. Tonsils were crushed in a 100 μm nylon mesh and washed extensively in PBS. After isolation of mononuclear cells by density gradient (Lymphoprep), B-cells were isolated by depletion of $CD43^+$ cells with CD43-microbeads (Miltenyi Biotec). This procedure yielded a population with typically 15% (± 9%) $CD5^+$ cells and < 2% contaminating $CD3^+$ cells (denoted as naïve B-cells). FACS-sorting using a FACSAria cell sorter was performed to further separate this cell population into IgD^+CD27^- and IgD^-CD27^+ fractions. B-cells isolated from tonsils were cultured in triplicate in a 96-well plate supplemented with or without DC supernatant (1:1) in culture medium and Goat anti-Human IgM (Jackson Immunoresearch Laboratories). After 48 h cells were supplemented with 0.5 μCi ^{3}H-thymidine per well and harvested with an automated harvester after another 18 h. ^{3}H-thymidine incorporation in the newly synthesized DNA from proliferating B-cells was measured in a Beta-counter (Wallac MicroBeta®).

Bacteria and LOS purification

Three *C. jejuni* strains were isolated from different GBS patients (GB2, GB11 and GB19). The carbohydrate structure of the *C. jejuni* LOS core antigen has been designed previously

by mass spectrometry (Table 1) (5, 10). Mutagenesis of the *cst-II* target gene in GB2, GB11 and GB19 has been described before (5, 9, 19). The corresponding *cst-II* mutants express a truncated LOS outer core due to the absence of sialic acids and lack ganglioside mimicry (Table 1) (5, 10). LOS was purified by an extended protocol described from the original hot-phenol method (35) as described in the *S3 Materials and Methods*. It was excluded that non-specific differences in LOS were compared since similar purity yields in a commercial silver staining (Invitrogen) and mass spectrometry of all tested batches were observed (Supplemental figures S4 and S5).

FACS analysis
FACS-analysis was performed on a Becton Dickinson FACscalibur. The following mAbs were used: CD86-PE, CD80-FITC, CD40-FITC, HLA-DR-PE, CD11c-Cy5, CD5-FITC, CD19-APC, IgD-PE, CD3-FITC, CD27-APC and CD43-PE. All monoclonal antibodies were purchased from BD Biosciences-Pharmingen.

HEK293/TLR4 cells
HEK293/TLR4 cells were grown in T75 flasks and harvested upon confluence, generally after 6 days of culture. Cells (10^5 cells/ml) were then plated into 96-well plates at 100 µl/well and stimulated with LOS or control medium for 24 h.

Cytokine detection by ELISA
Cytokines present in DC supernatants, including IL-6, IL-8, IL-10, IL12p40 and TNF-α, was determined by a capture ELISA according to the manufacturers' guidelines (Biosource).

LOS and ganglioside ELISA
The LOS ELISA was performed as described previously (36). For determining the adsorption of IgG anti-LOS activity, LOS was incubated with serum (diluted 1:100) for 3 hours at 4ºC. The supernatants were centrifuged and tested for residual anti-LOS activity. Percentage of inhibition was defined as:

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

The anti-ganglioside ELISA was performed as previously described (7).

Statistics
Significant differences in DC activation, cytokine production and B-cell proliferation were evaluated by non-parametric Wilcoxon-signed rank tests or Mann-Whitney U tests (GraphPad Prism 5 software). A p-value of <0.05 was considered to be significant.

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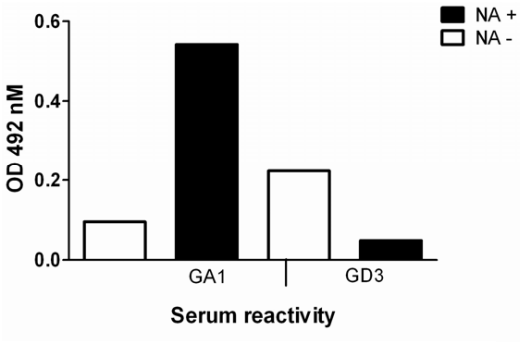

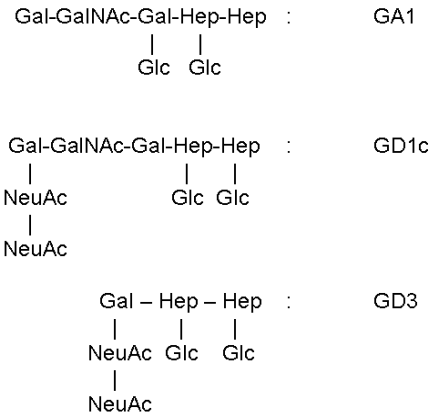

Figure S1. Effective enzymatic desialylation of LOS from C. jejuni GB19 WT.
LOS from GB19 WT shares with gangliosides GD1c and GD3 a terminal disialyl group (α(2-3)- and α(2-8)-linked Neu5Ac. LOS was incubated at 37ºC for 3 h with or without 0.8 U/ml of neuraminidase in sodium-acetate solution (pH 5.5). This enzymatic treatment resulted in effective desialylation of LOS, as indicated by loss of binding of an anti-GD3 mAb (TBG-3) and appearance of binding with serum from a patient with high titer anti-GA1 reactivity. Detection of serum reactivity to gangliosides was performed as described in the *Materials and Methods* section.

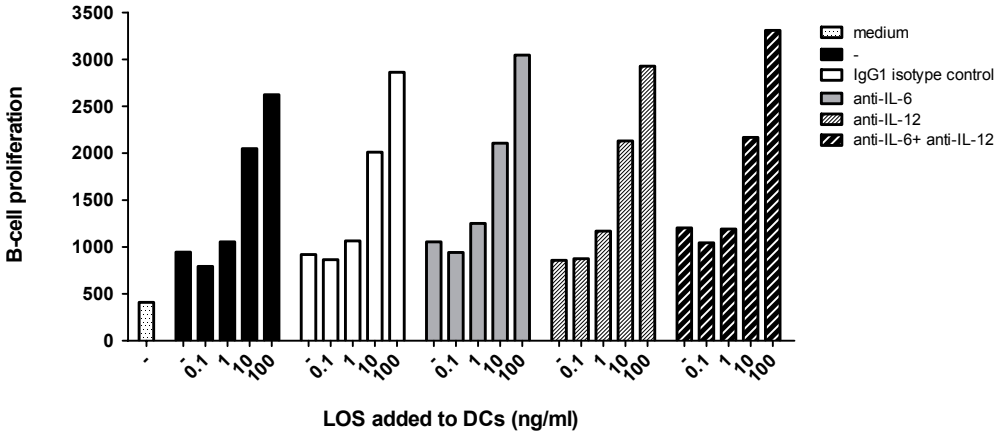

Figure S3. Neutralization of IL-6 and IL-12 does not affect the enhanced B-cell proliferation.
DCs were stimulated with various concentrations of LOS from GB11 WT, ranging from 0.1 to 100 ng/ml, after which supernatants were used to stimulate human CD43-depleted tonsillar B-cells. Neutralizing mouse mAb to human IL-6 (R&D systems, clone 6708, used at 5μg/ml), or to IL-12 (R&D systems clone 24910, used at 20 μg/ml), or a combination of both were added to the DC supernatants before incubation with the B-cells. The resulting B-cell proliferation, indicated by (^{3}H)-thymidine incorporation, did not differ from the situation when DC supernatant and an isotype IgG1 control mouse mAb (R&D systems clone 11711, used at 25 μg/ml) or when DC supernatant only was added to the B-cells. These results show that neutralization of IL-6 and IL-12 in the DC supernatant did not influence B-cell proliferation.

S3 Supplemental Material and Methods

Before LOS purification, 100 blood agar plates were inoculated with one of the six strains and cells were harvested in the log-phase. Next, bacteria were lyophilized and extracted three times with 90% hot-phenol (65ºC). The water phases were collected and combined. After dialysis with 3.5 kDa membranes, water phases were lyophilized again. LOS yield was determined and the material was treated at 37ºC with DNase (200 µg/ml, Sigma-Aldrich) and RNase (50 µg/ml, Sigma-Aldrich) subsequently followed by proteïnase K (1mg/ml, Sigma-Aldrich) treatment at 65ºC. After dialysis and lyophilisation, LOS yield was measured using a high precision balance.

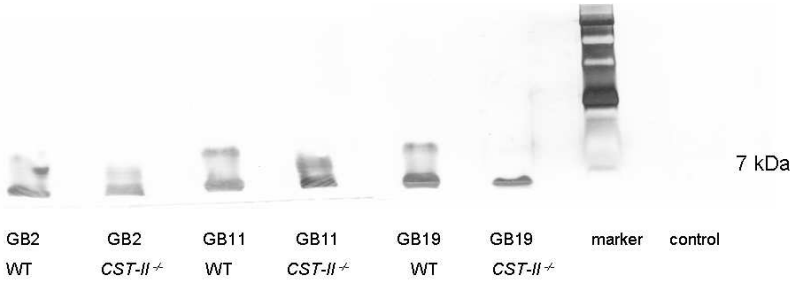

Figure S4. Purification of LOS from *C. jejuni* strains used in the study yields similar clean products.
LOS from *C. jejuni* isolates of GBS patients and corresponding *cst-II* mutant strains was purified by hot-phenol extraction and subsequent RNase and DNase treatments. No impurities were detected in a sensitive silver staining of SDS-PAGE gels loaded with large amounts of each LOS fraction (4 µg). LOS from the *cst-II* mutant strains is truncated and has a different degree of ionization and net charge and migrates faster in the gel than LOS from the WT strains.

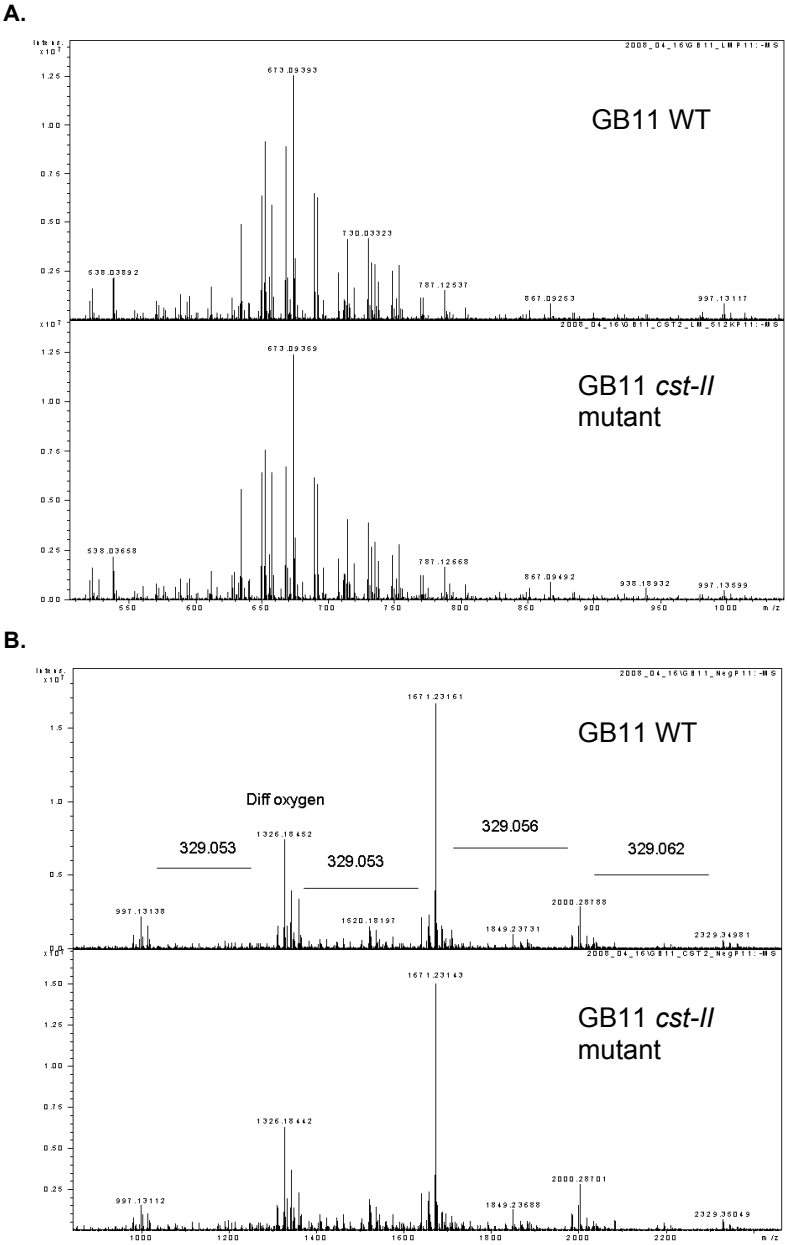

Figure S5.

(A) LOS from WT and cst-II do not contain significant amounts of contaminating peptides. Fourier Transform Mass spectrometry was performed in negative charge ionization mode. Peptide specific masses were not observed. Results in lower mass range (500 – 1000 Da) are visualized.

(B) Results in higher mass range (1000 – 2500 Da).

5

Serum anti-neural antibodies in GBS and other immune-mediated neuropathies

5.1

Reminder of important clinical lesson

Diagnostic value of anti-GQ1b antibodies in a patient with relapsing dysarthria and ataxia

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SUMMARY

Serum antibodies to the ganglioside GQ1b are associated with immune-mediated ophthalmoplegia and ataxia in patients with Miller Fisher syndrome (MFS) and Guillain-Barré syndrome. A patient with two clinically similar episodes of progressive bulbar signs, ophthalmoplegia and ataxia is reported here. During both episodes the patient required artificial ventilation. Serum anti-GQ1b antibodies were detected during the first episode compatible with MFS, but were absent during the second. Neuroradiological investigations during the second episode showed brain stem ischaemia and obstruction of the left posterior inferior cerebral artery. These findings illustrate that anti-GQ1b serology is a reliable and robust method that helped to distinguish between different causes of relapsing dysarthria and ataxia.

BACKGROUND

The clinical triad of ophthalmoplegia, ataxia and areflexia with a monophasic disease course is the classical description of Miller-Fisher syndrome (MFS) (1). However, patients with MFS often show only part of the triad, occasionally have other cranial nerve palsies or progress into the closely-related Guillain-Barré syndrome (GBS) if significant weakness of neck, shoulder and arm musculature is observed (1,2). In addition, relapses in MFS and GBS occur in up to 2% to 5% of patients, introducing a further diagnostic problem (3,4). When patients present with additional symptoms such as diplegia facialis, bulbar dysarthria or limb weakness, other causes should be considered in the diagnostic work-up. The presence of serum IgG and IgM antibodies to the ganglioside GQ1b may help to confirm or reject the diagnosis MFS, particularly when a patient with MFS has a recurrence of symptoms. We report a patient with relapsing dysarthria and ataxia in whom determination of serum anti-GQ1b antibodies helped to make the correct diagnosis. During the first episode the patient had MFS, but during the second episode the symptoms were caused by brain stem infarction.

CASE PRESENTATION

A vital 80-year-old man with a history of claudicatio intermittens developed double vision and unsteadiness of gait 1 week after a mild upper respiratory tract infection. Within 2 days he noticed difficulties with speech and swallowing. Neurological examination revealed a bilateral external ophthalmoparesis with normal light reactions, diplegia facialis and bulbar dysarthria with paresis of the pharyngeal muscles. Additionally, he had a symmetrical mild weakness of deltoid and biceps muscles, sensory ataxia, almost absent vibration sense and areflexia with normal plantar reflexes. The patient was unable to stand or walk unaided. The clinical symptoms were compatible with a diagnosis of MFS.

INVESTIGATIONS

This diagnosis was supported by the presence of an elevated cerebral spinal fluid protein content (0.77 g/litre, normal reference <0.58 g/litre) without pleiocytosis and a high serum antibody reactivity to GQ1b (IgG titre 3200 and IgM titre 1600). Cerebral CT scanning showed a small silent brain infarct in the left corona radiata.

TREATMENT

Immediately after admission the patient deteriorated and developed a paralysis of pharyngeal muscles followed by respiratory failure, for which he required mechanical ventilation. He was treated with a standard dose of intravenous immunoglobulins (0.4 g/kg/day for 5 days) after which he gradually improved.

OUTCOME AND FOLLOW-UP

At 4 weeks after admission the patient had a residual ataxia but was able to walk independently. The oculomotor movements also improved, leaving a mild bilateral ophthalmoparesis. The patient was discharged to a rehabilitation centre.

At 5 months later the patient developed a second episode with symptoms that were largely similar to the first episode. The patient again complained of progressive speech disturbances, double vision and unsteadiness of gait. This time the patient also complained of vertigo and nausea. The onset of this episode was possibly acute, although the symptoms fluctuated in severity and progressed within several hours. Neurological examination revealed normal consciousness and a residual external ophthalmoplegia with progression of impaired abduction on the right side without nystagmus. There was slight peripheral facial nerve palsy on the right. Bulbar dysarthria had worsened compared with the neurological examination at discharge. Visual fields were normal. The patient was unable to walk and showed respiratory distress. Tendon reflexes were absent and plantar reflexes were normal. Based on these findings, basilar artery thrombosis and recurrent MFS were considered as differential diagnoses. Cerebral CT scanning showed no new abnormalities compared to the CT scan of the previous episode. CT angiography showed occlusion of the intradural segment of the left vertebral artery (V4), compatible with acute thrombosis, and a normal basilar artery. Cerebral MRI showed a hypointensive area on T1 and a hyperintensive area on T2 in the left medulla oblongata, compatible with recent ischaemia due to occlusion of the left posterior inferior cerebellar artery (Figure 1). Moreover, anti-GQ1b serology was negative this time (Figure 2).

The patient rapidly developed respiratory failure again and required mechanical ventilation. He received a tracheostomy and was able to breathe independently after 3 weeks. He was discharged to a rehabilitation centre after 7 weeks, where he suddenly died of an unknown cause 2 months later.

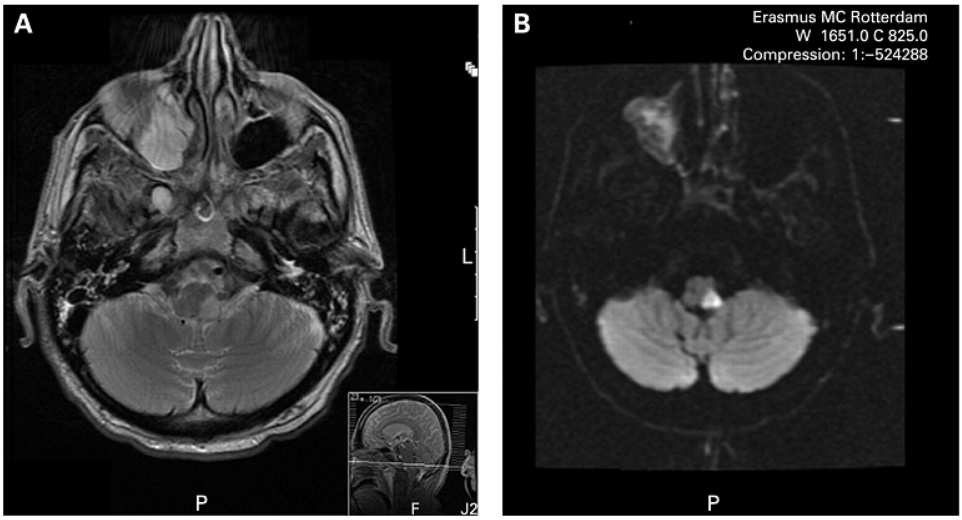

Figure 1. MRI scan of patient during second episode.
T2-weighted axial MRI shows a hypodense area in left brain stem corresponding to the drainage area of the posterior cerebellar inferior artery **(A)**. The ischaemic lesion is also visible in a diffusion weight MRI scan **(B)**.

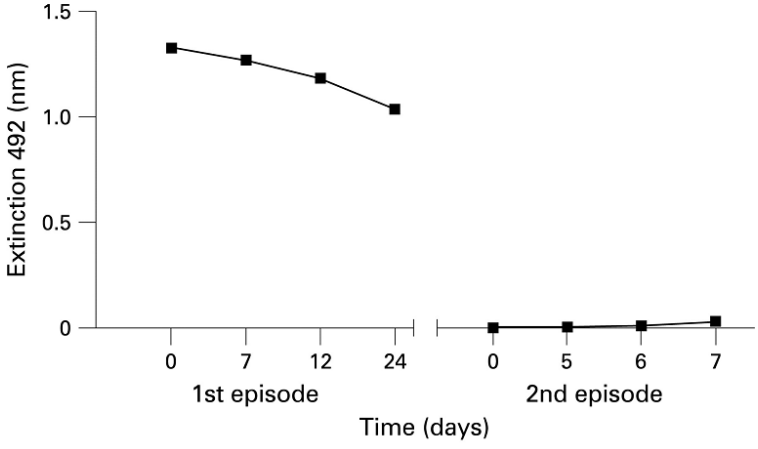

Figure 2. IgG serum reactivity to GQ1b in time during the first and second episode.
Serial measurements of serum reactivity to GQ1b in blood samples obtained during the first and second clinical episode.

DISCUSSION

This patient had two episodes within 6 months with almost similar clinical symptoms, but with different causes. The first episode was attributed to a MFS and the second to a brain stem infarction caused by occlusion of the left posterior inferior cerebellar artery. To our knowledge a relationship between MFS and brain stem infarction has not been described in the literature before. Moreover, in this patient the difference in pathogenesis was reflected by the high titres of serum anti-GQ1b antibodies in the first episode that were absent during the second.

Anti-GQ1b antibodies are specific for MFS and related syndromes such as GBS with ophthalmoplegia, CANOMAD (chronic acquired neuropathy associated with M protein, cold agglutinins and disialyl-ganglioside antibodies) and Bickerstaff encephalitis (5). Based on a series of patients from Japan, the sensitivity is higher than 95% for MFS and between 60 and 70% for Bickerstaff encephalitis (6,7). CANOMAD is defined by the presence of serum IgM antibodies to gangliosides with disialylated moieties, including GQ1b (5). The specificity of anti-GQ1b antibodies for these syndromes is also high (generally more than 90%). The GQ1b-related syndromes have in common that they are characterised by ophthalmoplegia and ataxia. This may suggest that in these patients anti-GQ1b antibodies interfere with normal function of the neurological circuits responsible for eye movement and coordination. High concentrations of GQ1b have been demonstrated in the extramedullar portion of the oculomotor nerve and positive immunostaining with monoclonal antibodies to GQ1b was found in large neurons of the dorsal root ganglia (6,8,9). In MFS, the antibodies are induced by preceding infections, including *Campylobacter jejuni* and *Haemophilus influenza*, which contain ganglioside-like moieties in lipo-oligosaccharides that induce a crossreactive antibody response (5,10). The precise cellular mechanism involved in this aberrant mucosal immune response and the breakdown of tolerance to self-antigens, however, is not well understood. In a mouse model, anti-GQ1b antibodies induced complement dependent disruption of presynaptic motor nerve terminals at the neuromuscular junction (11). These studies provide strong evidence that anti-GQ1b antibodies are pathogenic and are related to specific neurological deficits. Determination of serum anti-GQ1b antibodies in these patients may have important additional diagnostic value.

In general, serum IgG titres to ganglioside antibodies in GBS disappear after weeks to months as was also found in this patient. This unusual titre course may be a reflection of the aberrant immune response to infections in MFS. In this report, a standardised technique to determine anti-ganglioside antibodies was used. The interassay and intra-assay variation for this method has been validated and appears to be relatively robust for glycolipid assays (12,13). Anti-GQ1b serology is a reliable technique that can be used to perform serial measurements in patients with relapsing symptoms caused by different pathophysiological mechanisms. This may have diagnostic value in patients with relapsing symptoms caused by (postinfectious) immune-mediated polyneuropathies.

MFS is usually a monophasic illness but in rare instances recurrences may occur, even after long asymptomatic intervals (3). In the patient reported here, the symptoms of the second episode resembled the first. However, during the second episode there was possibly a more acute onset of symptoms and a fluctuating and progressive course. In addition, the patient also complained of vertigo, which is unusual in MFS. The time course and the vertigo is typical

of basilar artery occlusion (14). In patients with MFS and relapsing symptoms characteristic for MFS with negative anti-GQ1b serology, different causes than a MFS relapse should be considered.

LEARNING POINTS

- GQ1b serology is a reliable test to distinguish Miller Fisher syndrome from other causes of acute ophthalmoplegia and dysarthria.
- The classical triad of symptoms in Miller Fisher syndrome is often incomplete or complemented with other signs.
- Brain stem infarctions should enter the differential diagnosis in recurrent Miller Fisher syndrome.

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COMPETING INTERESTS

None.

PATIENT CONSENT

Patient/guardian consent was obtained for publication.

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5.2

Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA

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ABSTRACT

The Inflammatory Neuropathy and Treatment (INCAT) group developed a standardized ELISA method for the detection of serum anti-GM1 antibodies. The diagnostic value of anti-GM1 antibodies determined by this method has not yet been established in large groups of patients. We assessed the reproducibility, sources of variation, optimal cut-off values and evaluated the diagnostic relevance of the INCAT-ELISA in various groups of patients and controls (*N*=1232). The coefficient of variance was 11.2% for IgM and 3.8% for IgG. High IgG titers were only found in Guillain-Barré syndrome (GBS) and other inflammatory polyneuropathies. High IgM titers were associated with GBS and multifocal motor neuropathy. Low IgM titers had no additional diagnostic value. The INCAT-ELISA is a reliable test with additional diagnostic value in specific clinical situations.

INTRODUCTION

Determining antibodies to the ganglioside GM1 as a diagnostic marker for immune mediated neuropathies remains controversial because they have been reported in a wide spectrum of neurological diseases, autoimmune disorders and in healthy controls. The frequency of anti-GM1 antibodies in these groups of patients and controls also varies considerably between reports. These variations could largely be explained by differences in techniques used to detect anti-GM1 antibodies (1).

To facilitate the comparison of results from different laboratories a standardized enzyme-linked immunosorbent assay (ELISA) for measuring serum anti-GM1 antibodies was proposed by the Inflammatory Neuropathy and Treatment (INCAT) group (2). The INCAT initiative consists of a group of European neurological centers with the aim to develop standardized laboratory and clinical protocols to optimize research, diagnosis and treatment of inflammatory neuropathies. In a previous study of the INCAT-ELISA protocol, the variation between six laboratories was determined. This study showed that 41% of this variation was generated by the intra-laboratory variation (2). The sources of variation and the frequency of anti-GM1 antibodies in large groups of patients and controls using this standardized INCAT method have not yet been established.

In this study, as an intra-laboratory quality control, the reproducibility and sources of variation of the INCAT-ELISA were established. In addition, the frequency of anti-GM1 antibodies in large groups of patients was determined for the first time using this method. Based on the INCAT-ELISA, this study also provides the positive and negative predictive values for neurological disorders related with anti-GM1 antibodies assessing the diagnostic value of these antibodies in clinical practice.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 1232 patients and controls. These serum samples were retrospectively studied for the presence of IgM and IgG anti-GM1 antibodies. The study included patients with multifocal motor neuropathy (MMN, *N*=52), Guillain-Barré syndrome (GBS, *N*=471), chronic inflammatory demyelinating polyneuropathy (CIDP, *N*=82), paraprotein-related polyneuropathy (PP-PNP, *N*=102), other inflammatory polyneuropathy (I-PNP, *N*=40), non-inflammatory polyneuropathy (*N*=74), motor neuron disease (MND, *N*=78), multiple sclerosis (MS, *N*=38), other neurological diseases (OND, *N*=47), other autoimmune diseases associated with autoantibodies (AID, *N*=138) and healthy controls (HC, *N*=110).

All GBS serum samples were obtained pre-treatment and within 2 weeks of onset of weakness. All MMN serum samples were obtained prior to treatment with intravenous immunoglobulins. In the CIDP group the serum samples of 12 patients were obtained in the initial phase of disease and prior to any treatment. The GBS patients included in this study participated in one of the Dutch Guillain-Barré trials or survey studies and were described in detail elsewhere (3-6). Eight GBS patients that did not participate in one of the former

studies also fulfilled the diagnostic criteria for GBS (7). In 133 out of 233 GBS patients that participated in the Dutch Guillain-Barré trial published by van Koningsveld *et al.* (6) electrodiagnostic examination was performed. Acute motor axonal neuropathy (AMAN) syndrome and acute inflammatory demyelinating polyneuropathy (AIDP) were classified according to published electrodiagnostic criteria (8).

The I-PNP group included patients with paraneoplastic polyneuropathy (*N*=5), vasculitic polyneuropathy (*N*=6), mononeuritis multiplex (*N*=5), multifocal demyelinating polyneuropathy (*N*=6) and polyneuropathy associated with systemic autoimmune disease (*N*=18). The AID group included patients with myasthenia gravis (*N*=24), Lambert-Eaton myasthenic syndrome (*N*=3), dermatomyositis (*N*=1), celiac disease (*N*=20), systemic lupus erythematosus (*N*=20), Sjögren syndrome (*N*=30), Wegener granulomatosis (*N*=20) and untreated hyperthyroidism (*N*=20).

In the MND group, patients with upper and lower motor neuron involvement (*N*=26) were diagnosed according to El Escorial criteria (9) as amyotrophic lateral sclerosis (ALS) and adult patients with lower motor involvement (*N*=51) were diagnosed as spinal muscle atrophy type IV (SMA type IV). One patient in this group had a mild paresis that could be attributed to post-poliomyelitis.

Serum samples from most of the patients were obtained from the out-patient clinic of the Erasmus MC, Rotterdam, The Netherlands. The local Sanquin Bloodbank South West Region, The Netherlands, provided the serum samples obtained from healthy controls. All samples were stored at -20°C or -80°C before being tested.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum IgM and IgG anti-GM1 ganglioside antibodies were determined, without knowledge of diagnosis, by ELISA according to the method previously described (10) and standardized by the INCAT group (2). To determine anti-GM1 reactivity we used the mean difference of optical densities (d-OD) of two GM1 coated wells and two uncoated wells. To evaluate cut-off values for positive IgM and IgG anti-GM1 reactivity a receiver-operating characteristic (ROC) curve was made. The ROC-curves were based on the available d-OD results of MMN patients for IgM and the available d-OD results of GBS patients for IgG because these patient subgroups show relative highest and most frequent reactivity for these isotypes in the literature and in this study. To determine the ROC-curves the d-OD results of healthy controls was used as a control group for both isotypes. Positive serum samples were titrated using two-fold serial dilution series starting at 1:100. The reciprocal of the highest dilution that resulted in an OD higher than the cut-off value was taken to be the titer. In each experiment a positive and negative control sample was included.

Experiments for analysis of variance factors

Zsample obtained from a GBS patient with high IgM and IgG anti-GM1 reactivity (IgM titer 1600, IgG titer 51200). Anti-GM1 reactivity in this serum was confirmed previously in thin-layer chromatography (TLC) overlay (10). Two experienced technicians tested this positive serum sample on two consecutive days in two plates per day using 12 series of two GM1 coated wells and two uncoated wells to determine the d-OD for each isotype. To compare the variation of this serum we performed an experiment using 2 plates in which 24

GM1 coated wells were incubated for 1 hour with 1 μg/ml of peroxidase-conjugated cholera toxin β-subunit (Sigma). In addition, the variation in a serum sample with low anti-GM1 reactivity and low titer, as well as a serum sample without detectable anti-GM1 reactivity was tested. For each serum one plate was used to determine d-OD results (N=24 d-OD results per plate).

Statistic analysis

A nested analysis of variance was performed to determine the variances of technicians, days, plates and wells within the plates and their relative contribution in the overall intra-laboratory assay variance (11). The variation was also expressed as the coefficient of variance (CV) defined as the standard deviation (SD) divided by the overall mean.

Ruling-in and ruling-out gains were defined as the gain of a positive and negative test result, respectively, in diagnosing a patient correctly (1). The gain of a test was calculated by subtracting the positive predictive value and the negative predictive value by prior probabilities. The positive and negative predictive values of the test depend on the prior probability. Because the predictive values are related to the sensitivity and specificity these values also depend on the test results for specific patient subgroups, including the definition of controls. In Figures 4A and 4B the groups of HC, OND, AID and MS (total *n*=333) were used as a control group to determine the sensitivity and specificity.

Statistical analysis was performed using The SAS System for Windows version 8.2 (SAS Institute Inc., Cary, USA) and SPSS for Windows release 11.0.1 (SPSS Inc, Chicago, USA).

RESULTS

Analysis of variance factors in INCAT anti-GM1 ELISA

The results of the experiments to determine the relative contribution of the variation between technicians, days, plates and wells within the plates to the overall variation are shown in Figure 1. The CV of the overall intra-laboratory assay variance was 11.1% for IgM and 3.9% for IgG. The largest contributive variance factor for IgM was the variation between plates (62% of total variance) and wells within the plates (38% of total variance). For IgG, the largest contributive factor was the variation between wells within the plates (77% of total variance). There was no additional variance between technicians for IgM and IgG. The additional variance between days for IgG was low (15% of total variance) whereas for IgM the additional variance was negligible. The variance in days was also analyzed retrospectively by comparing the d-OD results of the same positive control serum used in 34 routine diagnostic experiments in a period of 4 years. The CV of the retrospective d-OD results was 17.2% for IgM and 14.9% for IgG. Two plates were incubated with cholera toxin β-subunit that binds to GM1 with high affinity. In these plates the CV's were 4.5% and 4.0% respectively.

The variability in test results with the serum with high anti-GM1 reactivity was compared with that of a serum without anti-GM1 reactivity and a serum with low anti-GM1 reactivity. The variability of a serum without anti-GM1 reactivity (SD IgG: 0.02, IgM: 0.01) was lower (Levene test $p<0.001$) than of a serum with low anti-GM1 reactivity (SD IgG: 0.06, IgM: 0.05) and a serum with high anti-GM1 reactivity (SD IgG: 0.09, IgM: 0.07).

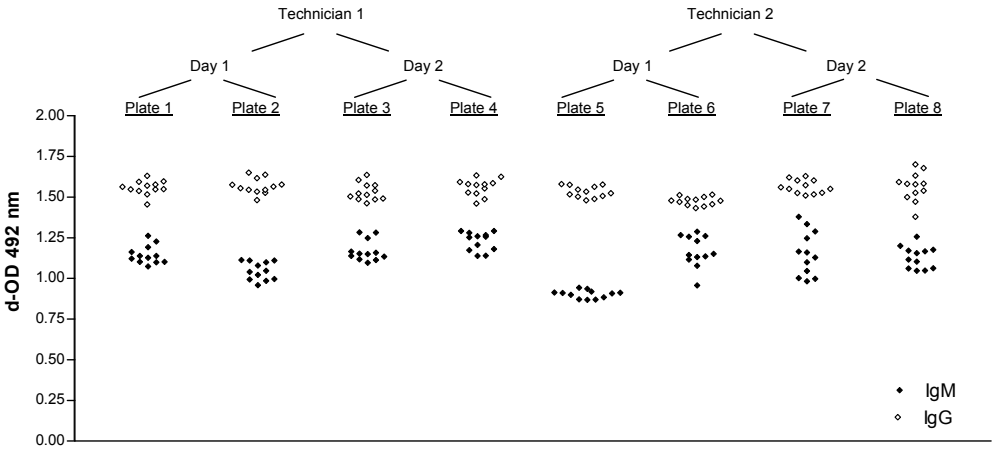

Figure 1. Assessment of intra-laboratory variation assay using a single positive serum sample.
Two experienced technicians tested a single positive serum sample positive for IgM and IgG reactivity to GM1 in ELISA on two consecutive days using two plates per day with 12 series of 4 wells (2 coated and 2 uncoated wells) per plate.

Cut-off values used to determine positive reactivity

To determine the optimal cut-off values for positive anti-GM1 reactivity and to discriminate between patients and controls we performed a ROC analysis. We used the d-OD results from healthy controls as a negative state variable and for respectively IgM and IgG the d-OD results of MMN and GBS patients as a positive state variable (Figure 2). For further analyses we used a cut-off value of 0.30 for IgM and 0.20 for IgG. ROC analysis indicated a corresponding

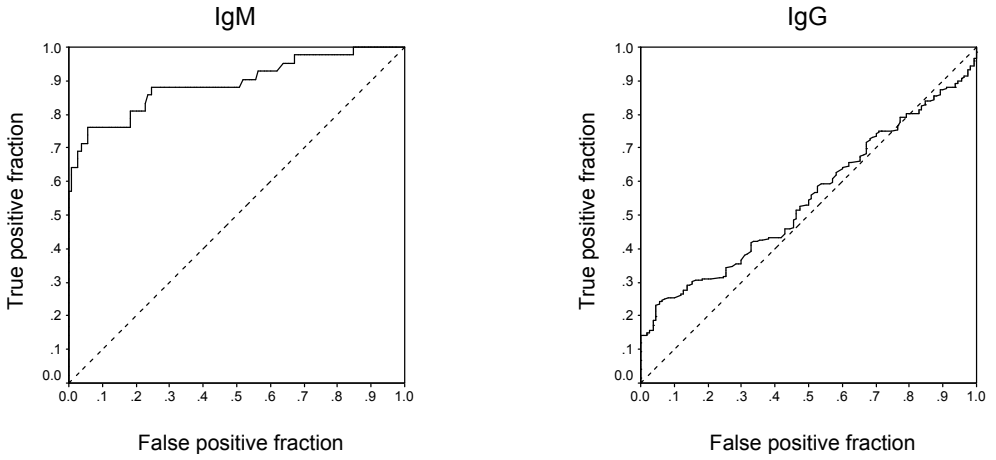

Figure 2. ROC curves for anti-GM1 reactivity.
ROC-curves for cut-off values of positive anti-GM1 IgM and IgG reactivity, based on the delta of optical densities (d-OD). The d-OD´s obtained from MMN and GBS patients were used respectively for IgM and IgG. For both isotypes healthy controls were included as a negative state variable.

specificity of 100% for IgM and a specificity of 100% for IgG with sensitivities of 45% and 13% respectively. The area under the curve for IgM was 0.89 and for IgG 0.55.

Anti-GM1 antibodies in patient subgroups

A total of 1232 patient serum samples were tested for the presence of IgM and IgG anti-GM1 antibodies. Of these, 155 (12.6%) were either positive for IgM and / or IgG, 119 (9.7%) for IgM only and 69 (5.6%) for IgG only (Table 1). The positive samples were further titrated (Figure 3).

IgM anti-GM1 reactivity was demonstrated in nearly all subgroups except for MS and HC. IgM titers higher than 200 were observed in patients with MMN (14%), GBS (5%), CIDP (1%), PP-PNP (1%), MND (3%) and OND (<1%). The four PP-PNP patients with positive IgM anti-GM1 reactivity had paraproteins of different isotypes and light chains. One patient had a monoclonal IgM-κ paraprotein, another patient had a monoclonal IgM-λ paraprotein, a third patient had both a biclonal IgM-κ paraprotein and a monoclonal IgG-κ paraprotein and a fourth patient both a monoclonal IgM-λ paraprotein and a biclonal IgG-λ paraprotein. The IgM anti-GM1 positive patients categorized as MND included 2 patients with ALS, 5 patients with SMA type IV and one patient with a mild paresis attributed to post-poliomyelitis. The OND patient with higher IgM reactivity (titer 400) presented with an oculomotor cranial nerve palsy without anti-GQ1b antibodies. IgG anti-GM1 reactivity was highly specific for GBS patients (13%). Other patients with positive IgG reactivity included five patients with CIDP (6%), one patient with a chronic inflammatory sensory-motor polyneuropathy of unknown cause (1%, titer 100), one patient with a slowly progressive distal sensory-motor axonal polyneuropathy (1%, titer 100) and a patient with a chronic pure motor neuropathy of unknown cause (1%, titer 100). Two IgG positive CIDP patients had an atypical clinical presentation. One of these patients had additional symptoms of ataxia; the other had additional proximal demyelination and cranial nerve involvement.

Anti-GM1 reactivity in GBS patients was associated with both diarrhea previous to onset of illness (p<0.001) and positive serology for a recent *Campylobacter jejuni* infection (p<0.001). Anti-GM1 antibodies were identified in 3 of 4 (75%) patients with AMAN, 9 of 40 (23%) with AIDP and in 17 of 89 (19%) patients who did not fulfill the criteria for AMAN or AIDP.

Table 1. Frequency of anti-GM1 antibodies in various patient subgroups.

Diagnosis	MMN	GBS	CIDP	PP-PNP	I-PNP	NI-PNP	MND	MS	OND	AID	HC	Total
N	52	471	82	102	40	74	78	38	47	138	110	1232
IgM positive												
N	23	64	6	4	5	3	8	0	4	2	0	119
%	44	14	7	4	13	4	10	0	9	1	0	9.7
IgG positive												
N	0	61	5	0	1	2	0	0	0	0	0	69
%	0	13	6	0	3	3	0	0	0	0	0	5.6

See figure 3 for used abbreviations. The total number and percentages of positive IgM and IgG anti-GM1 reactive sera as well as the total number of tested sera are listed.

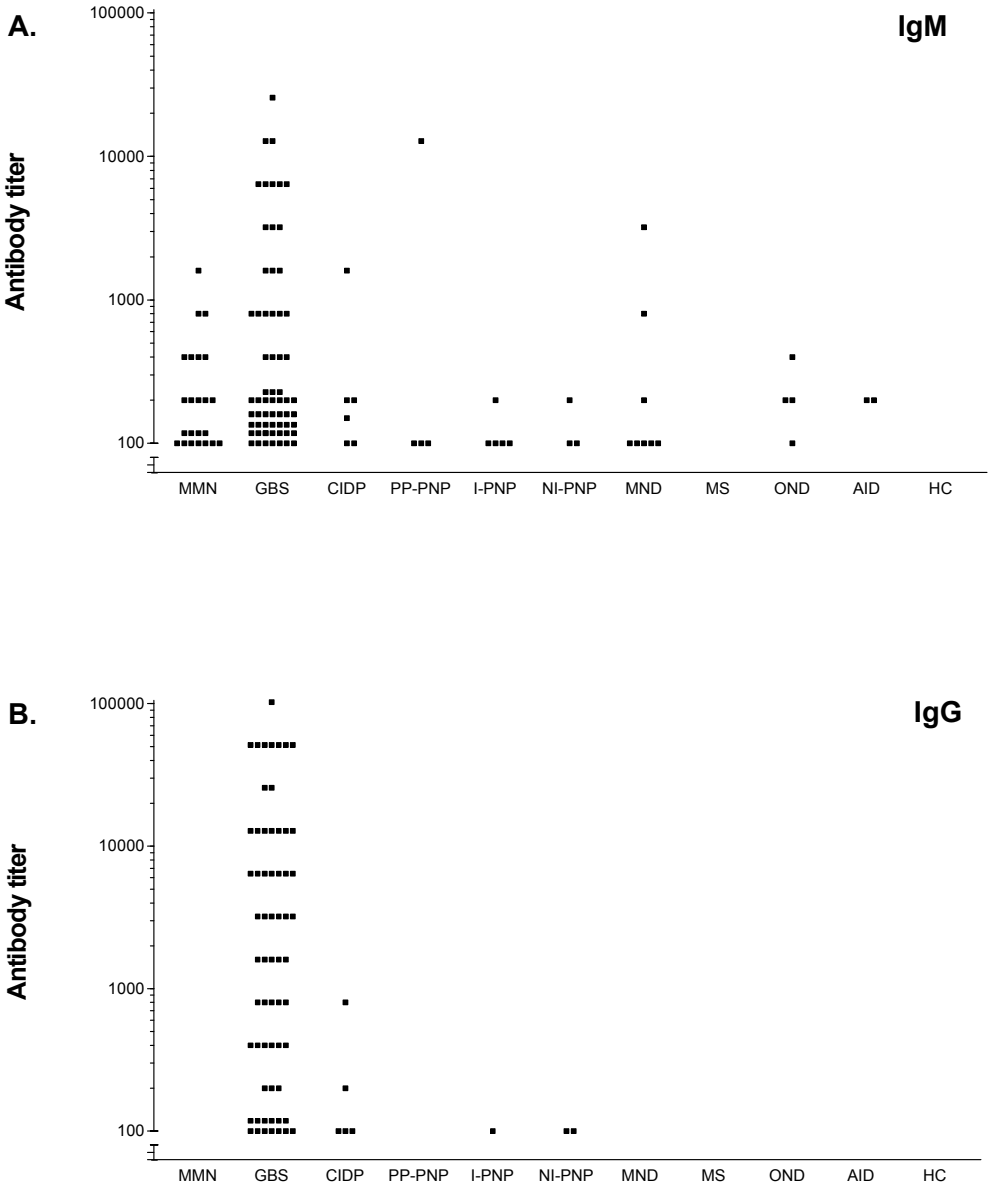

Figure 3. Titers of antibodies in anti-GM1 reactive sera.
(A) IgM anti-GMI antibody titers. (B) IgG anti-GMI antibody titers. Abbreviations used: MMN = multifocal motorneuropathy, GBS = Guillain-Barré syndrome, CIDP = chronic demyelinating polyneuropathy, PP-PNP = paraprotein-related polyneuropathy, I-PNP = other inflammatory polyneuropathy, NI-PNP = non-inflammatory polyneuropathy, MND = motor neuron disease, MS = multiple sclerosis, OND = other neurological disease, AID = other autoimmune disease, HC = healthy controls. Only sera with anti-GM1 reactivity are shown in this figure, see table 1 for total number of tested sera.

Predictive value of anti-GM1 antibodies

The sensitivity and the specificity of the assays were calculated based on the frequencies of anti-GM1 reactivity in the various patient subgroups and controls. These were used to determine the gain of the test shown in Figure 4.

The gain of a positive test to diagnose a patient correctly based on the test result was highest for MMN when the prior probability was between 10% and 40% (Figure 4A, ruling-in gain). For example, when the prior probability is 20% and a positive test result is obtained (gain of test 65%) the chance for a patient having MMN will change to 85% (post-test probability). The ruling-out gain of a negative test result was lower. A negative test will help to exclude the diagnosis in a presumable MMN patient optimally when the prior probability was between 40% and 70% (Figure 4A). The chance for a patient having MMN will change from e.g. 60% (prior probability) to 46% (60% -14%) when the test result is negative (Figure 4A). To determine the gain of a test result in the total group of inflammatory

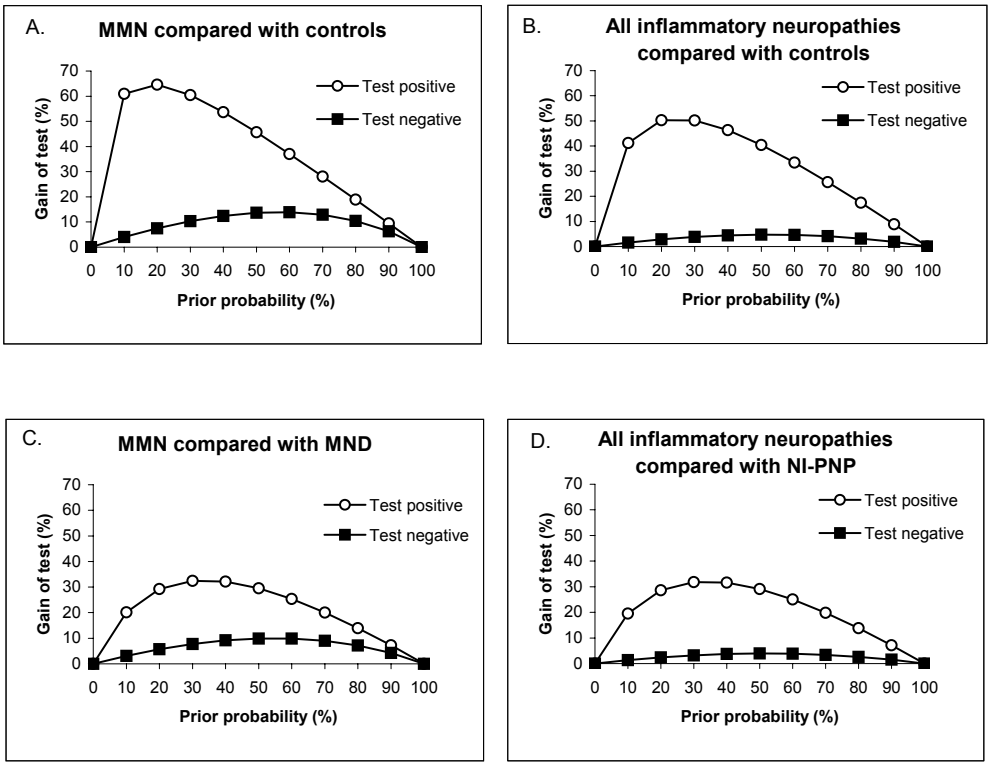

Figure 4. Ruling-in and ruling-out gains for prior probabilities.

Ruling-in and ruling-out gains plotted, as percentages, against the prior probability for patients with either MMN (Figure 4A, *n*=52) or an inflammatory polyneuropathy (Figure 4B including GBS, CIDP, MMN, PP-PNP and I-PNP patients, *n*=747). The control groups used for figures 4A and 4B included healthy controls, OND, AID and MS (*n*=333). For example, if the hypothetical prior probability for a patient having an inflammatory polyneuropathy is 30%, after a positive test the post-test probability for a patient having an inflammatory polyneuropathy is 80% (30% + 50%).

neuropathies we combined the subgroups MMN, GBS, CIDP, PP-PNP and I-PNP (total *n*=747) (Figure 4B).

When different groups are used to determine the specificity of the test, the gain of a test can be used as a measure to distinguish between patient subgroups. The gain of the test in distinguishing a MMN patient from a MND patient was relatively low (Figure 4C). When all inflammatory neuropathies were to be distinguished from non-inflammatory polyneuropathies the gain of the test was also lower (Figure 4D).

DISCUSSION

The INCAT-ELISA demonstrated good intra-laboratory reproducibility. The overall variability in our laboratory expressed as CV was 11.1% for IgM and 3.8% for IgG, which is lower than the findings in the previous INCAT study (2). In that study the intra-laboratory variance was determined less extensively and estimated based on results obtained from various laboratories. In a single experiment we determined the relative contribution that may influence the intra-laboratory variance of technicians, days, plates and wells within plates. This study showed that the inter-well variation within plates is the most important factor. The experiment used to determine the relative contribution of variance factors can be considered as a quality control for individual laboratories.

The most likely explanation for the relatively high inter-well variation in the INCAT-ELISA is related to the low titers and low affinity of anti-GM1 antibodies (12-14), that makes detection of these antibodies susceptible for small variations in the environment. This is especially the case for low titer IgM anti-GM1 antibodies that can be found in healthy donors and other controls. This notion is supported by the fact that the IgM variability was more than twice the IgG variability. Further experiments showed that cholera toxin, which has a high affinity to GM1, gives a CV of 4%. This is theoretically the lowest variation that can be obtained using the INCAT protocol. Differences between batches of plates have previously been suggested as a variation source (13). Differences in optical densities within separate regions of the plates (border versus center of plate) in our study were not significant (data not shown). To optimize the detection of antibodies of lower affinity the INCAT protocol recommends plates with high binding capacity for glycolipids like GM1, in duplo tests, long serum incubations at low temperatures and avoidance of detergents. To further improve the inter-laboratory variance individual laboratories should determine the factors that influence this variation and cooperate in exchanging control serum samples.

Using the INCAT-ELISA we found high frequencies of IgM anti-GM1 antibodies predominantly in MMN and GBS patients. These findings are in accordance with results from other studies using similar protocols as the INCAT-ELISA (1,15,16), but differ considerably with those of studies using other methods (17). In particular, we found no evidence for high titers of IgM or IgG anti-GM1 antibodies in serum from patients with MS or other autoimmune diseases. This difference in results from other studies most likely can be attributed to the use of different ELISA methods, although differences in patient populations can also play a role. This makes it difficult to discuss whether this method is the optimal one to use. To improve the comparison of different studies, we recommend the use of standardized methods such as proposed by the INCAT.

The presence of low titers of IgM anti-GM1 antibodies in control groups may indicate that anti-ganglioside antibodies are normal constituents of the human natural antibody repertoire (18). These antibodies could also represent a genetic predisposition to produce autoantibodies or may be related to polyclonal B-cell proliferation or to an autoimmune response secondary to tissue damage. In contrast, a proportion of patients with GBS, CIDP, MMN, PP-PNP and I-PNP have high titers of polyclonal or monoclonal IgM or IgG antibodies that may play a significant role in the pathogenesis of the immune mediated damage of peripheral nerve fibers (19,20). In an attempt to discriminate the disease related antibodies from i.e. naturally occurring antibodies we assessed the optimal cut-off value based on a ROC analysis of the d-OD's in samples from healthy controls versus patients with MMN and GBS. This analysis showed that these values correspond to an excellent specificity but a low sensitivity. The use of a lower cut-off value will increase the sensitivity but also result in an unwanted higher false positive fraction. We recommend a lower false positive fraction in favor of a higher sensitivity, especially because of the lack of discriminative power of the assay in general.

The diagnostic value of a laboratory assay in clinical practice also depends on the *a priori* chance of these diseases that determines the positive and negative predictive value. To determine the gain of the test, based on a range of *a priori* chances of diseases and predictive values, we assessed the frequency of anti-GM1 antibodies in serum from a large spectrum of patients and controls. The presence of anti-GM1 antibodies using the INCAT-ELISA showed a significant positive predictive value for MMN and the combined inflammatory neuropathies (GBS, CIDP, MMN, PP-PNP and I-PNP) when compared to controls (Figure 4A and 4B). However, the positive predictive value was lower when comparing clinically similar groups of patients, which is a better reflection of the situation in clinical practice. A relatively low additional gain of a positive test was found in discriminating MMN from motor neuron disease (gain of a positive test 33%, Figure 4C) and in discriminating inflammatory polyneuropathy from non-inflammatory polyneuropathy (gain of a positive test 32%, Figure 4D). In both situations however, the optimal gain for a positive test was obtained when there was still reasonable doubt for the diagnosis (*a priori* chance 20 to 50%). After adjustment for the availability of alternative assays (including electromyography, CSF and laboratory examination) to discriminate these disorders, the additional informative value of the INCAT-ELISA is presumably lower. A negative test result is therefore not helpful for excluding a diagnosis based on the test due to the low frequencies of these antibodies in specific disorders.

The application of anti-GM1 antibody assessment in the diagnostic work-up of inflammatory neuropathy patients can be informative, especially when there is reasonable doubt about the diagnosis and when positive results are obtained. It may be beneficial to treat patients with other forms of neuropathy with intravenous immunoglobulins if anti-GM1 antibodies have been demonstrated. To confirm this additional research is certainly needed. The ELISA method as suggested by the INCAT taskforce is a reliable and reproducible test that could be used as diagnostic tool in this matter.

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5.3

Detection of anti-MAG antibodies in polyneuropathy associated with IgM monoclonal gammopathy

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ABSTRACT

Background: Detection of serum antibodies to myelin-associated glycoprotein (MAG) by Western blot (WB) is a valuable assay to diagnose a distinct type of demyelinating polyneuropathy with IgM monoclonal gammopathy. In this study, the diagnostic accuracy of a new and more practical ELISA to detect these antibodies was validated.
Methods: Routine WB from two independent laboratories and ELISA were used to detect anti-MAG IgM in serum from 207 neuropathy patients and controls. The sensitivity and specificity of these assays were compared and related to the patient clinical and electrophysiological characteristics.
Results: In ELISA, anti-MAG antibodies were found in serum from 49 (72%) of 68 patients with demyelinating polyneuropathy and IgM monoclonal gammopathy. However, in this subgroup of patients only 30 (44%) and 37 (54%) were positive in the two WBs. All of the patients positive in the two WBs were also positive in ELISA. A high correlation was found for IgM activity in ELISA to MAG and sulfate-3-glucuronyl paragloboside (SGPG) (Spearman's rho 0.72, $p < 0.0001$), supporting the notion that the shared sulfated glucuronic acid moiety of MAG and SGPG is preserved. Most patients positive in anti-MAG ELISA had a slowly progressive sensory-motor demyelinating polyneuropathy, even if the WB was negative. In control groups, however, four WB negative patients with a non-demyelinating monoclonal gammopathy related polyneuropathy were positive in anti-MAG ELISA. The remaining samples were negative in ELISA.
Conclusion: ELISA is more sensitive than WB to diagnose anti-MAG related polyneuropathy, although a positive serology may be found in other forms of polyneuropathy as well.

INTRODUCTION

Patients with an IgM monoclonal gammopathy may develop a polyneuropathy if the monoclonal antibody binds to peripheral nerve antigens. In about half of these patients serum antibodies to myelin-associated glycoprotein (MAG) can be detected by Western blotting (WB) (1). Most patients with anti-MAG antibodies have a slowly progressive, distal, sensory or sensory-motor demyelinating polyneuropathy (2-4). These antibodies recognize the HNK-1 carbohydrate epitope on MAG, which is also present on other peripheral nerve glycoconjugates, including sulfate-3-glucuronyl paragloboside (SGPG) (5). The majority of patients with antibodies to MAG therefore also have serum antibodies to SGPG (3,4,6). In clinical practice, assays to detect anti-MAG and anti-SGPG antibodies are valuable diagnostic tools to diagnose a distinct subset of patients with chronic demyelinating polyneuropathy and IgM monoclonal gammopathy.

Anti-MAG WB serology, however, may be negative in patients who have otherwise the typical phenotype of the anti-MAG related polyneuropathy. This may indicate that anti-MAG antibodies are present in these patients, but that the sensitivity of the WB is insufficient. Recently an ELISA was developed to determine serum anti-MAG antibody reactivity (7). In general, ELISA is a highly reproducible and sensitive technique in which the antibody reactivity can be more easily quantified. At present it is unknown if ELISA is more sensitive than WB to detect anti-MAG antibodies and if testing for anti-SGPG antibodies has additional diagnostic value.

In the current study we used the ELISA to determine the frequency of anti-MAG antibodies in patients with various forms of chronic polyneuropathy and monoclonal gammopathy. These results were compared with those in anti-MAG WB and anti-SGPG ELISA, and analyzed in relation to the clinical and electrophysiological characteristics following the criteria as proposed by the Standards for Reporting of Diagnostic Accuracy (STARD) guideline (8).

METHODS

Patients

The study population comprised 154 patients with a chronic polyneuropathy who were recruited and diagnosed by neuromuscular specialists at the Departments of Neurology of the University Medical Center of Utrecht (UMCU) and the Erasmus MC in Rotterdam, The Netherlands, between 1986 and 2005. Medical history, physical examination, electrophysiology, laboratory results, including immunoelectrophoresis and immunofixation, were obtained according to a predefined diagnostic protocol and eligibility criteria as reported elsewhere (9).

Of these polyneuropathy patients, 87 had an IgM monoclonal gammopathy and were screened for (pre)malignancies. These studies demonstrated non-Hodgkin lymphoma in one patient, Waldenström's disease in two patients and breast cancer in one patient. No other causes for the neuropathy were found in the other 83 patients. Disease course was distinguished as either 'moderate progressive' (deterioration reaching endpoint within one year) or 'slowly progressive' (deterioration reaching endpoint at more than one year) (10). Endpoint was defined as a progression of the neuropathy leading to disability decrease of the

Rankin disability score of one point or decrease of sensory function or strength according to scales as published previously (11,12). The clinical phenotype was categorized as either pure sensory, sensory-motor or pure motor. Sensoric ataxia was defined as disturbance of gait or limb movements, which intensified when the eyes were closed (13). Nerve conduction and concentric needle examination identified a predominantly demyelinating neuropathy in 68 (77%) of these patients, according to previously described criteria (14).

The remaining 67 patients with a chronic polyneuropathy had an IgG monoclonal gammopathy (*N*=26), chronic inflammatory demyelinating polyneuropathy (CIDP, *N*=30) and chronic idiopathic axonal polyneuropathy (CIAP, *N*=11). For control studies we also included 19 patients with an IgM monoclonal gammopathy without polyneuropathy and 34 healthy blood donors.

Data collection

Consecutive cases with polyneuropathy associated with a monoclonal gammopathy and patients with IgM monoclonal gammopathy without polyneuropathy were previously included (*N*=132) (15). Recruitment of other patient groups was performed in a diagnostic

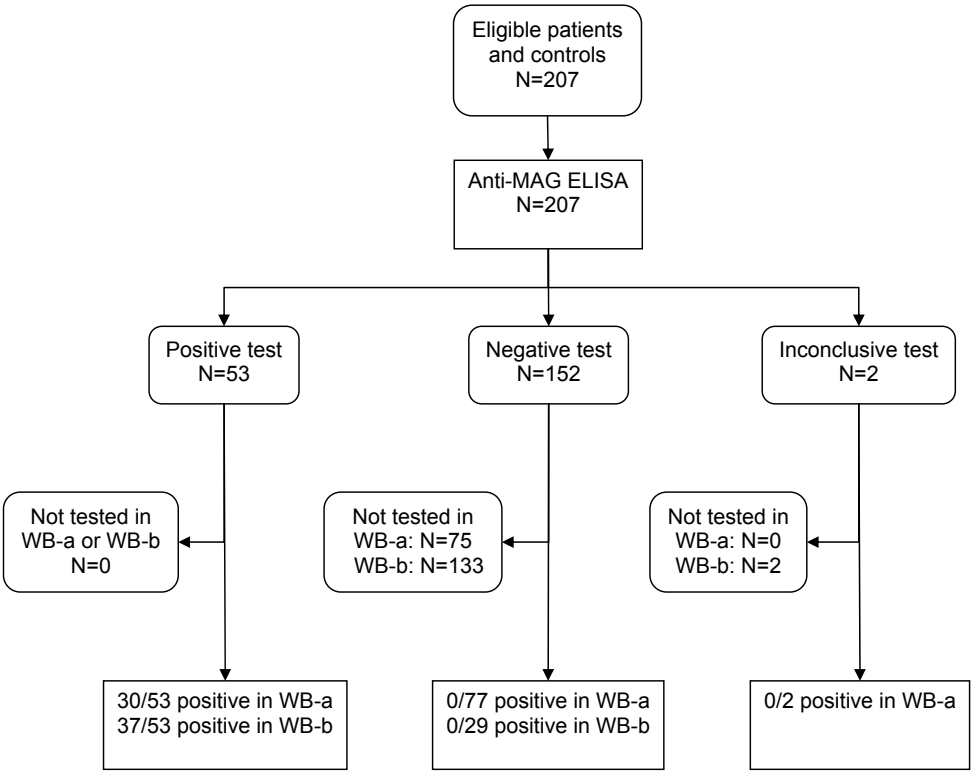

Figure 1. Flowchart of study.

Flowchart of initial data collection according to STARD criteria. WB-a and WB-b were used as reference test and applied on a selection of sera as depicted.

work-up context. These patients were selected randomly and not tested in anti-MAG ELISA before. Serum samples were tested in routine diagnostic WBs in two independent laboratories (reference standards WB-a and WB-b). All sera positive in anti-MAG or anti-SGPG ELISA and all patients with a demyelinating polyneuropathy and IgM monoclonal gammopathy (*N*=74) were retested in WB-b. Data for the ELISA and WB-b were collected prospectively and for WB-a retrospectively. The flowchart of this study is presented in Figure 1.

Standard Protocol Approvals, Registrations, and Patient Consents

Institutional approval from ethical standards committees of the Erasmus MC and the UMCU on human experimentation was received for experiments using human subjects (Erasmus MC METC 2004-242, UMCU METC 02/321). Participants gave written informed consent.

Anti-MAG serology

Pre-treatment serum samples from all 207 patients and controls were tested for anti-MAG IgM activity using an ELISA (Bühlmann Laboratories AG, Switzerland), according to the manufacturing instructions. Briefly, strips of wells pre-coated with human brain derived MAG purified by monoclonal antibodies were incubated in duplicate with serum samples diluted in incubation buffer at 1:1000 for two hours at 4°C. After washing, wells were incubated with anti-human IgM conjugate solution for two hours at 4°C. Next, the wells were rinsed and incubated with tetramethylbenzidine substrate solution for 30 minutes at room temperature. The reaction was stopped with an acidic stop solution within 30 minutes and the extinctions were read at 450 nm using a multiscan reader (Bio-Rad, Hercules, USA). Serum antibody activity was determined by using a standard calibration run and expressed as Bühlmann titer-units (BTU).

Serum IgM antibodies to SGPG were determined in all patients and in 17 of the 34 healthy controls using an ELISA (Bühlmann Laboratories AG, Switzerland). Instructions were the same as for the anti-MAG ELISA with a few modifications. Wells were pre-coated with SGPG purified from bovine cauda equine, also containing small amounts of lactosaminyl homologue (SGLPG), and sera were tested in 1:1000 dilutions. Serum anti-SGPG antibody activity was expressed as the mean optical density ratio of the patient sample and the calibration sample.

The method of WB-a was previously described (4). In short, human brain derived myelin protein fraction was separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and transferred to nitro-cellulose blots. The blots were incubated with 1:500 serum dilutions, washed and subsequently incubated with peroxidase-conjugated rabbit anti-human IgM antiserum. Positive sera, defined by the presence of a 100 kDa band, were titrated by serial two-fold dilutions until negative. The titer was defined as the highest serum dilution that showed the anti-MAG band. In WB-b, myelin was isolated from human brain by a protocol modified from Norton and Poduslo and loaded in 1 mg/ml in 10% SDS-PAGE (16). Gels were run at 200 volts for 3-4 hours and subsequently transferred to nitrocellulose blots. Next, patient sera diluted 1:500 were incubated at room temperature and after washing visualized by horseradish peroxidase anti-human IgM and stained by enhanced chemiluminescences on Kodak x-ray films.

All studies were performed blinded for clinical data and results in other assays. Three individuals blinded for each other's observations screened WB-b readings.

Positivity for WB results was then decided on consensus. WB-b was independently screened by three co-workers and final scores were reached by consensus.

Statistical analysis

Receiver operating characteristic (ROC) curve analysis was used to determine optimal cut-off values in the anti-MAG and anti-SGPG ELISA to discriminate between patients with typical anti-MAG related polyneuropathy versus the other patients or healthy controls. Subgroups of patients defined by the test results in anti-MAG WB and ELISA were compared using the Chi-square test or Fishers´ exact test. Spearman correlation coefficients and kappa values were used to compare the anti-MAG antibody activity found in ELISA and WB. Statistical analysis was performed with SPSS for Windows version 14.0. *P*-values <0.05 were considered to be significant.

RESULTS

Reproducibility and validation of anti-MAG and anti-SGPG ELISA

ROC curves for serum anti-MAG and anti-SGPG IgM activity were constructed based on the 68 cases with demyelinating polyneuropathy and IgM monoclonal gammopathy *versus* the 139 other patients and controls (Figure 2). A high discriminative power for the anti-MAG ELISA (area under the curve 0.84) and anti-SGPG ELISA (area under the curve 0.87) was found. The ROC analysis established that the optimal diagnostic cut-off value for the anti-MAG ELISA was 1500 BTU and for the anti-SGPG ELISA was a ratio of 1.0. The coefficient of variance was 6.8% for the anti-MAG ELISA and 6.1% for the anti-SGPG ELISA. The reproducibility of the anti-MAG ELISA was further determined by testing 64 patients

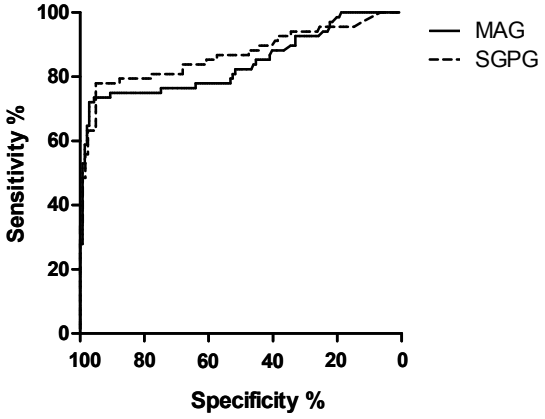

Figure 2. Receiver operating characteristic curve for the presence of serum IgM antibodies to MAG and SGPG determined by ELISA.

Receiver operating characteristic (ROC) curves are based on patients with demyelinating polyneuropathy associated with IgM monoclonal gammopathy patients (*N*=68) versus controls (*N*=139). Dotted line (- -) represents the anti-SGPG results, the solid line (–) the anti-MAG results.

with polyneuropathy and IgM monoclonal gammopathy a second time in which 62 (97%) had the same test result. In a third measurement the two discordant serum samples were negative and were further classified as such (Figure 1).

Frequency of serum anti-MAG and anti-SGPG IgM antibodies in patients and controls
Using this diagnostic cut-off value for the anti-MAG ELISA , 53 (26%) of the 207 samples were positive (Figures 1 and 3). From these, 49 (92%) had demyelinating polyneuropathy and IgM monoclonal gammopathy, three (6%) non-demyelinating polyneuropathy and IgM monoclonal gammopathy and one (2%) non-demyelinating polyneuropathy and IgG monoclonal gammopathy. The sensitivity of the anti-MAG ELISA was 72.1% for demyelinating polyneuropathy and IgM monoclonal gammopathy and 100% for the subgroup positive in anti-MAG WB. The specificity of the anti-MAG ELISA was defined in the combined groups of patients with chronic polyneuropathy, excluding patients with a paraprotein without polyneuropathy and healthy controls, to reflect the situation in clinical practice. The specificity of the anti-MAG ELISA was 95.3% for identifying a demyelinating polyneuropathy and IgM monoclonal gammopathy.

The test results in ELISA were compared with the results from the two routine diagnostic anti-MAG WBs (WB-a and WB-b). In the 68 patients with demyelinating polyneuropathy and IgM monoclonal gammopathy, WB-a was positive in 30 (44%) patients, WB-b in 37 (54%) patients, whereas anti-MAG ELISA was positive in 49 (72%) patients. All patients with a positive test result in WB-a or WB-b were also positive in the anti-MAG ELISA. In the 38 patients from this subgroup that were negative in WB-a, 19 (50%) were positive in anti-MAG ELISA. From the 31 patients negative in WB-b, 12 (39%) were positive in ELISA. The two routine WB-a and WB-b showed only a moderate agreement (kappa 0.62): three patients were positive in WB-a only and 10 patients in WB-b only. WB-b showed a weak positive band in the patient with a non-demyelinating polyneuropathy and IgG monoclonal gammopathy that was positive in anti-MAG ELISA.

Anti-SGPG IgM antibodies were found in 55 (29%) of the 190 serum samples tested (Figure 3). From these, 49 (89%) had a demyelinating polyneuropathy and IgM monoclonal gammopathy, four (7%) a non-demyelinating polyneuropathy and IgM monoclonal gammopathy, one (2%) a non-demyelinating polyneuropathy IgG monoclonal gammopathy and one (2%) an IgM monoclonal gammopathy without polyneuropathy. The sensitivity of the anti-SGPG ELISA for demyelinating polyneuropathy and IgM monoclonal gammopathy was 72.1% (and for the subgroup with a positive anti-MAG WB 100%). The specificity of the anti-SGPG ELISA for demyelinating polyneuropathy and IgM monoclonal gammopathy defined in all patients with a chronic neuropathy was 94.2%.

There was a high correlation between the serum IgM activity determined in the anti-MAG ELISA and anti-SGPG ELISA (Spearman's rho 0.72, $p < 0.0001$) (Figure 4). Comparing the positive and negative test results of the anti-MAG ELISA and anti-SGPG ELISA, we found a high kappa of 0.92. Four anti-SGPG positive patients were negative in anti-MAG ELISA. From these, one patient had a non-demyelinating polyneuropathy and IgM monoclonal gammopathy, one patient a IgM monoclonal gammopathy without polyneuropathy and two patients a demyelinating polyneuropathy and IgM monoclonal gammopathy (Figure 3). Two anti-SGPG negative patients were positive in anti-MAG ELISA and both had a

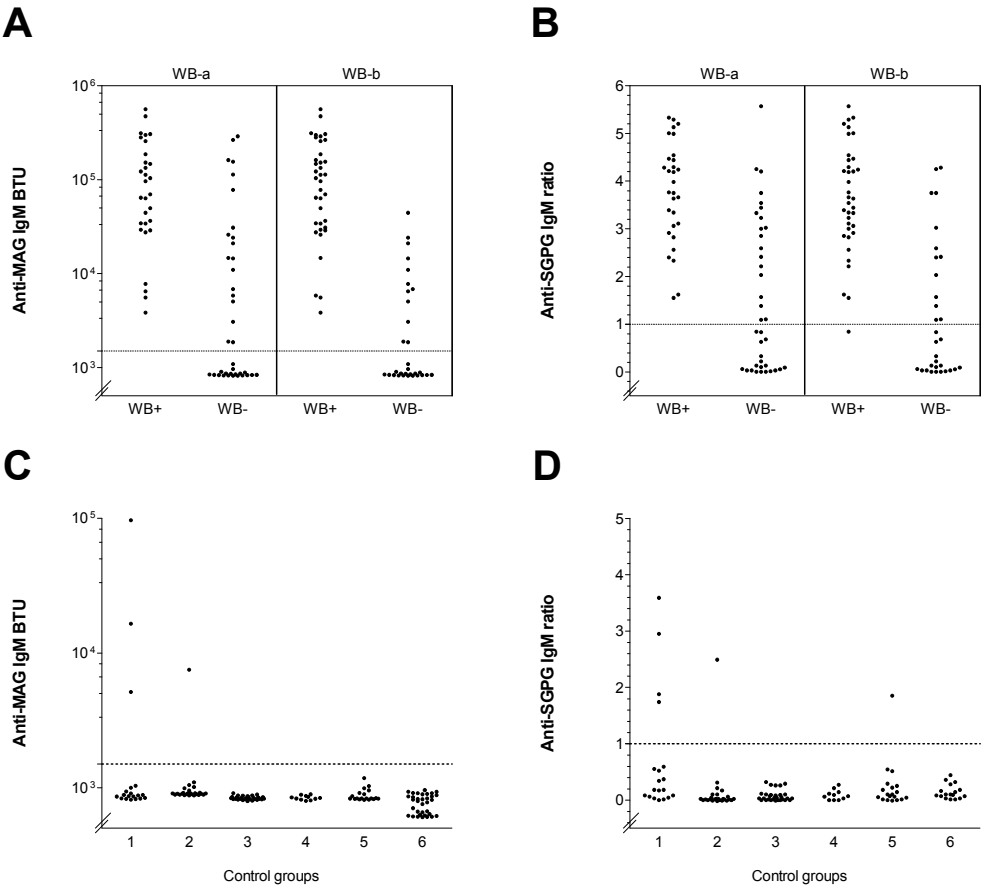

Figure 3. IgM antibodies to MAG and SGPG in serum from patients and controls (*N*=207).
Serum from patients and controls was tested in ELISA for the presence of IgM antibody activity to MAG expressed as Bühlmann titer units (BTU) and to SGPG expressed as antibody ratios. Abbreviations used: WB-a: Western blot performed in laboratory a, WB-b: Western blot performed in independent laboratory b. Dotted lines represent cut-off values for positivity.

(A) Anti-MAG IgM activity in ELISA is shown for patients with demyelinating polyneuropathy and IgM monoclonal gammopathy (*N*=68) and compared with activity in two routine diagnostic WBs (WB-a and WB-b), being positive (WB+) or negative (WB-). The left panel compares the activity in ELISA with the results in WB-a, the right panel with the results in WB-b.
(B) Identical to (A) but for anti-SGPG IgM activity.
(C) Anti-MAG IgM activity of control groups including patients with (1) non-demyelinating polyneuropathy and IgM monoclonal gammopathy (all negative in WB, *N*=19), (2) polyneuropathy and IgG monoclonal gammopathy polyneuropathy (all negative in WB, *N*=26), (3) chronic inflammatory demyelinating polyneuropathy (*N*=30), (4) chronic idiopathic axonal polyneuropathy (*N*=11), (5) monoclonal gammopathy without polyneuropathy (all negative in WB, *N*=19), and (6) healthy controls (*N*=34).
(D) Identical to (C) for anti-SGPG IgM activity.

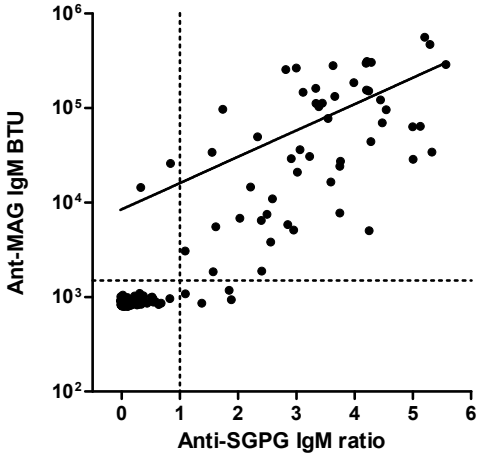

Figure 4. Correlation between IgM antibody activity to MAG and SGPG in serum from polyneuropathy patients and controls (*N*=190).
Scatter plot of serum IgM antibody activity to MAG and SGPG in ELISA, which shows that these activities are strongly correlated (Spearman's rho 0.72, $p < 0.0001$). Dotted lines represent cut-off values for positivity. Solid line represents nonlinear regression line.

demyelinating polyneuropathy and IgM monoclonal gammopathy. All sera positive with WB-a were positive in anti-SGPG ELISA. There was one patient positive in WB-b but negative in anti-SGPG ELISA. This patient had a demyelinating polyneuropathy and IgM monoclonal gammopathy and was also positive in anti-MAG ELISA.

Clinical characteristics of patients with anti-MAG serum antibodies in ELISA
The clinical characteristics of the patients with a demyelinating polyneuropathy and IgM monoclonal gammopathy were compared in relation to the test results in anti-MAG ELISA, WB-a and WB-b (Table). Slow progression of disease was more frequently found in patients with positive anti-MAG serum reactivity in both ELISA and WB-a or WB-b compared to those negative in both tests (Table). None of the other patient characteristics was associated with the presence of anti-MAG or anti-SGPG antibodies in either ELISA or WB. In addition, there were no differences between patients positive in ELISA only (negative in WB-a or WB-b) compared to patients positive in WB-a or WB-b. One of the patients that was positive in ELISA only was a 64 year-old female with a slowly progressive demyelinating sensory-motor polyneuropathy. Immunohistological investigation of a sural nerve biopsy in this patient showed the presence of IgM deposits at the myelin sheets, a finding frequently seen in patients with anti-MAG polyneuropathy.

Four patients from the other chronic neurpathy groupswere also positive in anti-MAG ELISA, but negative in WB-a. Three patients had a non-demyelinating polyneuropathy and IgM monoclonal gammopathy. One of these patients developed Waldenström's disease and another amyloidosis. The electrophysiological studies performed in these patients did not

Table. Characteristics of 68 patients with a demyelinating polyneuropathy and IgM monoclonal gammopathy in relation to serum anti-MAG reactivity in WB and ELISA.

	WB-a		WB-b		WB-a or WB-b
	WB^+ $ELISA^+$	WB^- $ELISA^+$	WB^+ $ELISA^+$	WB^- $ELISA^+$	WB^- $ELISA^-$
	N=30	*N*=19	*N*=37	*N*=12	*N*=19
Demography					
Mean age, y (SD)	60 (10)	58 (11)	59 (10)	61 (11)	61 (8)
Gender (W:M)	7:23	7:12	11:26	3:9	5:14
Slow progression (%)	21 (70) *	8 (42)	24 (65)†	5 (42)	5 (26)
Ataxia (%)	15 (50)	6 (32)	15 (41)	6 (50)	5 (26)
Classification (%)					
Sensory	0 (0)	1 (5)	0 (0)	1 (8)	1 (5)
Sensory-motor	30 (100)	17 (90)	36 (97)	11 (92)	17 (90)
Motor	0 (0)	1 (5)	1 (3)	0 (0)	1 (5)
IgM light chain (%)					
Kappa	22 (74)	14 (74)	29 (78)	7 (58)	13 (68)
Lambda	4 (13)	3 (16)	4 (11)	3 (17)	3 (16)
Both	4 (13)	2 (11)	4 (11)	2 (25)	3 (16)
Anti-SGPG positive (%)	30 (100)	17 (89)	36 (97)	11 (92)	2 (11)‡

WB: Western blot, WB-a: Western blot performed in laboratory a, WB-b: Western blot performed in independent laboratory b, y: year, SD: standard deviation, W: women, M: men. * p=0.003 compared to WB^- $ELISA^-$, † p=0.006 compared to WB^- $ELISA^-$, ‡ p<0.0001 compared to WB^+ELISA^+ and WB^-ELISA^+ in WB-a and WB-b.

show signs of demyelination. The other patient also had a non-demyelinating polyneuropathy but with an IgG monoclonal gammopathy and a non-Hodgkin lymphoma.

DISCUSSION

The current study showed that in patients with demyelinating polyneuropathy and IgM monoclonal gammopathy serum anti-MAG antibodies are more frequently detected by ELISA compared to WB. More than 70% of these patients were positive in ELISA while 44 to 54% were positive in the two routine diagnostic WBs, a percentage comparable with previous reports on WB (2,4). Our study indicates that patients who are positive in ELISA but negative in WB, may have a polyneuropathy with the typical anti-MAG phenotype. First, these patients had a similar slowly progressive, sensory or sensory-motor demyelinating polyneuropathy

as seen in the anti-MAG WB positive patients. Secondly, nearly all ELISA positive but WB negative patients had additional IgM serum antibodies to SGPG (96%), indicating that the antibodies are directed to the shared sulfated glucuronic acid moiety in MAG and SGPG, which is typical for patients with anti-MAG polyneuropathy (4,6). Third, a sural nerve biopsy from these patients positive in ELISA only showed myelin sheet IgM deposits in myelin sheets, a finding frequently seen in anti-MAG polyneuropathy (17-21). Together these observations suggest that ELISA is more sensitive than WB for identifying patients with an anti-MAG related polyneuropathy.

At present however, the WB method is considered to be the golden standard technique to determine serum anti-MAG antibodies (22). An important advantage of WB compared to ELISA is the possibility to verify that the antibodies are directed to the typical 100 kDa protein and not to a contaminant in the purified myelin fraction. The ELISA validated in the current study, however, uses a highly purified MAG fraction, showing no contaminants in silver staining, Coomassie blue staining or immunoblotting (Figure e-1 and Figure e-2). Disadvantages of WB compared to ELISA are the difficulties to control the quality of the used myelin fractions and to quantify the staining band. This may limit not only the sensitivity to detect anti-MAG antibodies, but also the inter-laboratory reproducibility, as illustrated in the comparison between WB-a and WB-b in our study. Previous studies comparing ELISA and WB also reported the moderate agreement in antibody activity to MAG measured by the two techniques (4,6). Detection of serum antibodies may be influenced by differences in ELISA and WB to capture MAG and present reactive epitopes. Our finding that all patients positive in WB have high anti-MAG antibody activity in ELISA, indicates that ELISA at least can be used as a first screening method in the clinical work-up of patients with chronic demyelinating polyneuropathy and IgM monoclonal gammopathy. Following the guidelines of the European Federation of Neurological Societies and the Peripheral Nerve Society, it may be useful to confirm the anti-MAG ELISA positive sera in WB (23).

The anti-MAG ELISA was also positive in four patients with a non-demyelinating polyneuropathy. These patients all had a monoclonal gammopathy (three IgM and one IgG), but were negative in anti-MAG WB. The additional positive serology for SGPG in these patients, suggests that their serum antibodies recognized the shared sulfated glucuronic acid moiety in MAG and SGPG. Previous studies indicate that in exceptional cases patients with axonal neuropathy can be positive for anti-MAG antibodies, even in WB (24). Serum antibodies from anti-MAG positive patients may bind to peripheral nerve axons, especially if there is additional serum reactivity for SGPG (25). This staining pattern may reflect the presence of SGPG in human peripheral nerve axons (26). These findings indicate that electrophysiological studies are required in the diagnostic work-up and cannot be replaced by anti-MAG serology. If the routine testing for anti-MAG antibodies is restricted to patients with a demyelinating form of polyneuropathy, this may not influence the specificity of the ELISA in the diagnosis of the classical anti-MAG polyneuropathy.

Disease markers for chronic immune mediated neuropathy are required to classify diseases and predict the response to therapy. The presence of serum IgM antibodies to MAG defines a distinct type of neuropathy, in which these antibodies are probably involved in the pathogenesis of disease (18,27-29). Previous studies using WB indicate that about half of the patients with demyelinating polyneuropathy and IgM monoclonal gammopathy

have anti-MAG antibodies (2,4). The current study indicates that anti-MAG antibodies are found in more than 70% of patients with a typical IgM monoclonal gammopathy related demyelinating polyneuropathy. This may indicate that the group of patients with demyelinating polyneuropathy and IgM gammopathy is more homogenous than previously thought. Patients with a non-demyelinating polyneuropathy or with a demyelinating polyneuropathy without detectable IgM gammopathy incidentally may have anti-MAG antibodies (17,24). Nerve biopsy studies are needed to determine whether these patients also have the typical anti-MAG related immunopathology. Detection of anti-MAG antibodies is also relevant because it may help to target immunotherapy. It has been shown that IgM anti-MAG neuropathy usually responds poorly to most conventional immunomodulatory therapies (30). Recent studies in IgM anti-MAG neuropathy, however, indicated promising results using rituximab, a chimeric monoclonal that targets B-cells, in which anti-MAG titers decay with clinical improvement (31-34). Interestingly, most clinical improvement was reported in patients with the lowest baseline anti-MAG titers (31), further illustrating the clinical relevance of a sensitive technique to demonstrate anti-MAG antibodies. Our study shows that the ELISA can be used as a sensitive and reliable screening method for determining anti-MAG antibodies.

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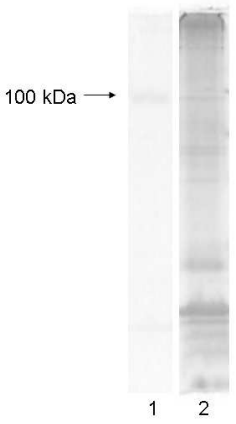
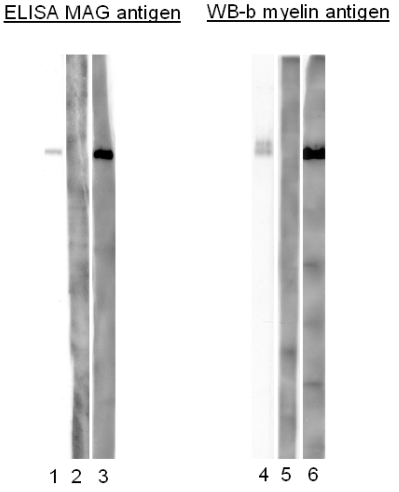


Supplementary Figure e-1. Comparison of MAG preparation used in ELISA and myelin preparation used in Western blot.
Coomassie staining of MAG preparation used in ELISA (lane 1) and myelin preparation used WB-b (lane 2). No additional bands are visualized in MAG antigen used in ELISA.



Supplementary Figure e-2. Binding of antibodies to the MAG preparation used in anti-MAG ELISA and the myelin preparation used in Western blot.
Immunoblotting with monoclonal antibody Leu-7 (Becton Dickinson, NJ, USA) recognizing the HNK-1 epitope in MAG (lanes 1 and 4) reveals a band at 100 kD both in the blot with the MAG preparation used in ELISA (lane 1) and the myelin preparation used in WB-b (lane 4). No band was seen after incubation with serum from a healthy control (lanes 2 and 5). Serum from a patient with a demyelinating polyneuropathy and IgM monoclonal gammopathy showed a single band with the ELISA MAG preparation (lane 3), and several bands, including the 100 kD band, with the myelin preparation used in WB-b (lane 6).

6

General Discussion

GENERAL DISCUSSION

GBS is often a devastating disease for which currently limited effective treatment is available. Artificial respiration for several weeks or months is required in 20% of patients. Around 5% of patients are hospitalized for more than a year and require years of rehabilitation for their residual deficits. This prolonged process involves an extraordinary patience and endurance of patients and their relatives. The severity of the disease varies considerably and is related to the type of preceding infection and genetic background of the patient. The most severe forms of GBS are particularly associated with antecedent *C. jejuni* infections. How does an infection caused by *C. jejuni*, which usually leads to a self-limiting and mild gastroenteritis, in exceptional cases (1:1,300) trigger the onset of GBS? The challenge for performing the research described in this thesis was to further unravel the etiology and pathogenesis of GBS in order to improve the prognosis, especially for the grave subgroup with *C. jejuni*-related GBS.

In this thesis we aimed to dissect the factors involved in the interaction between *C. jejuni* and other specific pathogens and the hosts (the patients) that determines the aberrant immune response preceding the development of GBS. We addressed whether the risk of developing GBS in The Netherlands is related to the demography and seasonality of *C. jejuni* infections and to what extent infections caused by CMV may be involved in triggering GBS. For investigating the mechanisms that determine the outcome of host-pathogen interactions, we especially focused on *C. jejuni* because this is the predominant preceding infection in GBS and is relatively best characterized within GBS pathogenesis. Molecular techniques to characterize epitopes in the LOS structure of *C. jejuni* were used and related to the specificity of anti-ganglioside antibodies and clinical symptoms in GBS patients. Furthermore, the serum cross-reactivity to new molecular targets in *C. jejuni* LOS composed of combinations of ganglioside mimics or ganglioside complexes was determined. The clinical relevance of these targets was addressed by determining the diagnostic value of anti-neural antibodies in GBS patients as well as in other immune-mediated neuropathies. Furthermore, we assessed whether the immune response is modulated by these targets and by interactions between human dendritic cells (DCs) and *C. jejuni.* We also addressed the hypothesis that genetic variation in CD1 molecules expressed by DCs is related to a higher risk of developing GBS.

These comprehensive objectives were addressed in the studies that are described in chapters 2 to 5 by using one of the largest GBS datasets currently available in the world. In this chapter the results of these studies will be outlined and discussed in relation to available literature. In addition, suggestions for further research will be put forward where applicable.

METHODOLOGICAL PERSPECTIVE AND EPIDEMIOLOGY OF PRECEDING INFECTIONS IN GBS

Investigating GBS in relation to the objectives of this thesis requires patient material and clinical data preferably from a large number of patients to strengthen the general applicability of its observations. GBS is a relatively rare disease with an incidence of approximately

1 per 100.000 persons per year (1). As a consequence, well-organized and large-scale clinical studies that systematically include GBS cases and recruit patient materials are required. At the Erasmus MC, in the past twenty-five years several clinical trials and national survey studies have been coordinated, which has resulted in the presence of a large bio-bank with serum, DNA, cerebrospinal fluid samples and extensive clinical information from more than 500 GBS patients. In addition, more than 25 GBS-related *C. jejuni* isolates have been assembled between 1986 and 2005 from efforts by various microbiologists and neurologists in The Netherlands (2-6). Control samples from healthy individuals but also from patients with other neurological illnesses and *C. jejuni*-related gastroenteritis were also collected. The studies performed in the past have identified several important host- as well as pathogen-related factors involved in the etiology of GBS and showed the efficacy of various treatment regimens (3-7). The extensive body of data obtained during these studies was available for performing the research described in thesis and constituted a unique opportunity to address the objectives stated in the Introduction.

Detection and epidemiology of preceding infections in GBS

The most commonly identified triggers for GBS found in case-control studies are preceding infections caused by *C. jejuni,* CMV, EBV and *Mycoplasma pneumoniae* (8). Combined, these infections explain only about half of the cases, despite the fact that GBS is generally considered to be a post-infectious disease. Determining the frequency of preceding infections is complicated by the fact that in many GBS cases, infections lead to mild symptoms or remain subclinical. In addition, most GBS patients have already cleared their infection by the time they develop neurological symptoms, making it often impossible to isolate the pathogen causing the infection. These factors explain that preceding infections are not detected in a large proportion of GBS patients. Possibly the techniques to identify these infections are not sensitive enough, or these negative patients have other types of preceding infections. Improvement of techniques to detect and study the epidemiology of preceding infections in GBS is relevant because the type of preceding infection may determine the GBS variant and clinical outcome. For example, diarrhea in GBS is associated with poorer prognosis and the AMAN variant is highly associated with preceding *C. jejuni* infections (9-11). In addition, the type of infection may also determine the mechanism leading to development of GBS.

In a recent Austrian report a highly sensitive PCR technique was used showing that 40% of GBS patients of a small cohort (n=42 patients) contained CMV DNA in cerebrospinal fluid whereas only 12% had positive IgM serum reactivity to CMV (12). This could suggest that CMV plays a more significant role in AIDP, the predominant form of GBS in European countries, than the 15% as previously considered based on case-control studies (8,12). CMV infections are thought to induce GBS as a result of a cross-reactive immune response to neural targets such as GM2 (13-15). The detection of CMV DNA in cerebrospinal fluid samples suggests that either a local reactivation of CMV or a primary CMV infection in peripheral nerves directly causes nerve dysfunction. Because GBS patients show no symptoms of central nervous system dysfunction, the presence of CMV DNA in cerebrospinal fluid may represent passive transfer of CMV across a damaged blood nerve barrier or retrograde transport from peripheral nerves. The finding of a highly frequent associated pathogen for GBS in Europe, perhaps in analogy to the situation of *C. jejuni* and AMAN, requires further attention and

suggests that new sensitive PCR techniques play an important role in identifying potential new clinical subgroups. In contrast, we describe in **Chapter 2.1** that only one out of 170 GBS patients (<1%) displayed CMV DNA in cerebrospinal fluid, whereas 22 patients (14%) had positive IgM serum reactivity (16). Remarkably, a similar PCR technique was used and both studies were performed in reference laboratories for detection of CMV infections. We accordingly could not identify a new relevant clinical subgroup associated with CMV infections.

Possible explanations for this discrepancy include selection bias, geographic difference in circulating genotypes of CMV and poor specificity of the PCR technique (16). Molecular techniques such as PCR have the advantage of being highly sensitive but their specificity for CMV has been estimated 76% (17). Even when CMV is cultured from cerebrospinal fluid, this does not rule out the possibility that this occurred in absence of active disease as is seen in CMV viraemia (18). The cross-reactivity of CMV induced anti-GM2 antibodies from GBS patients and clinical homogeneity in CMV IgM seropositive GBS patients are highly suggestive of a causal relationship (13-15,19). Although these observations clearly indicate that CMV can trigger GBS, the use of PCR techniques to demonstrate CMV DNA in cerebrospinal fluid samples from GBS patients did not contribute to a better understanding of the significance of this infection in terms of relative frequency yet.

In general, the detection of CMV infections and distinction between recent infection, active disease and reactivations of CMV is difficult. To discriminate primary CMV infections, advanced serological assays that determine the avidity of IgG antibodies to CMV could be applied in large groups of GBS patients (20,21). New developments in assays to detect CMV viraemia such as the NASBA pp67 RNA assay (22), which is more specific but less sensitive than the real-time PCR currently used, should be validated in specific clinical situations such as GBS. Since it is likely that most if not all AIDP or GBS patients have preceding (subclinical) infections that trigger the disease, it is recommended to search for new pathogens and, in compliance with the molecular mimicry model for *C. jejuni*-related GBS, focus on those known to express neural antigens. Screening for new pathogens could be performed by random PCR amplification strategies known as RAP-PCR that enables obtaining sequence information on unknown viruses (23-25). More information about the frequency of CMV infections and of other types of infection in GBS is important to identify new epitopes present in pathogens that may induce a cross-reactive immune response. Further characterization of the mechanism and involved immune response may subsequently lead to development of new therapies.

In addition to increasing our knowledge about the frequency and type of infection preceding GBS, describing the epidemiology of specific infections could lead to identification of new risk factors by empirical cause and effect analysis. One of the crucial questions remains why only 1 in 1,000 to 5,000 individuals develops GBS after enteritis caused by *C. jejuni* (26,27). Host-, pathogen- but also environmental factors may be involved in this exceptional outcome of *C. jejuni* infection. GBS occurs more frequently at older age and a male predominance is found, which could be related to acquired immunity to *C. jejuni* and riskier practices as regards to food handling in men causing more frequent infections by *C. jejuni* compared to women (1,28). It has been suggested that in the industrialized world, handling fresh poultry is the most common cause of infection by *C. jejuni* (29). Seasonal changes may influence

the predominance of specific circulating strains of *C. jejuni* that could be more pathogenic (30,31). Comparing the demography and seasonality of *C. jejuni*-related gastroenteritis and GBS could lead to identification of possible environmental risk factors.

In **Chapter 2.2** the demography and seasonality of culture proven *C. jejuni*-related gastroenteritis (*n*=16,621) and GBS patients (*n*=26) as well as serology-proven *C. jejuni*-related GBS patients (n=128) was compared. Interestingly, a striking inverse seasonal pattern of *C. jejuni*-related GBS and gastroenteritis was found. This study suggests that *C. jejuni* infections occurring during the winter season seem to have a higher risk for the post-infectious development of GBS (**Chapter 2.2**). In The Netherlands a higher chance of becoming infected does apparently not correlate to a higher chance of developing GBS. We also found that *C. jejuni* infections in GBS patients occurred more often in age groups above 60 years in contrast to *C. jejuni*-related gastroenteritis, a pattern that was also independent of changes in age pyramids of the general population during 1986-2006 (1). Accordingly, older people have a higher risk to develop GBS after *C. jejuni* infections than young people, possibly as a result of encountering rare serotypes or by becoming sensitized (32).

The collecting process of GBS patients and gastroenteritis patients limit concise statistical comparisons by confounding selection bias. However the strong inverse patterns that were observed justifies speculation about the causes of the discrepancy between the seasonality and age distribution of *C. jejuni*-related GBS and gastroenteritis. It is possible that subclinical infections occurred more frequently in the serologically proven *C. jejuni*-related GBS patients compared to the *C. jejuni*-related gastroenteritis patients as medical help for diarrhea was sought and stools were taken for examination in the *C. jejuni*-related gastroenteritis group. Therefore we determined whether clinical differences between culture-proven and serologically proven *C. jejuni*-related GBS cases biased our observations but could not demonstrate significant discrepancies between these two groups. The culture-confirmed *C. jejuni*-related GBS cases are limited in number but worldwide provided a unique set of isolates, which has been thoroughly investigated for several genetic and phenotypic features of *C. jejuni* probably involved in GBS pathogenesis (**Chapter 3.1**).

A potential association was demonstrated between specific *C. jejuni* serotypes and *Pulsed Field Gel Electrophoresis* (PFGE) restriction profile types during winter and summer months in New Zealand, Sweden and the North West of England (33-35). Application of universal typing methods such as *multilocus sequence typing* (MLST) for seasonality of *C. jejuni* infections should be encouraged to confirm these interesting observations (36). New functional studies that investigate the pathogenicity of *C. jejuni* strains and compare strains isolated during winter or summer may identify new intrinsic virulence factors for *C. jejuni* with possible relevance for medical treatment, especially in the elderly. During winter months, the pathogenicity of *C. jejuni* is possibly influenced by the local flora at mucosal surfaces and due to dual hit infections may lead to a boost of immune responses. Comparing immune responses to antigens between GBS patients with both diarrhea and upper-respiratory tract infections and a control group hit by the same infections but who did not develop GBS, may indicate whether such a boost of immune response likely occurs in GBS. New detection methods for demonstrating recent infections, including PCR based molecular techniques and serological assays for *C. jejuni* or other pathogens, may also increase the chance of finding multiple infections occurring at the same time (37-39). A nationwide survey study that is

designed to identify each GBS patient after a positive *C. jejuni* culture will promote functional studies and increase the number of available GBS-related *C. jejuni* isolates (40).

In conclusion, new developments in techniques to detect infections are important for understanding the pathogenesis of GBS and for defining new subgroups of patients. Moreover, investigating the demography and seasonality of these subgroups may help to determine new risk factors of developing GBS after common infections.

CROSS-REACTIVITY, SPECIFICITY AND CLINICAL RELEVANCE OF ANTI-NEURAL ANTIBODIES

The expression of ganglioside mimics in *C. jejuni* LOS has been reported in several case-reports of *C. jejuni*-related GBS patients and has been associated with the presence of pathogenic cross-reactive anti-ganglioside antibodies (41-43). More convincing evidence supporting this molecular mimicry theory is obtained by chemical characterization of ganglioside mimics in series of *C. jejuni* isolates from GBS patients and relating these to clinical symptoms and host-related antibody responses. Identification of the genetic mechanisms that are involved in the variation of expression of ganglioside mimics in LOS also increases our understanding of host-pathogen interactions that determine why patients develop GBS after a *C. jejuni* infection (**Chapter 3.1**).

Using mass spectrometry we found a high frequency of mixtures of ganglioside mimics such as GM1- and GD1a-like LOS, which was found in 36% of 26 GBS-associated *C. jejuni* isolates, an association which was also later confirmed in a Japanese report (44,45). Remarkably, Kaida *et al.* recently reported patients having antibody responses to ganglioside complexes formed by mixtures of two gangliosides (46,47). In combination, both observations suggest that the mixtures observed in LOS are related to induction of ganglioside complex antibodies. Next, we searched for anti-ganglioside complex antibodies in the *C. jejuni* culture positive group of GBS patients and found 4 of 26 patients with anti-GM1/GD1a or anti-GQ1b/GD1a complex antibodies that all cross-reacted with LOS from the autologous isolated *C. jejuni* strain, expressing similar ganglioside mimics in three of these isolates (48). These results indicate that mixtures of ganglioside-like epitopes in LOS form conformational epitopes and induce anti-ganglioside complex antibodies in GBS (**Chapter 3.2**). The clinical relevance of ganglioside complex antibodies in GBS patients and other immune-mediated neuropathies requires further exploration.

2.1. Clinical relevance and detection and of anti-neural antibodies

Several combinations of ganglioside mimics were identified by mass spectrometry, which in part correlated with specific clinical variants (**Chapter 3.1**). For example, in GBS patients with ophthalmoplegia and MFS patients the presence of GD1c-like LOS was predominantly found. In addition, the presence of GM1/GD1a complex antibodies was associated with a pure motor form of GBS and the presence of GQ1b/GD1a ganglioside complex antibodies with a GBS overlap syndrome with ophthalmoplegia (**Chapter 3.2**). Likewise, ganglioside complex antibodies to GM1/GD1b, GQ1b/GD1a, GQ1b/GM1 and GalNAc/GD1a are associated with distinct forms of GBS characterized by specific neurological deficits (46,47,49). It will

be interesting to asses if antibodies to different types of combinations of ganglioside and glycolipids or glycoproteins exist and to determine which combinations are immunogenic and have a clinical correlate. The detection of anti-ganglioside complex antibodies is intricate because the environment in which these glycolipids reside influences the avidity or capacity of antibody binding by masking the antigenic epitope on glycolipids (50). This concept of new anti-neural targets formed by complexes of glycolipids in immune-mediated neuropathies, demands a critical look at diagnostic assays for antibody detection.

Previous studies have reported variable frequencies of anti-ganglioside antibodies in different immune-mediated neuropathies and other diseases using different techniques, which has caused uncertainty about the clinical relevance and correlates of these antibodies (51,52). In general, most immune-mediated neuropathies may be diagnosed also without determining anti-neural antibodies. However, it can be difficult to discriminate an immune-mediated cause of polyneuropathy from other causes of disease, especially at the onset of disease. Since these polyneuropathies are often progressive, cause a significant amount of disability and are treatable, sensitive and robust assays are required for early detection of antibodies that should be validated by testing large groups of patients and controls.

In **Chapter 5.2** we validated the European standardized INCAT ELISA technique by testing more than 1,000 patients and showed that antibodies to GM1 were highly specific for GBS (IgG and IgM 13-14%) and other inflammatory neuropathies such as MMN (IgM 44%) (51,53). A typical illustration of the diagnostic value of this ELISA is shown in **Chapter 5.1.** Here we present a case with MFS and high titers of IgG to GQ1b that after follow-up developed a clinically similar episode of dysarthria and ataxia but was in this case caused by a brainstem infarction with negative GQ1b serology. In line with our observations the gain of positive high titers (>1/640) IgM anti-GM1 serology for correctly diagnosing MN patients was recently reported in Italy (54). This study used an identical assay technique and addressed the diagnostic value of IgM antibodies to gangliosides and other neural antigens, including myelin-associated glycoprotein (MAG), in chronic forms of immune-mediated neuropathy (54). What we may learn from these studies is that in patients with reasonable doubt about the cause, IgG and high titers of IgM antibodies to GM1 strongly increases the chance that an immune-mediated cause defines the type of neuropathy. There is a rationale for starting or adjusting immunomodulating treatment for such patients although further research is required (55,56).

One of the hallmarks of anti-GM1 serology is that in spite of the generally low sensitivity the assay is highly specific with a specificity up to 100% (53). Developments in using synthetically produced gangliosides for immunoadsorption therapies with ganglioside coated columns (57,58), also emphasize the importance of adequate detection of anti-ganglioside antibodies without identification of false-positives for individual patients. If such therapies become available antibody titers should also be monitored accurately. This requires clinically validated, robust and reproducible assays. At present the INCAT ELISA complies best for these demands.

At Erasmus MC, the department of Immunology collaborates closely with the clinical Neurology department and has collectively set up a Neuroimmunology unit, which is specialized in the development and validation of anti-neural antibody assays. As part of this initiative we have also invested in evaluating the diagnostic value of the anti-MAG serum

antibodies in polyneuropathies associated with monoclonal gammopathies. The typical clinical picture of anti-MAG polyneuropathy is a slowly progressive distal weakness with sensory deficits and ataxia, predominantly occurring in the elderly. Electrophysiology mainly shows a demyelinating form of polyneuropathy with marked prolonged distal latencies. This study shows that 72% of patients with polyneuropathy associated with an IgM monoclonal gammopathy have IgM anti-MAG antibodies when tested in ELISA compared to 44-54% when tested using two different western blot techniques (**Chapter 5.3**). We propose to use ELISA as a first screening method because it is a much less laborious and robust technique that identifies patients with a similar clinical picture of a slowly progressive sensory-motor demyelinating polyneuropathy (59).

In light of emerging new therapies such as rituximab for this group of patients, which specifically target B-cells, these findings have practical implications as well (60-62). For example, antibody levels can be monitored accurately by ELISA which could be relevant for follow-up of patients treated by rituximab (61,63,64). The identification of patients with a non-demyelinating polyneuropathy with anti-MAG antibodies in ELISA also suggests that a broader clinical spectrum is associated with anti-MAG antibodies (**Chapter 5.3**). Given the high sensitivity we may need to extend the indication for testing these antibodies beyond only patients with polyneuropathy associated with an IgM monoclonal gammopathy, also to further determine the specificity of anti-MAG antibodies (59). Professional experience, centralization and standardization of the experimental methods for demonstration of anti-neural antibodies are important to improve our ability to recognize these treatable diseases.

With respect to the origin of anti-MAG antibodies, it is possible that a clonal B-cell population during B-cell development aberrantly expands and produces monoclonal antibodies reactive to peripheral nerve antigens. It has also been suggested that these antibodies arise in response to bacterial antigens as is the case in *C. jejuni*-related GBS (65). Future studies should confirm whether CMV infections or other infections play significant roles in these patients (66,67)**.**

This thesis emphasized the afferent phase during the immune response in GBS, highlighting the origin and detection of anti-neural antibodies. During the efferent phase, in which anti-ganglioside antibodies bind to peripheral nerves, complement activation through the classical pathwayplays or via the lectin pathway plays a crucial role in disruption of motor nerve terminals (68,69). Inhibition of complement activation is a potential therapeutic option and its efficacy in mice and rabbit models has recently been shown (70,71). Further demonstrating the pathogenicity of anti-ganglioside complex antibodies in complement-mediated nerve injury strengthens the clinical implication of the detection of these antibodies (72).

Accurate (diagnostic) assays to detect antibodies to neuronal targets composed of glycolipids, glycoproteins or combinations of these are desirable. With the extending number of possible targets formed as ganglioside complexes, ELISA techniques become impractical because only a limited number of combinations can be tested in a single run. Glycan microarray techniques have been developed in which antibody binding to hundreds of glycolipids covalently bound to glass slides can be assessed (73). This attractive approach could be adapted for detection of anti-ganglioside complex antibodies for patients with immune-mediated neuropathies. One has to keep in mind the that solid phase environments of assays, which in the case for ELISA (polystyrene) is highly negatively

charged, limits the flexibility and as a consequence the accessibility for antibody binding of masked epitopes formed by complexes of gangliosides (74). Recently, a glycolipid array for antibody detection to ganglioside complexes has been developed that makes use of an automated thin-layer chromatography system in a non-solid phase polyvinylidine fluoride environment. This assay enables antibody binding to ganglioside complexes in a non-immobilized fashion and showed interesting differences between ELISA results for antibody specificity of serum from GBS patients (74). Validation of these methods in groups of patients with immune-mediated neuropathies is necessary to evaluate the value of these new assays in clinical practice.

***C. jejuni* mimicry and specificity of anti-ganglioside antibodies in GBS**

Previous serological studies indicated a strong relationship between antibody specificity to gangliosides in GBS patients and ganglioside mimics in LOS from autologous *C. jejuni*. Remarkably, we observed that the overlap between antibody specificity in patients and the ganglioside mimics found by mass spectrometry in *C. jejuni* LOS was not complete (**Chapters 3.1 and 3.2**). In addition, in 6 of 26 GBS-related *C. jejuni* strains no ganglioside mimics were found at all. This study provides molecular evidence that ganglioside mimics in LOS induce anti-ganglioside antibodies, which is in accordance with indirect observations from the previous serological reports (44,75). The LOS biosynthesis class A LOS locus was associated with GBS and the expression of GM1-like structures, whereas the class B LOS locus was associated with MFS and the expression of GQ1b-like structures. Mechanisms that explain the observed discrepancy and genetic pathways in this process have been addressed in **Chapter 3.1**. Specific base deletions and frame-shift mutations in *sialyltransferase* genes of the LOS biosynthesis cluster in three strains without ganglioside mimicry were found that may have occurred during colonization or culture. Alternatively, a different *C. jejuni* strain may have been isolated from patients that were infected by multiple strains (76). Interactions between hosts and pathogens during colonization and the immune response could be involved in this process.

Relating the anti-ganglioside antibody specificity to the LOS carbohydrate composition in terms of the number of carbohydrate molecules and attached sialic acid groups is an oversimplification of the actual interactions between host receptors, bacterial epitopes and antibodies. For example, the presence of bacterial GD1c-like LOS is highly associated with induction of anti-GQ1b antibodies. This can be explained because GD1c and GQ1b share the presence of terminal di-sialylated carbohydrates. Nevertheless, GD1c and GQ1b are not identical structures (Table 2, Introduction). A different example is that a proportion of serum anti-GM1 antibodies bind to cryptic GM1 which is only accessible after the unmasking of neighboring gangliosides such as GD1a (50). Gangliosides reside within glycosynapses in which nearby factors such as other adjacent glycolipids or (glyco-) proteins alter electrostatic charge and hydrophilic interactions and modulate signaling pathways (77). The proximity of different gangliosides in membranes enables the formation of so called conformational epitopes that are comprised of contiguous but physically discontinuous components of the immunogenic molecule. The presence of such conformational epitopes in the *C. jejuni* cell wall could well explain the presence of ganglioside complex antibodies in a subgroup of GBS patients (78). Chemical characterization of the exact molecules to which anti-ganglioside

complex antibodies bind in *C. jejuni* LOS or in immunoassays may be essential to further demonstrate this and would be supplementary to the observed serum cross-reactivity described in **chapter 3.2**. Whether the cellular wall of *C. jejuni* and the host cell membrane forms a so called glycosynapse and coordinates downstream signaling events should be determined. Carbohydrate microarrays and synthetically produced glycolipids are essential new tools that can be exploited to investigate this hypothesis (58,79). Revealing new host and pathogen interactions, possibly at the level of a glycosynapse, increases our understanding of bacterial strategies for survival and infection and could lead to new therapeutic or preventative developments.

Although the mimicry function of *C. jejuni* to express gangliosides may have evolved to evade immune recognition, in GBS it seems to result in an exaggerated immune response to self-structures (80). Several host-pathogen interactions, as listed in the Table, may interfere with recognition of pathogens during the aberrant immune response in GBS. Underlying mechanisms such as adherence and entry of pathogens, dissemination of infection, multiplication and immune evasion or modulation all involve specific interactions between host and pathogen (81). Sialylation of bacterial or host surfaces plays a critical role in these mechanisms and is an interesting field for further research. Epithelial cell invasion by *C. jejuni* depends on sialylation of LOS and sialylation is involved in the pathogenicity of various other bacteria and viruses such as *Neisseria meningitidis* and *Influenza viruses* (82-84). *Haemophilus Influenza*, also thought to be involved in triggering GBS in a substantial proportion of GBS patients (85), incorporates host-derived sialic acids into its LPS increasing its virulence (86). Pathogen-derived sialidases also modulate immune responses by freeing the pathogen from host surfaces (83). In addition, variation in sialidase gene transcription leads to a modulation of the capacity of antigen presenting cells to stimulate B-cells through IL-4 (87,88). Thus, variation in genes encoding sialidases with a functional relevance for sialidase activity could theoretically modulate immune responses at mucosal surfaces to *C. jejuni* and additionally lead to differences in the self repertoire of gangliosides in the nervous system. Comparing SNPs in genes encoding sialidases in GBS patients and healthy individuals therefore is an interesting approach to assess whether such SNPs increase host susceptibility to develop GBS (87). Given the crucial role of sialic acid in various host-pathogen interactions it is interesting to further determine the effect of sialylation of *C. jejuni* and other GBS-related pathogens in human immune responses to understand how cross-reactivity is induced in GBS.

In conclusion, the presence of microbial targets consisting of more than one molecule has been demonstrated in LOS of a unique set of 26 GBS-related *C. jejuni* strains and were found to induce ganglioside-complex antibodies. Variability of antibody responses and clinical symptoms could be related to variable expression of ganglioside mimics in LOS due to genetic changes in *C. jejuni*. Interactions between pathogens and hosts in which multiple risk factors simultaneously influence the chance of inducing a cross-reactive immune response may determine the development of GBS. Further studies that characterize these interactions may lead to new targets for prevention or treatment of GBS.

Table. Examples of factors that may interfere with host-pathogen recognition during host immune response to pathogen.

Process	References
Double infection with additional *C. jejuni* strain or microorganism with different pathogenicity	(76)
Genetic changes in LOS locus during colonization, influencing the expression of ganglioside mimics in *C. jejuni*	(44,89)
Bacterial invasiveness and pathogen-pathogen interactions influencing local flora and inflammatory environment	(84,90)
Uptake of sialic acids or secretion of sialidases influencing receptor-ligand interactions between pathogen and host or between pathogens	(86,87)
Variability between individuals in distribution and self-repertoire of ganglioside expression	(91-94)
Clustering of gangliosides and receptors that change the composition in microdomains of lipid rafts	(77)

HOST IMMUNE RESPONSE TO GANGLIOSIDE MIMICS IN GBS-RELATED *C. JEJUNI*

Interactions between host and pathogens take place at several stages during immune responses and because the innate immune response is defined as the host's first barrier for entry of pathogens, studying innate responses to *C. jejuni* may be of particular interest for understanding GBS pathogenesis. Innate immunity has evolved to swiftly respond to evolutionary conserved microbial structures such as lipid A, also present in *C. jejuni* LOS, and coordinates suitable types of adaptive immune responses (95). The characterization of the adaptive immune response and identification of cross-reactive serum antibodies and relationship with antecedent infections in GBS has received much attention during the past two decades but the host-related receptors, cells and pathways involved in this aberrant immune response are largely unknown. Reducing this gap in our understanding of GBS pathogenesis may have general implications for understanding other post-infectious diseases.

An important cell type of innate immunity is the dendritic cell (DC), a professional antigen-presenting cell (APC) type abundantly present underneath the epithelial cell layer in the gut. In our focus on *C. jejuni*-related GBS this anatomical localization is of particular interest and gut DCs are presumably one of the first immune cells to interact with invading pathogens such as *C. jejuni* (96). DCs can directly interact with B-cells providing signals to class switch antibody isotype without T-cell help (96,97). As such, these important features position DCs at a critical checkpoint for interactions between host and pathogen that could be of relevance for the induction of the aberrant immune response in GBS. Accordingly, we aimed to construct an *in vitro* model for humans, which would enable to study functional interactions between DCs and *C. jejuni* in relation to host- (i.e. antigen presentation) as well as pathogen- (LOS sialylation) related factors in GBS pathogenesis.

Sialylation of *C. jejuni* LOS boosts human immune response

In **chapter 4.2** we investigated the human DC response to *C. jejuni,* the influence of LOS

sialylation and whether DCs provide signals to B-cells in relation to stimulation with *C. jejuni* LOS. We found that *C. jejuni* strains from GBS patients activated DCs and showed that highly purified LOS from these strains activated DCs at very low concentrations. This activation of DCs was partly mediated by signaling through TLR4, a pattern recognition molecule that binds to lipid A, which is present in both LOS and LPS. Sialylation of LOS had strong immunomodulatory effects on DCs; enhanced DC cytokine release was found when DCs were stimulated with sialylated LOS from a *C. jejuni* strain obtained from a GBS patient compared to a genetically mutated strain lacking sialic acid (*cst-II* knockout mutant strain). A tenfold higher concentration of the non-sialylated mutant strain compared to the wild-type was required in order to obtain similar cytokine levels secreted by DCs after stimulation with LOS (**Chapter 4.2**). In addition, we found that supernatant from *C. jejuni*-stimulated DCs contained soluble factor(s) that increased human naïve B-cell proliferation. The immunomodulatory effect of sialic acid on DC activation correlated with increased B-cell proliferation. These results indicate that sialylation of *C. jejuni* LOS causes a boosting of the *in vitro* immune response, rather than functioning as a scavenger molecule for evasion of immune recognition (80). This boost of the immune response may lead to improper antigen presentation and a cross-reactive B-cell response, which in susceptible hosts might cause GBS. In the next paragraphs three possible pathways related to this process are discussed and related to available data and literature.

Receptor-mediated uptake of sialylated glycolipids

The correct balance of the amount of sialic acid expression by pathogens appears crucial for immune detection by host cells sensing sialylated surfaces by specialized receptors called lectins (98). Antigen presenting cells also express several receptors such as TLRs that recognize microbial conserved structures and trigger cell activation. We observed that DC activation by *C. jejuni* LOS was partly inhibited when TLR4 signaling was prevented by a blocking mAb. In addition, a significantly lower response in HEK/TLR4 cells after enzymatic removal of sialic acid residues from LOS was shown, indicating a potential role for TLR4 in the differential activation of DCs by sialylated LOS. In line with these findings it was found that gangliosides, containing sialic acid residues by definition, alter TLR4 expression in astrocytes and trigger inflammatory responses (99). However, the mechanism by which sialic acid may alter TLR4 binding or the configuration of the lipid-A carbohydrate complex is unknown. In analogy to the situation in which functional units are formed by TLR4 and either MD-2 or CD14, which subsequently binds to smooth or rough LPS, conformational and electrostatic differences in sialylated LOS may cause that binding to TLR4 occurs in a different functional unit or context (100).

Such functional units for LOS binding may be formed by specific sialic acid binding lectins called Siglec's (sialic acid binding Ig-like lectins) and other calcium dependent C-type lectins. These receptors bind to carbohydrate molecules and theoretically may bind ganglioside mimics in LOS. Siglec's are thought to be involved in monitoring self-antigens by detecting changes in expression of sialylated proteins- and glycolipid rich surfaces (101-103). Two subsets of Siglec's have been characterized that differ in the presence of an anchored cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) that suppresses immune activation, or the absence of this motif such as seen in Siglec-14, -15 and -16 (98,104).

Binding of *C. jejuni* LOS expressing ganglioside mimics to Siglec-7 has been demonstrated but the functional effects on DCs and the immune response is unknown (105).

Siglec's contribute to endocytosis of antigens after *trans* interactions (interactions with ligands on other cells) with pathogens (82,98,106). In theory, activation of DCs through TLR4 by LOS may lead to unmasking *cis* interactions (interactions with ligands on the same cell surface) of endogenous sialic acids with Siglec's, which can facilitate access or binding of sialylated LOS to Siglec's and as a result increase endocytosis of *C. jejuni* by DCs. Interestingly, a link between Siglec's and TLRs has been suggested for Siglec-H which associates with immunoreceptor tyrosine-based activation motif (ITAM) through DAP12 adapter molecules after stimulation with TLR-9 agonists (107) (108). Whether functional units formed by TLRs and Siglec's may explain our observed modulation of TLR-4 signaling by sialylated LOS is unknown but would be an interesting question for further research. Unraveling these complex mechanisms for activation of DCs by LOS helps to elucidate the question of how immune responses are boosted by sialylated LOS and how this leads to a derailed co-stimulation of auto-reactive B-cells in GBS.

Antigen presentation of ganglioside-like LOS

After antigen uptake by innate immune cells such as DCs, one of the crucial events during the immune response is the presentation of these microbial antigens to other immune competent cells. Antigen presentation is a molecular process in which self- and non-self antigens are continuingly processed by molecules including the major histocompatibility complex (MHC) system and CD1 group and is designed to prevent harmful auto-reactive immune responses. It has been shown that dendritic cells expressing CD1 induce proliferation of GM1-restricted γ-δ T-cells when stimulated by *C. jejuni* (109). Moreover, dendritic cells secrete de novo produced self-gangliosides upon stimulation with *C. jejuni,* possibly further recruiting surrounding GM1-specific T- or B-cells. Because CD1 molecules present glycolipids including GM1 (110), the aberrant immune response to *C. jejuni* in GBS may be due to an inaccurate antigen presentation by CD1 molecules in particular.

Genetic studies in GBS patients mainly identified disease modifying factors influencing the severity of the disease and outcome (Table 3 in the Introduction) but the host-factors that determine the susceptibility to develop GBS after antecedent infections are poorly defined and could be related to an aberrant presentation of antigens (111). In support of this hypothesis, a recent study in an Italian GBS cohort consisting of 65 patients showed an increased susceptibility for GBS associated with *CD1A* and *CD1E* SNPs (112). This association also seemed specific for GBS because it could not be found in other immune-mediated neuropathies (113). These results implicate that an important host-related factor was identified for development of GBS. Reproducing these results in a different GBS cohort was therefore relevant especially when using a much larger cohort, enabling subgroup analysis for patients with anti-ganglioside antibodies and antecedent *C. jejuni* infections.

In **chapter 4.1**, the genetic variation in CD1 molecules was studied in currently the largest cohort of GBS patients in the world assembled in The Netherlands (*n*=312), in which the association between *CD1A* and *CD1E* SNPs and GBS susceptibility could not be replicated (114). This difference was probably related to sample size differences. The last study was five times larger and consequently has a stronger statistical power. A definite role for *CD1A*

and *CD1E* SNPs in GBS susceptibility cannot be claimed. Larger databases of GBS patients are required, which can only be established by extensive international collaborations and platforms (115).

A functional role for CD1 could also not be established in an elegant study using CD1d knockout mice (110). Cross-reactive anti-ganglioside antibodies (IgG1, IgG2b, and IgG3 isotypes) in response to *C. jejuni* infection were similar in wild-type mice and in CD1d knockout mice, suggesting that at least this group of CD1 molecules are not critically involved. In contrast to humans, mice do not express CD1e (110). Further addressing the role of this molecule in GBS is recommended because this intracellular protein processes complex glycolipids and is involved in loading glycolipids onto CD1b in late endosomes and lysosomes. Although it has not been demonstrated yet that CD1e transports gangliosides, crystallographic imaging showed the presence of GM2 within the binding groove of the CD1b molecule (116,117). Interestingly, mutations in *CD1E* have been reported with functional effects on antigen presentation, supporting the suggestion that sequencing *CD1E* genes from groups of GBS patients may reveal unknown but possibly relevant mutations (118). Exploring the functional effects of such mutations by siRNA methods, which enables switching off single genes, could be an interesting alternative for *in vitro* cell culture systems with *C. jejuni* strains from GBS patients. These studies help to answer the question whether cross-reactive immune responses to *C. jejuni* are caused by a host-dependent aberrant presentation of ganglioside-like antigens through CD1.

Coordination of the adaptive immune response

The boost of the immune response elicited by sialylated *C. jejuni* LOS was also reflected in enhanced B-cell proliferation in the presence of supernatant from stimulated DCs (**Chapter 4.2**). In order to determine how B-cell proliferation is regulated by DCs, the role of B-cell activating molecules such as IL-6 and IL-12, which were also present in supernatant of stimulated DCs (119,120)**,** was studied by using neutralizing antibodies. These cytokines were not found to be critical for boosting the B-cell response in our model, which was in contrast to results from a previous study that investigated the DC and B-cell response to *E. coli* LPS, however the study protocol in that case included T-cell help (120). Since anti-ganglioside antibodies induced by *C. jejuni* LOS are often isotype-class switched antibodies (IgA,IgG), which is uncommon for antibodies to carbohydrate antigens that are mostly T-cell independent, an alternative stimulation of B-cells by DC-derived secreted factors may be involved.

Factors secreted by DCs such as BAFF, APRIL, and C4b binding protein (C4BP) are known to induce isotype-class switch without T-cell help and TLR agonists may also synergize and deliver direct signals to B-cells for isotype-class switching (97,121-124). Increased serum BAFF levels occur in autoimmune diseases such systemic lupus erythematosus, primary Sjogren's syndrome and rheumatoid arthritis and are associated with increased serum anti-nuclear antibodies (125). Likewise, the induction of auto-reactive anti-ganglioside antibodies by antecedent infections in GBS may be associated with increased circulating levels of BAFF and APRIL, which are mainly secreted by DCs but also other cell types such as gut epithelial cells (126). C4BP is a regulator component of the classical complement pathway and can bind to CD40 on B-cells, is upregulated by monocytic cells and could reduce the amount of CD40L

needed to activate and class-switch B-cells (121,122,127,128). The number of other possible soluble interacting factors that modulate the B-cell response in relation to DCs stimulated by *C. jejuni* or other GBS-associated pathogens is daunting. In addition, it is unknown whether the observed effects only lead to a general impulse for B-cells or whether this may also lead to detectable (auto-reactive) antibody production. From a strategic point of view, implementing screening methods that diminish the number of possible causative factors like dialysis and microarray techniques is desirable. Results from such studies possibly bring us closer to the cause of the breakdown of B-cell tolerance, sometimes occurring after a relatively benign infection in GBS, but more generally leading to more long-lasting antibody-mediated autoimmune diseases such as anti-MAG polyneuropathy, MMN or Sydenham's chorea. When GBS patients develop their neurological symptoms the production of auto-reactive antibodies may be an ongoing process, as the titers of antibodies in some patients decay after several months whereas the half-life of antibodies is shorter. Investigating the regulation of auto-reactive B-cells or plasma cells by DCs may lead to new therapeutic developments that may be implemented in the earliest stages of disease.

Experimental limitations and directions for future studies

Other pathogens than *C. jejuni* are involved in GBS, which were not addressed in the current study (**Chapter 4.2**). Further limitations related to the conclusions of this study are caused by the methodology and use of an *in vitro* model to study a human disease. The monocyte-derived DC used is an artificial cell type that is skewed by exogenous cytokines and growth factors and may function differently than DCs present in the lamina propria of the gut (129). The advantage however, is that the results are less likely to depend on variation in DC phenotypes existing for example in peripheral blood or in the gut. The human tonsillar naïve B-cells used in the study also represent an approximation of mucosal B-cells but included relevant cell types such $CD5^+$ B1 cells responding to thymus-independent antigens.

Elegant and suitable animal models with rabbits have been developed that can in part overcome these shortcomings but require laborious and intensive protocols (130,131). Earlier studies by different groups failed to induce weakness in *C. jejuni* immunized mice, rats and rabbits (132-135). The advantage of applying an animal model is that it enables investigation of pathological changes at several anatomical localizations such as peripheral nerves and the intestine. For example, it would be interesting to use fluorescently traceable *C. jejuni* strains for infecting these rabbits and to investigate by fluorescence light-microscopy methods the localization, spreading and surrounding immune cells of *C. jejuni* invasion. This could answer the question whether cross-reactive *C. jejuni* responses arise in the gut or as a result of a systemic infection in secondary lymphoid tissues. However, caution should be taken in extrapolating the results from animal studies to the human situation, especially in relation to human B-cell responses to carbohydrate antigens in which important species differences exist (136-138). Therefore the development of a human experimental system in which host- and pathogen related factors could be addressed is an additional relevant method for studying the pathogenesis of GBS.

The next step forward would be to obtain DCs from GBS patients and compare immune responses with healthy individuals or gastroenteritis patients that did not develop GBS after *C. jejuni* infections. Ideally, assessment of DC responses to autologous *C. jejuni* isolates from

GBS patients is performed by using DCs isolated from the lamina propria or DCs generated from peripheral blood from GBS patients (during the acute stage of disease and after recovery). These studies could identify whether constitutional differences in GBS patients exist regarding functional DC responses to ganglioside-expressing microbes explaining their susceptibility of developing a cross-reactive immune response. Less than 5% of GBS patients have recurrences, an interesting subgroup for further study in this context (139). Characterization of the intra-cellular pathways by micro-array techniques may subsequently lead to strategies for developing new therapies.

CONCLUSION

GBS is preceded by infections and pathogen-related factors in part cause the disease. Host-related factors such as the induction of cross-reactive anti-ganglioside antibodies cause damage of peripheral nerves. It is important to consider that GBS is a heterogeneous disease triggered by different pathogens that are incompletely characterized. The most important infection is caused by *C. jejuni* during the winter months. Combinations of ganglioside-like structures in *C. jejuni* LOS form conformational epitopes and induce cross-reactive anti-ganglioside complex antibodies. The clinical relevance of these antibodies has been validated and may become clearer by developing new diagnostic assays to detect anti-ganglioside antibodies. An important functional interaction between host and pathogen was observed in which sialylation of *C. jejuni* LOS results in boosting of DC and subsequent B-cell responses. Characterization of the pathways involved in this process (Figure 1) could contribute to the development of new therapies for GBS. Progress in understanding the pathophysiology of the different variants and levels of severity of GBS depends critically on further unraveling the mechanisms of interactions between pathogens and patients.

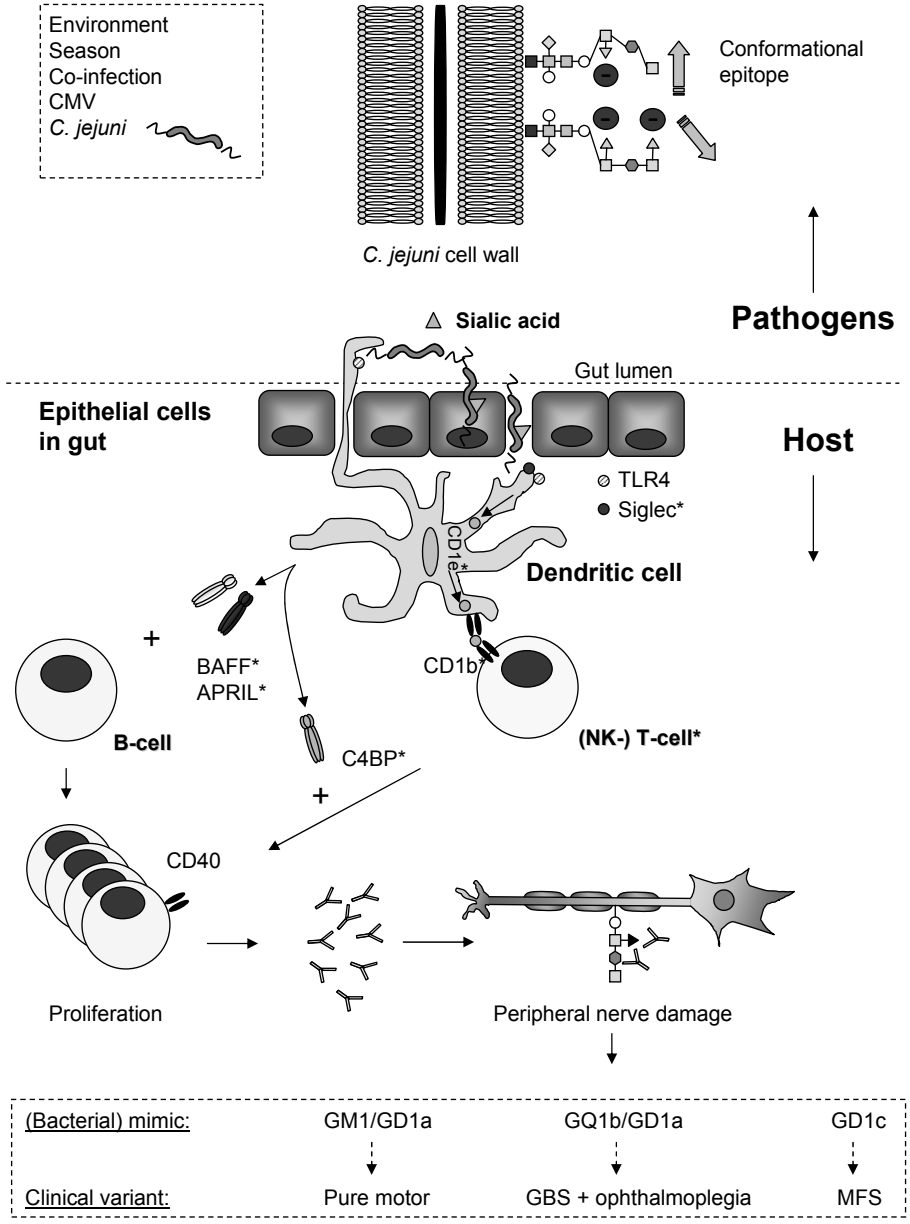


Figure 1. Hypothetical model of host-pathogen interactions in GBS.
Conformational epitopes present in the outer cell wall of *C. jejuni* LOS induce cross-reactive antibodies leading to GBS. *C. jejuni* invades intestinal epithelium from the lumen of the intestine. Pattern recognition receptors and sialic-acid binding Ig-lectins (siglec's) on DCs may bind to LOS and induce an inflammatory response and enhance B-cell proliferation. The conformation of the mimics present in *C. jejuni* LOS are related with the clinical variants of GBS. TLR4: Toll-like receptor 4, BAFF: B-cell activating factor, APRIL: a proliferation inducing ligand, C4BP: C4b binding protein, *: Putative pathways.

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SUMMARY

Guillain-Barré syndrome (GBS) is a neurological disorder characterized by rapid progressive and symmetrical limb muscle weakness and loss of tendon reflexes. Sensory deficits and cranial nerve involvement is observed to a variable extend. Some patients have additional weakness of respiratory miscles for which they may require prolonged ventilation at an intensive care unit. The disease is caused by a post-infectious immune mediated polyradiculoneuropathy and has a monophasic course with a variable and often incomplete recovery. The Miller Fisher syndrome (MFS) is a variant of GBS that is characterized by double vision, inability to coordinate movements and loss of tendon reflexes. *Campylobacter jejuni* is the most frequent cause of preceding infection in GBS and MFS, and is identified in 20-50% of patients. Preceding *C. jejuni* infections are associated with a more severe clinical course and poorer outcome. Other infections that can precede the development of GBS include *Mycoplasma pneumoniae* and viruses such as cytomegalovirus and Epstein-Barr virus. An immune response orchestrated to eradicate these infections follows an aberrant course in GBS, which eventually leads to destruction of peripheral nerves and muscle weakness. It has been shown that *C. jejuni* isolated from individual GBS patients bear specific structures on the outer surface that resemble structures (gangliosides) present on human nerves. This resemblance is called molecular mimicry and is held responsible for the induction of antibodies that cross-react to *C. jejuni* and peripheral nerves.

Previous studies identified several **host-related factors** associated with GBS, including demographic features, variation in genes of the immune system and presence of serum antibodies to gangliosides. Other studies found **pathogen-related factors** involved in the pathogenesis of GBS, including distinct types of antecedent infection, such as *C. jejuni*, and of molecular mimicry with gangliosides. The aim of the research described in this thesis was to identify mechanisms by which these host and pathogen factors induce GBS and delineate the clinical relevance of these factors.

First, the epidemiology of specific infections associated with GBS are investigated. In **Chapter 2.1** we assessed whether CMV infections are as frequent as *C. jejuni* in GBS as was suggested in a previous study performed by another research group based on analysis of cerebrospinal fluid samples from patients using a sensitive molecular technique to detect the virus genome. A large set of cerebrospinal fluid samples from Dutch GBS patients was tested for comparison using a similar technique. In contrast to the previous report, only 3 of 171 samples were found positive for a recent CMV infection. Therefore, it is too early to suggest that CMV plays a similar important role as *C. jejuni* preceding the development of GBS. The difference between our results and previous published data may be related to the selection of patients and the tested material.

The remaining chapters focus on the most frequent preceding infection in GBS caused by *C. jejuni*. Food products, especially from chicken and shell fish, may contain these gut bacteria and cause gastroenteritis, with severe diarrhea, or remain unnoticed. In **Chapter 2.2** it is described whether age and season of infection by *C. jejuni* are risk factors for development of GBS. A striking inverse pattern was observed when the age distribution of patients with or without development of GBS after a *C. jejuni* infection was compared. Similarly, an inverse pattern was also observed for the season of becoming infected. *C. jejuni*-related GBS patients

tended to be older and more frequently infected during winter months suggesting that both environmental and host-related factors may play a role for the risk to develop GBS after a *C. jejuni* induced gastroenteritis which processes underlie this phenomenem are unknown.

In **Chapter 3.1** the combined results are described from molecular and genetic studies of a set of 26 *C. jejuni* strains, which were isolated from or associated with GBS patients. Never before a large group of *C. jejuni* strains was available for such an extensive investigation of the molecular mimicry theory. The compilation of strains and patients now also enabled the possibility to investigate the clinical relevance of the presence of ganglioside-like structures on the outer surface of *C. jejuni*. We found that a majority of the *C. jejuni* strains express combinations of ganglioside-like motifs although strains without ganglioside-like motifs were also found. Genetic mechanisms responsible for the lack of ganglioside expression were found in part of these strains. In **Chapter 3.2** is described that four of these GBS patients had antibodies to combinations of gangliosides, so called ganglioside complex antibodies, which were also induced by similar combination of ganglioside-like structures present in these bacteria.

In **Chapter 4** we move forward to the role of host-related factors in the development of GBS. We investigated whether variation in CD1 genes is a susceptibility factor for GBS. CD1 binds to certain gangliosides and is amongst others expressed by dendritic cells, a specific antigen-presenting cell of the innate immune system and is one of the major coordinators of the type of required adaptive immune response. A previous study in a cohort of 65 Italian patients with GBS reported that specific variations occurring in two genes of CD1 (CD1A and CD1E) increase the chance to develop GBS. This important association for understanding the susceptibility to develop GBS was also studied in the Dutch cohort of almost 400 patients, but these variations were not associated with specific subgroups of patients (**Chapter 4.1**). This inconsistency illustrates that genetic association studies for diseases like GBS need large number of patients and well defined cases records and strict guidelines for performing such studies. The role of dendritic cells as possible coordinator of the aberrant immune response in GBS is addressed in **Chapter 4.2**. Here we investigated whether sialic acid, present in the outer membrane of *C. jejuni* and an important constituent of gangliosides, influences the extent of activation of dendritic cells. It was found that *C. jejuni* strains expressing sialic acid were 10 times more efficient in activation of dendritic cells compared to genetically mutated strains lacking the expression of sialic acids. In addition, dendritic cells activated by *C. jejuni* were found to secrete factors that enhance the growth of B-cells, precursor cells of antibody producing plasma cells. The involvement of specific receptors in activation of dendritic cells was investigated and the possible mechanisms related to the activation of dendritic cells, growth of B-cells and modulation by sialic acids are highlighted in the general discussion. These results contribute to the understanding of the initial steps in the pathogenesis of GBS and may eventually lead to the development of new preventative strategies or therapies.

Chapter 5 is dedicated to the detection of antibodies to peripheral nerve carbohydrate structures, including glycolipids and glycoproteins, and the role that antibody detection may have in clinical practice. Antibodies to gangliosides are mainly found in GBS but also in other immune-mediated diseases of peripheral nerves. For example, antibodies of the IgM isotype to the GM1 ganglioside are frequently found in multifocal motor neuropathy, a progressive disease with asymmetric weakness in limb muscles, especially of the arms, with

a patchy distributed lesion of the peripheral nerves, causing a slow but severe functional decline. In **Chapter 5.2**, a technical validation of the ELISA technique used for detection of anti-ganglioside antibodies is performed. Next, an analysis of more than 1,000 patients tested for anti-ganglioside antibodies is done in which the diagnostic value was assessed by comparing test results between patients with immune-mediated causes of polyneuropathy and different control groups. Although antibodies to gangliosides are found infrequently, an additional diagnostic value was especially found for patients with a low *a priori* chance of an immune-mediated cause for their neuropathy. In **Chapter 5.1**, a typical example of the diagnostic value of antibodies to the GQ1b ganglioside is described in a patient with recurrent symptoms resembling Miller Fisher syndrome but seemed to be mistaken for a brainstem infarction. In **Chapter 5.1**, a typical example is presented that illustrates the diagnostic value of the detection of antibodies to the GQ1b ganglioside. The patient had two episodes with very similar neurological symptoms. The first episode was caused by the Miller Fisher syndrome and serum obtained at that time point contained high titers of IgG antibodies to GQ1b. After this the patient showed an almost complete recovery. In the second episode these antibodies were absent and an MRI scan of the brain identified a brainstem infarction. In **Chapter 5.3**, a new technique was validated to test sera for the presence of antibodies to myelin-associated glycoprotein (MAG). This antibody is not related to GBS, but to another distinct form of immune-mediated neuropathy in which patients have a slowly progressive form of loss of sensation, weakness, often causing severe problems in walking and other coordinating intended movements. This new ELISA technique was compared to the existing golden standard technique of Westernblotting, and showed that ELISA detected anti-MAG antibodies more frequently in patients with this neurological phenotype and is a more sensitive diagnostic assay.

SAMENVATTING

Het Guillain-Barré syndroom (GBS) is een neurologische aandoening die wordt gekenmerkt door snel progressieve symmetrisch verdeelde spierzwakte in ledematen en verlies van peesreflexen. Gevoelsstoornissen en betrokkenheid van hersenzenuwen komt ook in een variabele mate bij patiënten voor. Sommige patiënten hebben een additionele zwakte van de ademhalingspieren waardoor zij soms langdurige kunstmatige beademing nodig hebben op een intensive care unit. De ziekte wordt veroorzaakt door een post-infectieuze polyradiculoneuropathie en heeft een monofasich beloop met een variabel en meestal incompleet herstel. Het Miller Fisher syndroom (MFS) is een variant van GBS die wordt gekenmerkt door dubbelzien, onvermogen om bewegingen te coördineren en verlies van peesreflexen. GBS wordt meestal voorafgegaan door een infectie waarvan *Campylobacter jejuni* de frequentste oorzaak is. *C. jejuni* komt voor bij 20-50% van de patiënten en is geassocieerd met een ernstiger klinisch beloop en een slechtere uitkomst. Andere infecties die voorafgaan aan de ontwikkeling van GBS zijn *Mycoplasma pneumoniae* en virussen zoals het cytomegalovirus en Epstein-Barr virus. Een storing in de afweerreactie welke in gang wordt gezet om deze infecties te bestrijden, leidt in GBS patiënten tot destructie van perifere zenuwen en spierzwakte. Het is aangetoond dat *C. jejuni* geïsoleerd uit individuele GBS patiënten specifieke structuren bevat die lijken op structuren (gangliosiden) aanwezig op de menselijke zenuwen. Deze gelijkenis of moleculaire mimicry wordt verantwoordelijk gehouden voor de ontwikkeling van antilichamen tegen *C. jejuni* die kruisreacties geven met perifere zenuwen en in dit proces ernstige schade aanrichten.

Patiënten met GBS fungeren als gastheer voor bacteriën en andere pathogenen. Vanuit dit concept is uit eerdere studies gebleken dat verschillende gastheer-gerelateerde factoren, met inbegrip van demografische kenmerken, variatie in genen van het immuunsysteem en de aanwezigheid van antilichamen tegen gangliosiden, een rol spelen bij het ontstaan en beloop van GBS. In verschillende studies werd gevonden dat pathogeen-gerelateerde factoren betrokken zijn bij de pathogenese van GBS, waaronder de verschillende soorten voorafgaande infecties, zoals *C. jejuni*, en de moleculaire mimicry met gangliosiden. De doelstelling van dit promotie-onderzoek was het identificeren van mechanismen waarmee deze gastheer- en pathogeen-gerelateerde factoren GBS veroorzaken waarbij getracht is de klinische relevantie van deze factoren af te bakenen.

Als eerste wordt de epidemiologie van specifieke infecties die geassocieerd zijn met GBS beschreven. In **Hoofdstuk 2.1** wordt behandeld of CMV-infecties net zo frequent voorkomen in GBS als *C. jejuni*, zoals onlangs werd gesuggereerd in een studie uitgevoerd door een andere onderzoeksgroep en was gebaseerd op een analyse van hersenvocht monsters uit patiënten waarin gebruik werd gemaakt van een gevoelige moleculaire techniek om het virus genoom aan te tonen. Een grote verzameling monsters van hersenvocht afkomstig van Nederlandse GBS patiënten werd ter vergelijking door ons getest met een identieke techniek. In tegenstelling tot het eerdere rapport, was in deze studie 1 van de 171 monsters positief voor CMV. Hoewel er bewijzen zijn dat in ongeveer 15% van de GBS patiënten CMV infecties een rol spelen, kunnen we de suggestie dat CMV een vergelijkbare rol speelt als *C. jejuni* (ongeveer 30-40% van alle GBS patiënten) voorafgaand aan het ontstaan van GBS nog niet onderbouwen. Het verschil tussen onze resultaten en de eerdere gepubliceerde gegevens kan te maken hebben met de selectie van patiënten en het geteste materiaal.

In de overige hoofdstukken wordt de aandacht gericht op de meest voorkomende infectie voorafgaand aan GBS die wordt veroorzaakt door *C. jejuni.* Voedselproducten, voornamelijk kippenvlees en schaaldieren, kunnen deze darm geassocieerde bacteriën bevatten en veroorzaken een gastro-enteritis die met ernstige diarree gepaard kan gaan maar ook onopgemerkt kan verlopen. In **Hoofdstuk 2.2** wordt beschreven of leeftijd en het seizoen waarin een infectie door *C. jejuni* ontstaat risicofactoren zijn voor het ontwikkelen van GBS. Een opvallend omgekeerd patroon werd waargenomen voor seizoen om besmet te raken en leeftijd bij het vergelijken van een grote groep patiënten met *C. jejuni*-gerelateerde gastro-enteritis met en zonder GBS. *C. jejuni*-gerelateerde GBS patiënten waren doorgaans ouder en werden vaker besmet tijdens de wintermaanden. Dit suggereert dat zowel omgevings- en gastheer-gerelateerde factoren een rol kunnen spelen in de ontwikkeling van GBS na *C. jejuni* veroorzaakte gastro-enteritis. Welke processen hieraan ten grondslag liggen is vooralsnog onduidelijk.

In **Hoofdstuk 3.1** worden de gecombineerde resultaten beschreven van moleculaire en genetische studies uit een reeks van 26 *C. jejuni* stammen, die werden geïsoleerd uit of samenhang hadden met GBS patiënten. Nooit eerder was een groep van *C. jejuni* stammen beschikbaar voor een dergelijk uitgebreid onderzoek naar de moleculaire mimicry theorie en de klinische relevantie ervan. Dit laatste bleek uit associaties tussen specifieke ganglioside-achtige structuren op de buitenkant van *C. jejuni* en uitvalsverschijnselen bij besmette patiënten. Verder werd gezien dat een meerderheid van de *C. jejuni* stammen uitdrukkelijke combinaties van ganglioside-achtige motieven bevatten, hoewel ook stammen zonder ganglioside-achtige motieven werden gevonden. De genetische mechanismen die verantwoordelijk zijn voor het ontbreken van deze ganglioside-achtige motieven werden geïdentificeerd in een deel van deze stammen. In **Hoofdstuk 3.2** wordt beschreven dat sommige van deze GBS patiënten antilichamen hadden tegen combinaties van gangliosiden, de zogenaamde antilichamen tegen ganglioside complexen. Door middel van kruisreactie experimenten werd bewezen dat deze antilichamen werden veroorzaakt door besmetting met een *C. jejuni* stam met een vergelijkbare combinatie van ganglioside-achtige structuren aanwezig in deze bacteriën.

In **Hoofdstuk 4** wordt gekeken naar de rol van gastheer-gerelateerde factoren in de ontwikkeling van de GBS. Onderzocht werd of het hebben van specifieke variaties in CD1 genen een rol speelt in het risico voor het ontwikkelen van GBS. CD1 moleculen kunnen binden aan bepaalde gangliosiden en worden onder andere gevonden in en op dendritische cellen, een specifieke antigeen-presenterende cel van het immuunsysteem en een belangrijke coördinator als het gaat om het aansturen van het type immuun-respons voor verschillende lichaamsvreemde gevaren. Een eerdere studie in een Italiaans cohort van 65 GBS patiënten toonde aan dat specifieke variaties in twee genen van CD1 (CD1A en CD1E) een verhoogd risico gaven om GBS te krijgen. Deze belangrijke associatie voor het begrijpen van de gevoeligheid om GBS te krijgen, werd in onze Nederlandse database van bijna 400 GBS patiënten niet gevonden en kon ook niet geassocieerd worden met specifieke subgroepen van patiënten (**Hoofdstuk 4.1**). Deze inconsistentie illustreert dat genetische associatie studies voor ziekten als GBS, grote groepen patiënten met goed gedefinieerde gevallen en strikte richtlijnen voor het uitvoeren van dergelijk onderzoek vereisen. De rol van dendritische cellen als mogelijke coördinator van de afwijkende immuunrespons bij GBS wordt behandeld in **Hoofdstuk 4.2**. Hiervoor is onderzocht of siaalzuur, aanwezig in het buitenste membraan van

C. jejuni en een belangrijk bestanddeel van gangliosiden, de mate van activatie van dendritische cellen beïnvloedt. Er werd vastgesteld dat *C. jejuni* stammen die siaalzuur bevatten 10 keer efficiënter zijn in activatie van dendritische cellen in vergelijking met genetisch gemuteerde stammen die geen siaalzuur bevatten. Daarnaast vonden we dat door *C. jejuni* geactiveerde dendritische cellen factoren afscheiden die de groei van B-cellen bevorderen; voorlopercellen van antilichaam producerende plasmacellen. De betrokkenheid van specifieke receptoren bij de activatie van dendritische cellen werd onderzocht en de mogelijke mechanismen met betrekking tot de activatie van dendritische cellen, de groei van B-cellen en modulatie door siaalzuur is gemarkeerd in de algemene discussie (**Hoofdstuk 6**). Deze bevindingen geven inzicht in de eerste stappen in de pathogenese van GBS en kunnen uiteindelijk leiden tot de ontwikkeling van nieuwe preventieve strategieën of therapieën.

Hoofdstuk 5 is gewijd aan de rol van antilichamen die binden aan perifere zenuw koolhydraatstructuren, waaronder glycolipiden en glycoproteïnen, en de rol van antistof detectie voor de klinische praktijk. Antilichamen tegen gangliosiden worden voornamelijk gezien in GBS maar ook in andere immuun-gemedieerde ziekten van perifere zenuwen. Zo worden antilichamen van het IgM isotype tegen het GM1 ganglioside vaak gevonden in multifocale motorische neuropathie, een progressieve ziekte met zwakte in verschillende spieren met een fragmentarische distributie die langzame maar functioneel ernstige achteruitgang veroorzaakt. **Hoofdstuk 5.2** beschrijft de uitvoering van een technische validatie van de ELISA-techniek gebruikt voor de detectie van anti-ganglioside antilichamen. Vervolgens werd een analyse verricht, in meer dan 1000 patiënten die getest zijn op anti-ganglioside antilichamen, waarin de diagnostische waarde beoordeeld is door vergelijking van de testresultaten tussen patiënten met immuun-gemedieerde polyneuropathie en verschillende controle-groepen. Hoewel antistoffen tegen gangliosiden niet vaak worden gevonden, werd een extra diagnostische waarde vooral gezien in patiënten met een lage a priori kans op het hebben van een immuun-gemedieerde oorzaak voor hun ziekte. In **Hoofdstuk 5.1** wordt een typisch voorbeeld gepresenteerd dat de diagnostische waarde van detectie van antilichamen tegen het GQ1b ganglioside illustreert. De patiënt had twee episoden met zeer vergelijkbare neurologische symptomen. De eerste episode werd veroorzaakt door het Miller Fisher syndroom en serum monsters verkregen op dat moment bevatte hoge titers van IgG-antistoffen tegen GQ1b. Hierna bleek de patiënt bijna volledig hersteld te zijn. In de tweede episode waren deze antistoffen afwezig en toonde een MRI-scan van de hersenen een hersenstaminfarct. In **Hoofdstuk 5.3** is een nieuwe techniek gevalideerd om sera te testen op de aanwezigheid van antilichamen tegen myeline-geassocieerde glycoproteïne (MAG). Dit antilichaam is niet gerelateerd aan GBS, maar aan een andere bijzondere vorm van immuun-gemedieerde neuropathie waarbij patiënten een langzaam progressieve achteruitgang hebben van het gevoel en zwakte ontwikkelen die vaak ernstige problemen met lopen veroorzaakt en daarnaast problemen hebben met het coördineren van juiste bewegingen. Deze nieuwe ELISA-techniek werd vergeleken met de huidige gouden standaard techniek, Westernblotting, en liet zien dat ELISA vaker deze anti-MAG antilichamen kon detecteren bij patiënten met dit neurologische fenotype en een gevoeliger diagnosticum is.

LIST OF FREQUENTLY USED ABBREVIATIONS IN THESIS

Abs	Antibody
APRIL	A Proliferation Inducing Ligand
BAFF	B-cell activating factor
BAFF-R	Receptor for B-cell activating factor
TACI	Transmembrane activator and calcium-modulator and cyclophilin ligand interactor
BLyS	B-cell activating factor
C. jejuni	*Campylobacter jejuni*
CE-ESI-MS	Electrospray ionization mass spectrometry
CIAP	Chronic idiopathic axonal polyneuropathy
CIDP	Chronic inflammatory demyelinating polyneuropathy
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
cst-II	*Campylobacter sialyltransferase II*
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBV	Epstein-Barre virus
ELISA	Enzyme-Linked Immuno Sorbent Assay
F-score	GBS disability score
GBS	Guillain-Barré syndrome
H. Influenza	*Heamophilus Influenza*
INCAT	Inflammatory Neuropathy and Treatment
I-PNP	Other inflammatory polyneuropathy
LOS	Lipo-oligosaccharide
LOS locus	LOS biosynthesis gene locus
LPS	Lipo-polysaccharide
M. pneumoniae	*Mycoplasma pneumoniae*
MAG	Myelin-associated glycoprotein
mDC	Monocyte-derived dendritic cell
MFS	Miller Fisher syndrome
MMN	Multifocal motor neuropathy
MND	motor neuron disease
MRC	Medical Research Counsil sumscore
MS	Mass spectrometry
MS	Multiple sclerosis
OD	Optical density
OND	Other neurological diseases
orf	*Open reading frame*
PCR	Polymerase Chain Reaction
PNP	Polyneuropathy
PP-PNP	Paraprotein-related polyneuropathy
PRR	Pattern-recognition receptor

RNA	Ribonucleic acid
SGPG	Sulfate-3-glucuronyl paragloboside
SNPs	Single Nucleotide Polymorphisms
STARD	Standards for Reporting of Diagnostic Accuracy
TLR-4	Toll-like receptor 4
TNF	Tumor necrosis factor
WB	Western blot

APPENDIX 1.

Diagnostic criteria of Guillain-Barré syndrome

Required for the diagnosis

1. Progressive motor weakness of more than one limb
2. Areflexia or marked hyporeflexia in very weak muscles (< grade 3 MRC)

Features strongly supportive of the diagnosis

1. Progression over days to a maximum of four weeks
2. Relative symmetry
3. Mild sensory signs or symptoms
4. Cranial nerve involvement
5. Onset of recovery 2-4 weeks after progression stops
6. Autonomic dysfunction
7. Initial absence of fever
8. Elevated CSF protein after the first week of symptoms
9. CSF cell counts of 10 or fewer mononuclear leucocytes/mm^3
10. Abnormal electrodiagnostics with conduction slowing or block
11. No other identifiable cause

Feautures that rule out the diagnosis

1. A current history of hexacarbon use
2. Abnormal porphyrin metabolism
3. A history or finding of recent diphtheric infection
4. Lead intoxication
5. The occurrence of a purely sensory syndrome
6. Diagnosis of poliomyelitis, botulism, hysterical paralysis or toxic neuropathy

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

APPENDIX 2.

STARD checklist for reporting of studies of diagnostic accuracy

(version January 2003)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	
Test methods	7	The reference standard and its rationale.	
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	
	13	Methods for calculating test reproducibility, if done.	
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	

	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	
	20	Any adverse events from performing the index tests or the reference standard.	
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	
	22	How indeterminate results, missing data and outliers of the index tests were handled.	
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	
	24	Estimates of test reproducibility, if done.	
DISCUSSION	25	Discuss the clinical applicability of the study findings.	

This checklist was developed by the STARD (STAndards for the Reporting of Diagnostic accuracy studies) group and published as Bossuyt PM, Reitsma JB, Bruns DE Gatsonis PP, Glasziou PP, Irwig LM, Moher D, Rennie D, De Vet HC, Lijmer JG; Standards for Reporting of Diagnostic Accuracy. The STARD statement for reporting of diagnostic accuracy: explanation and elaboration" in 2003 by two journals: *Clin. Chem.* 2003;49:7-18 and *Ann. Intern. Med* . 2003:138:W1-12. Checklist and directions can be found at http://www.stard-statement.org.

APPENDIX 3.

Antistoffen tegen myeline-geassocieerd-glycoproteïne (MAG)

Geschreven door M.L. Kuijf en B.C. Jacobs en verschenen als hoofdstuk in "Handboek medische laboratoriumdiagnostiek"; Prelum uitgevers, mei 2009, ISBN10:9085620139.

Doel
Diagnostiek bij verdenking op een gammopathie-gerelateerde polyneuropathie.

Benodigde klinische informatie
Standaardinformatie bij aanvraag laboratoriumonderzoek (o.a. identificatie, leeftijd, geslacht, klinische gegevens/vraagstelling).

Beschrijving methodes
IgM antistoffen tegen myeline-geassocieerd-glycoproteïne (MAG) worden bepaald door middel van een *Enzyme-Linked Immuno Sorbent Assay* (ELISA). Sera worden daarbij getest in ELISA platen waarvan de wells vooraf zijn gecoat met humaan MAG, zodat in het serum aanwezige anti-MAG antistoffen kunnen binden. De gebonden antistoffen worden vervolgens aangetoond door middel van geconjugeerd anti-humaan IgM antiserum en een enzymatische reactie, waarna een kleuromslag optreedt die gemeten wordt met een ELISA reader. De hoogte van de antistofreactiviteit tegen MAG welke wordt berekend aan de hand van een standaard calibratie curve en uitgedrukt in titer-units. Indien deze antistofreactiviteit hoger is dan de grenswaarde is het serum positief voor deze antistoffen.
Belasting voor de patiënt
Venapunctie.
Voorbereiding patiënt
Geen specifieke voorbereiding; afname kan op elk tijdstip van de dag plaatsvinden.
Materiaalafname/Fixatie
Afname van bloed middels een venapunctie (stolbuis). De bepaling wordt bij voorkeur uitgevoerd in serum. Scheiding van serum door middel van centrifugeren. Indien de bepaling plaatsvindt binnen vijf dagen kan het serum worden bewaard bij 2-8°C. Indien de bepaling later plaatsvindt kan het serum beter worden bewaard bij -20°C of -80°C. Verzending van serum naar een extern laboratorium kan bij kamertemperatuur.

Mogelijke toepassingen
Diagnostiek bij verdenking op een gammopathie-gerelateerde polyneuropathie.

Contra-indicaties
Geen.

Complicaties
Geen.

Interpretatie
Bij ongeveer de helft van de patiënten met een IgM gammopathie-gerelateerde polyneuropathie wordt in het serum IgM antistoffen tegen MAG gevonden. Patiënten met deze antistoffen zijn meestal ouder dan 60 jaar en hebben meestal last van distale sensibele stoornissen, met name in de vorm van sensore ataxie van de benen. De polyneuropathie is in het algemeen langzaam progressief. Bij electrofysiologisch onderzoek wordt in het beginstadium van de polyneuropathie kenmerken gevonden van demyelinisatie. Indien bij deze gammopathie een maligniteit is uitgesloten, wordt er gesproken van een "monclonal gammopathie of unknown significance" (MGUS). MAG speelt een belangrijke rol bij de myelinisatie van zenuwen en IgM antistoffen tegen dit glycoproteïne van 100 kD lijken een directe rol te spelen in de pathogenese van gammopathie-gerelateerde polyneuropathie.

Sensitiviteit/specificiteit
De sensitiviteit van anti-MAG antistoffen is 40-75% bij patiënten met een IgM gammopathie-geassocieerde polyneuropathie. De specificiteit is 99%.

Valkuilen
Geen.

Vergelijking andere methodes
Anti-MAG antistoffen kunnen ook worden bepaald door middel van immunoblotting. Deze techniek heeft een hoge specificiteit, maar mogelijk een lagere sensitiviteit dan de recent ontwikkelde ELISA voor het aantonen van deze antistoffen. Soms wordt er voor de diagnostiek gebruik gemaakt van een combinatie van beide technieken. De gevonden antistoftiters met deze twee methoden vertonen echter een matige correlatie.

Referentiewaarden
Titer-units boven de 1500 worden niet gevonden bij gezonde individuen.

Zie ook
Antistoffen tegen gangliosiden.

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APPENDIX 4.

Antistoffen tegen gangliosiden

Geschreven door M.L. Kuijf en B.C. Jacobs en verschenen als hoofdstuk in "Handboek medische laboratoriumdiagnostiek"; Prelum uitgevers, mei 2009, ISBN10:9085620139.

Doel
Diagnostiek bij verdenking op immuun-gemediëerde polyneuropathie.

Benodigde klinische informatie
Standaardinformatie bij aanvraag laboratoriumonderzoek (o.a. identificatie, leeftijd, geslacht, klinische gegevens/vraagstelling).

Beschrijving methodes
Antistoffen tegen gangliosiden worden bepaald door middel van een *Enzyme-Linked Immuno Sorbent Assay* (ELISA) volgens het gevalideerde protocol van de Inflammatory Neuropathy Cause and Treatment (INCAT) groep (1;2). Sera worden daarbij getest in ELISA platen waarvan de wells vooraf zijn gecoat met gangliosiden (gesialydeerde glycolipiden) zodat in het serum aanwezige anti-ganglioside antistoffen kunnen binden. De gebonden antistoffen worden vervolgens aangetoond door middel van geconjugeerd anti-humaan IgM en IgG antisera en een enzymatische reactie, waarna een kleuromslag optreedt die gemeten wordt met een ELISA reader. De sterkte van de verkleuring is een maat voor de hoogte van de antistofreactiviteit tegen gangliosiden in het serum. Indien deze antistofreactiviteit hoger is dan de grenswaarde is het serum positief voor deze antistoffen. Door middel van een verdunningsreeks van het serum kan ook de antistoftiter worden bepaald als maat voor de hoogte van de antistofreactiviteit.
Belasting voor de patiënt
Venapunctie.
Voorbereiding patiënt
Geen specifieke voorbereiding; afname kan op elk tijdstip van de dag plaatsvinden.
Materiaalafname/Fixatie
Afname van bloed middels venapunctie (stolbuis). De bepaling wordt bij voorkeur uitgevoerd in serum. Scheiding van serum door middel van centrifugeren. Indien de bepaling plaatsvindt binnen vijf dagen, dan kan het serum worden bewaard bij 2-8°C. Indien de bepaling later plaatsvindt kan het serum beter worden bewaard bij -20°C of -80°C . Verzending van serum naar een extern laboratorium kan bij kamertemperatuur.

Mogelijke toepassingen
Diagnostiek van immuun-gemediëerde en mogelijk behandelbare vormen van polyneuropathie.

Contra-indicaties
Geen.

Complicaties
Geen.

Interpretatie
In het serum van patiënten met een immuun-gemediëerde polyneuropathie kunnen IgM en IgG antistoffen tegen diverse gangliosiden worden gevonden. Gangliosiden zijn gesialydeerde glycolipiden die in hoge concentraties aanwezig zijn in neurale celmembranen. Voor de diagnostiek kunnen de antistoffen tegen de gangliosiden GM1, GD1a en GQ1b van belang zijn (zie tabel). Deze antistoffen lijken een directe rol te spelen bij het ontstaan van deze vormen van neuropathie (3). In het algemeen worden antistoffen tegen GM1 en GD1a gevonden bij patiënten met een belangrijke motore uitval, terwijl antistoffen tegen GQ1b voorkomen bij patiënten met een sensore ataxie en/of oogbolmotoriekstoornissen. Het isotype van de anti-ganglioside antistoffen is soms gerelateerd aan het type neuropathie. Deze antistoffen kunnen met verschillende technieken worden aangetoond, maar alleen van de ELISA volgens het INCAT protocol zijn gevalideerde studies beschikbaar (1;2).

De multifocale motore neuropathie (MMN) is sterk geassocieerd met de aanwezigheid van IgM antistoffen tegen GM1. MMN is een chronisch progressieve neuropathie die klinisch wordt gekenmerkt door het optreden van een asymmetrische spierzwakte in armen of benen en wordt veroorzaakt door een demyeliniserende neuropathie van perifere motore zenuwen (4). De patiënten hebben geen of slechts minimale sensore uitval. MMN kan worden behandeld met intraveneus toegediende immuunglobulinen (4).

Het Guillain-Barré syndroom (GBS) is een postinfectieuze acute polyneuropathie. Het GBS is een opvallend heterogene aandoening, die kan worden onderverdeeld in diverse subgroepen met een typische klinische en electrofysiologische presentatie. Er is er een associatie tussen deze subgroepen, het type voorafgaande infectie en de specificiteit van de anti-ganglioside antistoffen. Bij patiënten met een voorafgaande gastro-enteritis of andere aanwijzingen voor een infectie met de *Campylobacter jejuni* worden in de acute fase in het serum vaak IgG en/of IgM antistoffen tegen GM1, GD1a en GQ1b gevonden (5;6). Bij deze patiënten zijn de antistoffen primair gericht tegen het lipo-oligosaccharide van de *C. jejuni*, maar door moleculaire mimicry kunnen deze antistoffen kruisreageren met gangliosiden op humane perifere zenuwen (7). De antistoffen tegen GM1 en GD1a zijn geassocieerd met ernstige motore uitval, vaak zonder uitval van sensore- of hersenzenuwen. De antistoffen tegen GQ1b zijn sterk geassocieerd met de aanwezigheid van oogbolmotoriekstoornissen (waaronder ophthalmoplegie). Antistoffen tegen verschillende combinaties van gangliosiden kunnen ook voorkomen. Daarnaast worden antistoffen tegen gangliosiden gezien na andere typen infecties, waaronder bovenste luchtweg infecties met *Haemophilus influenzae* (3;6). Het GBS is behandelbaar met o.a. intraveneus toegediende immuunglobulinen (8). Meestal is er een monofasisch ziektebeloop, soms met ernstige restuitval. De anti-ganglioside antistoffen verdwijnen bij patiënten met GBS vrijwel altijd binnen enkele maanden uit het serum.

Serum IgG en IgM antistoffen tegen GQ1b zijn ook sterk geassocieerd met het Miller Fisher syndroom (MFS), dat wordt beschouwd als een variant van het GBS. Het MFS wordt gekenmerkt door het klinische trias oogbolmotoriekstoornissen, ataxie en areflexie (9). Overige aandoeningen waarbij anti-GQ1b antistoffen kunnen voorkomen zijn de Bickerstaff encefalitis en CANOMAD. Bickerstaff encefalitis is een fulminant verlopende ontsteking

van de hersenstam die gepaard gaat met bewustzijnsveranderingen (3). CANOMAD is een chronische neuropathie gekenmerkt door ataxie, ophthalmoplegie en de aanwezigheid van een IgM paraproteine tegen GQ1b en andere disialosyl-gangliosiden (10). Antistoffen tegen GM1 en GD1a kunnen ook voorkomen bij enkele andere zeldzame vormen van inflammatoire polyneuropathie.

In typische gevallen kan bij de meeste van deze aandoeningen de diagnose worden gesteld op basis van de klinische kenmerken en de bevindingen bij electrofysiologisch en overig aanvullend onderzoek. In atypische gevallen echter kan het bepalen van deze anti-ganglioside antistoffen een bijdrage leveren aan de diagnostiek. Het klinische belang van het stellen van de juiste diagnose is gelegen in het feit dat veel van deze antistof-gerelateerde vormen van neuropathie behandelbaar zijn, o.a. met intraveneus toegediende immuunglobulinen (4;8).

Sensitiviteit/specificiteit
De sensitiviteit voor de verschillende aandoeningen wordt weergegeven in onderstaande tabel. IgG antistoffen zijn specifieker dan IgM antistoffen en worden vrijwel uitsluitend gezien bij GBS en zuiver motorische vormen van inflammatoire polyneuropathie.

Valkuilen
Sera met een hoge achtergrondactiviteit kunnen vals negatief zijn.

Vergelijking andere methodes
De resultaten zijn sterk afhankelijk van de gebruikte methode. Alleen van de ELISA volgens het INCAT-protocol zijn momenteel gevalideerde resultaten bekend (1;2).

Referentiewaarden
Sera met een antistof-titer van 1:100 of hoger zijn positief (2).

Zie ook
Antistoffen tegen MAG.

Tabel. Frequentie van serum IgM en IgG antistoffen tegen de gangliosiden GM1, GD1a en GQ1b in relatie tot neuropathie.

	GM1		GD1a		GQ1b	
	IgM	IgG	IgM	IgG	IgM	IgG
MMN[1]	40-60%	-	-	-	-	-
GBS[2]	10-20%	10-20%	5-10%	5-10%	1-5%[3]	1-5%[3]
MFS[4]	-	-	-	-	20–50%	>90%
Bickerstaff encefalitis	-	-	-	-	10-30%	60-70%
CANOMAD[5]	-	-	-	-	100%	-
Inflammatoire motore PNP[6]	<5%	<5%	<5%	<5%	-	-
Gezonde bloeddonoren[7]	-	-	-	-	-	-

-, aanwezig bij <0.3% van deze patiënten of personen.
[1] Multifocale motore neuropathie.
[2] Guillain-Barré syndroom.
[3] Bij GBS met ophthalmoplegie bij > 90 %.
[4] Miller Fisher syndroom.
[5] Chronische Atactische Neuropathie met Opththalmoplegie, M-proteïne, koude Agglutininen en antistoffen tegen Disialyl-gangliosiden.
[6] Chronisch inflammatoire demyeliniserende polyneuropathie (CIDP), polyneuropathie (PNP) geassocieerd met paraproteïnen, mononeuritis multiplex, andere chronische vormen van inflammatoire PNP.
[7] Gebaseerd op een studie van 110 gezonde bloeddonoren uit regio Rotterdam (2).

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Achtergrondinformatie

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wel eens koffie-uurtjes en een lunchpauze een heus drie gangen menu. Toch heeft dit alles een basis gelegd voor een creatieve en betrokken groep collega's die allen zeer gedreven aan hun doelen bezig zijn en waarvan sommigen ongetwijfeld nu de vruchten plukken van die rust uit de beginjaren. Enkele namen van collega's die de afgelopen jaren met mij op de 22e gewerkt hebben en die ik allen dank voor de prettige werksfeer zijn Karin Geleijns, Maaike Dirks, Rinze Neuteboom, Marie-Claire de Wit, Eric van Breda, Bas ter Meulen, Naghmeh Jafari, Nadine van der Beek, Ilse Hoppenbrouwers, Krista Kuitwaard, Liselotte Ruts, Sonja van Nes, Christa Walgaard, Marcel Garssen, Heleen den Hertog, Immy Ketelslegers, Juna de Vries, Laura Donkerkaart, Harro Seelaar, Alex Korsten en Karin ter Meulen - Boer. Daarnaast wil ik de verpleging van de afdeling Neurologie, alle overige arts-assistenten van de afdeling Neurologie en alle neurologen bedanken voor hun flexibiliteit en medewerking aan het vervolgen van opgenomen patiënten met GBS die meededen met klinische studies.

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ABOUT THE AUTHOR

Mark Laurens Kuijf was born on the 1st of February 1977 in Arnhem, The Netherlands. After finishing high school he started his training in Medicine in 1995 at the Erasmus University of Rotterdam. During that period he worked from 1997 until 2000 as a nurse-assistant at the department of Neurology of the Erasmus MC and in 2001 completed a placement at the Neurology department of the University hospital of Juiz de Fora, Brazil. He graduated in 2002 on a research project about the diagnostic value of fine-needle biopsies in the rejection of liver transplantation under the supervision of Dr. H.J. Metselaar and Dr. J. Kwekkeboom at the department of Gastroenterology of the Erasmus MC, Rotterdam. After this he worked from 2002 until 2003 as a medical doctor at the department of Neurology of the Erasmus MC. In the winter of 2003 he started his PhD project on Guillain-Barré syndrome under the supervision of Dr. B.C. Jacobs and Professor P.A. van Doorn from the departments of Immunology and Neurology of the Erasmus MC and started his training for neurologist under supervision of Professor P.A.E. Sillevis-Smitt in 2005.

LIST OF PUBLICATIONS

1. Ang, CW, Krogfelt, K, Herbrink, P, Keijser, J, van Pelt, W, Dalby, T, Kuijf, M, Jacobs, BC, Bergman, MP, Schiellerup, P, and Visser, CE. **2007**. Validation of an ELISA for the diagnosis of recent Campylobacter infections in Guillain-Barre and reactive arthritis patients. *Clin. Microbiol. Infect.* 13:915-922.

2. Godschalk, PC, Kuijf, ML, Li, J, St, MF, Ang, CW, Jacobs, BC, Karwaski, MF, Brochu, D, Moterassed, A, Endtz, HP, Van, BA, and Gilbert, M. **2007**. Structural Characterization of Campylobacter jejuni Lipooligosaccharide Outer Cores Associated with Guillain-Barre and Miller Fisher Syndromes. *Infect. Immun.* 75:1245-1254.

3. Kuijf, ML, Kwekkeboom, J, Kuijpers, MA, Willems, M, Zondervan, PE, Niesters, HG, Hop, WC, Hack, CE, Paavonen, T, Hockerstedt, K, Tilanus, HW, Lautenschlager, I, and Metselaar, HJ. **2002**. Granzyme expression in fine-needle aspirates from liver allografts is increased during acute rejection. *Liver Transpl.* 8:952-6.

4. Kuijf, ML, van Doorn, PA, Tio-Gillen, AP, Geleijns, K, Ang, CW, Hooijkaas, H, Hop, WC, and Jacobs, BC. **2005**. Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. *J. Neurol. Sci.* 239:37-44.

5. Kuijf, ML, Ang, CW, van Doorn, PA, Niesters, HG, and Jacobs, BC. **2006**. Presence or absence of cytomegalovirus in cerebrospinal fluid from patients with Guillain-Barre syndrome? *J. Infect. Dis.* 193:1471-1472.

6. Kuijf, ML, Godschalk, PC, Gilbert, M, Endtz, HP, Tio-Gillen, AP, Ang, CW, van Doorn, PA, and Jacobs, BC. **2007**. Origin of ganglioside complex antibodies in Guillain-Barre syndrome. *J. Neuroimmunol.* 188:69-73.

7. Kuijf, ML, Geleijns, K, Ennaji, N, van Rijs, W, van Doorn, PA, and Jacobs, BC. **2008**. Susceptibility to Guillain-Barre syndrome is not associated with CD1A and CD1E gene polymorphisms. *J. Neuroimmunol.* 205:110-112.

8. Kuijf, ML, Eurelings, M, Tio-Gillen, AP, van Doorn, PA, van den Berg, LH, Hooijkaas, H, Stork, J, Notermans, NC, and Jacobs, BC. **2009**. Detection of anti-MAG antibodies in polyneuropathy associated with IgM monoclonal gammopathy. *Neurology* 73:688-695.

9. Mortensen, NP, Kuijf, ML, Ang, CW, Schiellerup, P, Krogfelt, KA, Jacobs, BC, Van, BA, Endtz, HP, and Bergman, MP. **2009**. Sialylation of Campylobacter jejuni lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes. Infect.* 11:988-994.

10. van Rijen, MM, Kuijf, ML, Metselaar, HJ, Tilanus, HW, Bouma, GJ, de Weger, RA, Jonker, M, and Kwekkeboom, J. **2002**. CD154 is expressed during treatment with calcineurin inhibitors after organ transplantation. *Transplantation* 73:1666-1672.

PHD PORTFOLIO DRS. MARK KUIJF

Name PhD student: Drs. Mark Kuijf Erasmus MC Department: Neurology and Immunology Research School: Molecular medicine postgraduate school		PhD period: 2004 - 2009 Promotor: Prof.Dr. P.A. van Doorn Supervisor: Dr. B.C. Jacobs	
1. PhD Training	Year	Workload	
		Hours	ECTS
General courses			
Classical methods for Data-analysis	2006		5.7
Animal research course according to Law on research animals, article 9	2007		4
Specific courses			
Pre-congress Teaching course, 5th European Society of Neuroimmunology, Venice, Italy	2004	16	
Molecular Immunology course	2005		2
Various research school courses and workshops	2004 - 2008		6
Seminars and Workshops			
Department Journal club and seminars	2004 - 2008	20	
Presentations			
5 Poster presentations at congresses or symposia	2004 - 2008		
3 Oral presentation at congresses	2004 - 2008		3
International conferences			
European Congress (poster presentation)	2004		1
International Congress (oral presentation)	2004		1
European Congress	2005		1
International Congress (oral and poster presentation)	2005		1
International Congress (Winner award `Best Young Investigator` for oral presentation)	2007		1
International Congress (2 poster presentations)	2007		1
2. Teaching			
Supervising master's thesis	2007	28	
TOTAL		64	26.7