# MOLECULAR MIMICRY IN THE GUILLAIN-BARRÉ SYNDROME

## Moleculaire mimicry bij het Guillain-Barré syndroom

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## PROMOTIECOMMISSIE

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| Promotor: | Prof. dr. F.G.A. van der Meché |
| Overige leden: | Prof. dr. R. Benner<br>Prof. dr. A.D.M.E. Osterhaus<br>Dr. H.J. Willison |
| Co-promotoren: | Dr. P.A. van Doorn<br>Dr. J.D. Laman |

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*Aan mijn ouders*

# MOLECULAR MIMICRY IN THE GUILLAIN-BARRÉ SYNDROME

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# Chapter 1

## INTRODUCTION

- The Guillain-Barré syndrome, infections and molecular mimicry
- Mechanisms of post-infectious immune-mediated disease
- The Guillain-Barré syndrome
- Outline of the thesis

# THE GUILLAIN-BARRÉ SYNDROME, INFECTIONS AND MOLECULAR MIMICRY

Damage to the axon or myelin sheath of peripheral nerves leads to dysfunction called neuropathy. The type of dysfunction depends on the localization of the injured nerve and the type of nerve (motor, sensory, autonomic) involved. In addition to damage such as caused by metabolic disturbances, toxic substances or degenerative processes, it is well recognized that components of the immune system such as antibodies, complement, T cells and macrophages can be found in affected nerves and are likely to be involved in the pathogenesis of neuropathies. This particular group of neurological disorders is called immune-mediated neuropathies. Patients with an immune-mediated neuropathy often have antibodies that react with components of peripheral nerves and respond favorably to immune-modulating therapies such as plasmapheresis and intravenous immunoglobulin (IVIg) treatment. The immune-mediated neuropathies can be further subdivided according to their course, although the distinction between acute and chronic disease is made arbitrarily (Table 1).

**Table 1. Immune-mediated neuropathies.**

| Acute | Chronic |
|---|---|
| Guillain-Barré syndrome (GBS) | Chronic inflammatory demyelinating polyneuropathy (CIDP) |
| *Primary axonal* | Multifocal motor neuropathy (MMN) |
| Acute Motor Axonal Neuropathy (AMAN) | Neuropathies associated with monoclonal gammopathies of undetermined significance (MGUS-PNP) |
| Acute Motor Sensory Axonal Neuropathy (AMSAN) | With anti-Myelin-Associated Glycoprotein (MAG) antibodies |
| *Primary demyelinating* | Without anti-MAG antibodies |
| Acute Inflammatory Demyelinating Polyneuropathy (AIDP) | |
| Cranial nerve variants of GBS | |
| Miller Fisher syndrome (MFS) | |
| Pharyngeocervicobracheal variant | |
| Acute oropharyngeal palsy | |

The Guillain-Barré syndrome (GBS) is the most frequent acute immune-mediated neuropathy. Approximately two-third of GBS patients report symptoms of an infectious illness in the weeks before the development of neurological symptoms (Hughes and Rees, 1997; Leneman, 1966; Van Koningsveld *et al.*, 2000b). Other precipitating events include surgery and vaccinations although this has been found difficult to confirm in case-control studies (Hughes, 1990; Winer *et al.*, 1988c).

The subject of this thesis will be the relation between antecedent infections and the development of GBS, with special reference to "molecular mimicry". This term refers to a structural resemblance of host-tissue and pathogens and is one of the proposed mechanisms for the induction of post-infectious immune mediated disease (Albert and Inman, 1999; Behar and Porcelli, 1995). Evidence for a role of molecular mimicry in the pathogenesis of GBS is provided by the detection of antibodies against peripheral nerve glycolipids such as ganglio-

sides GM1 and GD1a (Hughes *et al.*, 1999; Van der Meché and Van Doorn, 1995). These anti-glycolipid antibodies have the potential to cross-react with lipopolysaccharides (LPS) from Gram-negative bacteria, especially *Campylobacter jejuni* (Yuki, 1997).

The presence of cross-reactive anti-LPS/glycolipid antibodies in serum of GBS patients form the starting point of the work described in this thesis. Our hypothesis is that GBS is caused by antibodies and possibly also T cells that have been induced following an antecedent infectious agent due to molecular mimicry between the microbial agent and nervous tissue. In this introduction a short overview of possible mechanisms in the development of post-infectious immune-mediated diseases will be given. Subsequently, the pathogenesis of GBS will be discussed with special emphasis on the current knowledge of anti-glycolipid antibodies followed by a description of current animal models for immune-mediated neuropathies. At the end of this introduction the research questions for this thesis will be formulated.

## MECHANISMS OF POST-INFECTIOUS IMMUNE-MEDIATED DISEASE

Like GBS, other immune-mediated diseases are also triggered by infections or microbial products and a number of infectious diseases are linked to autoimmune phenomena. There is confusion about whether to use the term "autoimmune diseases" or "infectious disease" for infection-triggered immune-mediated diseases. Since little is known about the initial events that lead to autoimmune phenomena, all diseases with autoimmune phenomena may be triggered by infections and so the distinction between "autoimmune" and "infectious" diseases becomes blurred. In immune-mediated diseases with a suspected autoimmune origin such as multiple sclerosis and type I diabetes mellitus, infectious triggers have been suspected but firm epidemiological evidence for their causal role is still lacking.

GBS forms an excellent opportunity to study the relation between infections and the development of immune-mediated disease because there is a well-documented relation between the infectious event and the development of neurological symptoms (Figure 1). In addition, specific strains of bacteria and viruses that have triggered GBS in patients were isolated and are available to study the presence of GBS-specific characteristics.

### Molecular mimicry

The term molecular mimicry was coined by Damian in 1964 and refers to a sharing of antigens by infectious agents and self tissues (Damian, 1964). Although in this first publication Damian rejected the possibility of molecular mimicry as a mechanism for the induction of autoimmune diseases, it turned out to be a very attractive hypothesis (Table 2). Antibodies and/or T cells which are induced by the infection are initially directed against microbial antigens. Due to the structural resemblance of the microbial antigen and self antigens, the antibodies and/or T cells not only destruct the invading pathogen but also host tissue (Albert and

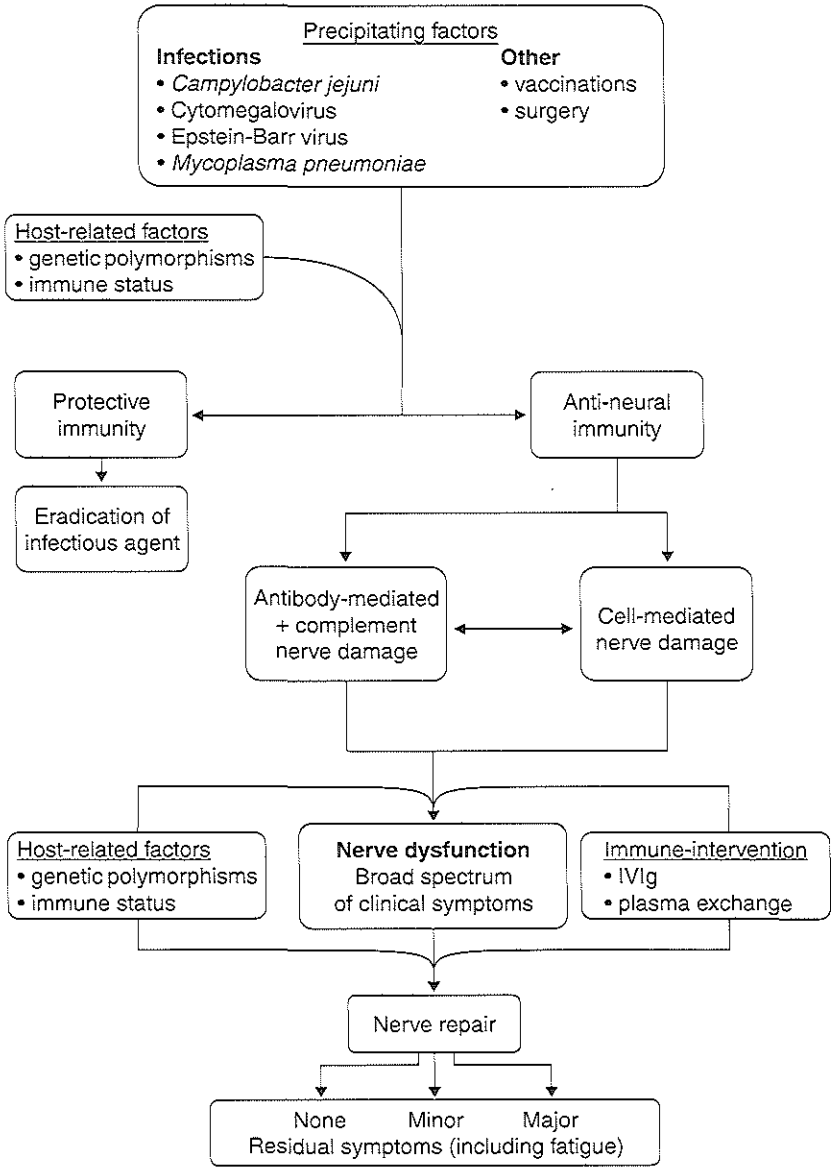


**Figure 1.** General scheme of the pathogenesis of the Guillain-Barré syndrome. See text for details.

Inman, 1999).

A classical example of a disease in which molecular mimicry is postulated to be the pathogenetic mechanism is rheumatic carditis, with a cross-reactive immune response towards pneumococcal polysaccharides and cardiac tissue, leading to rheumatic heart disease

**Table 2. Examples of diseases in which molecular mimicry has been implicated in the pathogenesis.**

| Immune mediated disease | Proposed autoantigen | Proposed pathogen/molecule | Antibody/T cell mediated |
|---|---|---|---|
| Cardiovascular disease | Alpha-myosin heavy chain | *Chlamydia* species | Antibodies/T cells |
| Celiac disease | Gliadin | Adenovirus/enteric microbes | Antibodies |
| Chagas' disease | Cardiac myosin heavy chain | *Trypanosoma cruzi*, B13 protein | T cells |
| Diabetes mellitus type 1 | GAD65, proinsulin carboxypeptidase | Coxsackievirus P2-C | T cells |
| Guillain-Barré/Miller Fisher | Gangliosides | *C. jejuni*, lipopolysaccharides | Antibodies |
| Graves disease | Thyrotropin receptor | *Yersinia enterocolica* | Antibodies |
| Herpes stromal keratitis | Corneal antigens | Herpes simplex virus type 1, UL6 | T cells |
| Lyme disease | LFA-1 | *Borrelia burgdorferi*, OSP-A | T cells |
| Multiple sclerosis | MBP/MOG | Multiple viruses, e.g. HHV-6, Epstein-Barr virus | Antibodies/T cells |
| Peptic ulcer | Gastric mucosa antigens | *Helicobacter pylori* | T cells |
| Primary biliary cirrhosis | Pyruvate dehydrogenase complex | *E. coli* PDC-E2 | Antibodies |
| Psoriasis | Keratin | Beta-hemolytic streptococcus, M-protein | T cells |
| Rheumatic heart disease | Cardiac glycoproteins | Beta-hemolytic streptococcus, M-protein | Antibodies |
| Rheumatoid arthritis | HLA-DRB1 | 40-kd heat shock protein (dnaJ) | Antibodies/T cells |
| Rheumatoid arthritis | Heat shock protein 60 | *Mycobacterium tuberculosis* HSP 65 | Antibodies/T cells |
| Spondyloarthropathies | HLA-B27 | *Chlamydia trachomatis*, Gram-negative enteric pathogens | Antibodies |

(Huber and Cunningham, 1996). Lyme disease, in which T cells directed against the outer surface protein of *Borrelia burgdorferi* cross-react with antigens in joints is another well-known example (Albert and Inman, 1999; Hemmer *et al.*, 1999). In addition, mimicry between glutamate decarboxylase (GAD65), an enzyme present in pancreatic β cells, and coxsackievirus P2-C has been demonstrated (Kaufman *et al.*, 1993). This mimicry might be responsible for β-cell destruction in patients with type I diabetes mellitus although convincing evidence for this in patients has yet to be found and other mechanisms such as bystander autoimmunity may also play a role (Atkinson, 1997; Atkinson *et al.*, 1992; Horwitz *et al.*, 1998; Richter *et al.*, 1994). More examples of diseases in which molecular mimicry has been implicated can be found in Table 2 although this list is not exhaustive.

*Cross-reactivity and molecular mimicry*

The original concept of molecular mimicry was a structural resemblance between a micro-organism and the host (Damian, 1964). This structural resemblance is often taken as a starting point for defining cross-reactive potential of antibodies and T cells. However, some developments have extended our view on cross-reactivity of antibodies and T cell receptors and have uncoupled structural resemblance and cross-reactivity.

Detailed analysis of monoclonal antibodies and T cell clones has revealed that all B and T cell receptors have the potential to recognize more than one antigen, and that there is only a gradual difference in strength of interactions (Hemmer *et al.*, 1999; Kersh and Allen, 1996; Van Regenmortel, 1998). The degeneracy of the T and B cell receptors implies that all antibodies have the potential to cross-react and that it is only possible to talk about "speci-

ficity" in a strictly defined situation. An observation that underscores this issue is the fact that approximately 3.5% of more than 600 mouse monoclonal antibodies that were raised to 11 different viruses reacted with mouse organs (Srinivasappa *et al.*, 1986). Furthermore, individuals without autoimmune disease also have so-called naturally occuring antibodies that react with self antigens (Avrameas, 1991; Guilbert *et al.*, 1982). The role of these natural auto-antibodies is unclear but they may be essential in the first line of defence against infectious invaders (Casali and Notkins, 1989; Ochsenbein *et al.*, 1999).

Knowledge of protein structure has led to a focus on amino acid sequence similarity as the major form of mimicry. Many studies have revealed nearly identical amino acid sequences in human tissue and bacteria and viruses (Fujinami and Oldstone, 1985; Roudier *et al.*, 1996). In recent studies, it was demonstrated that T cell clones are stimulated in vitro by different peptides with only a very limited homology at the primary amino acid level (Hemmer *et al.*, 1999; Wucherpfennig and Strominger, 1995). This complicates the use of protein databases to look for potential cross-reactive antigens. An additional problem of protein database searches, which is much harder to overcome, is that most pathogens which contain these potential cross-reactive peptides have never been epidemiologically linked with a particular autoimmune disease, making the role of mimicry in the pathogenesis of these diseases uncertain (Wucherpfennig and Strominger, 1995).

Much of the research in GBS has focused on the presence of antibodies against non-protein antigens. A large proportion of B and T cells are able to react with non-protein antigens (Fairhurst *et al.*, 1998; Heidenreich *et al.*, 1994; Jensen *et al.*, 1999; Zhu *et al.*, 1994). Although little is known about epitope-paratope interactions in the recognition of glycolipid and glycopeptide antigens the binding of a B or T cell receptor clearly depends on the three-dimensional structure and the charge of the antigen (Deck *et al.*, 1999; Ishioka *et al.*, 1992; Jensen *et al.*, 1999; Van Regenmortel, 1998). Cross-reaction is therefore not merely a function of similarity of amino acid sequence. This is exemplified by the fact that monoclonal antibodies not only react with peptides that are similar to the peptide they have been raised against, but also against completely unrelated peptides, so called mimotopes (Sparbier and Walden, 1999). An even more extreme situation is the presence of monoclonal anti-carbohydrate antibodies that react with peptide mimotopes. These peptide mimotopes are peptides which presumably have the same conformational structure as particular oligosaccharides (Kieber-Emmons, 1998).

We propose to use the term "mimicry" in an operational sense: a micro-organism and host tissue display mimicry when it is possible to detect antibodies and/or T cells which can recognize both the micro-organism and host tissue. Therefore, the detection of cross-reactive antibodies and/or T cells will be taken to be indicative for a role of mimicry in the induction of auto antibodies and self reactive T cells.

**Exposure of cryptic epitopes**

A special form of molecular mimicry is the exposure of cryptic epitopes. Following infection of a cell with a virus or a bacterium, the surface of the cell may be altered in such a fashion that epitopes that are unexposed in the healthy situation, can be recognized by the immune system (Behar and Porcelli, 1995; Lehmann *et al.*, 1992). These "new" epitopes are recognized as foreign and the immune system will mount a B cell and/or T cell response against these cells resulting in organ damage.

**Persistent infection/antigen exposition**

When a microbial agent chronically infects an organ or when microbial antigens persist in the tissue, the organ may be damaged by the immune response against the micro-organism. This process is called "bystander autoimmunity", as there is no real anti-self response although the outcome is the same: organ damage (Behar and Porcelli, 1995). Bystander autoimmunity as a mechanism for neuritis was first proposed by Wisniewski and Bloom (1975). In other immune-mediated diseases such as multiple sclerosis, type I diabetes mellitus and Chagas' disease it is controversial whether destruction of organ occurs due to organ-specific antibodies and/or T cells or to bystander autoimmunity (Giovannoni and Hartung, 1996; Horwitz *et al.*, 1998; Tarleton and Zhang, 1999). The presence of antibodies and/or T cells directed against auto-antigens in later stages of the disease process may result from an immune response against self-antigens that are released due to the tissue damage caused by the infection (Miller *et al.*, 1997).

**Superantigens**

Many pathogens have the ability to produce superantigens. These compounds have the ability to stimulate T cells via their T cell receptor independent from their T cell receptor specificity (Schiffenbauer *et al.*, 1998). The stimulation of a large number of T cells has been postulated to contribute to immune-mediated disease (Schiffenbauer *et al.*, 1998). In Kawasaki disease associated with *S. aureus* infections there is a selective expansion of a Vβ2 subset of T cells (Abe *et al.*, 1993; Leung *et al.*, 1995). Exacerbation of skin lesions in psoriasis has been linked to increased antibody titers to streptococci and an increased number of Vβ2 and Vβ5.1 T cells (Valdimarsson *et al.*, 1997).

Both staphylococci and group A streptococci are known to produce a number of superantigens (Fraser *et al.*, 2000; Renno and Acha-Orbea, 1996). In recent years, it has become clear that other bacteria like *Yersinia*, *Mycoplasma* species and also viruses like cytomegalovirus (CMV), Epstein-Barr virus (EBV) and mouse mammary tumor virus have superantigen-activity (Leung *et al.*, 1998; Torres and Johnson, 1998). However, despite their theoretical potential, the role of superantigens in the pathogenesis of post-infectious immune-mediated disease is not well defined.

**Interference with immune-regulation**

Many viruses and some bacteria have the capacity to interact with normal immuno-

logical processes. Examples are the production of MHC-like molecules by CMV infected cells, the mimicking of complement receptors by virus-infected cells and the production of molecules with cytokine-like properties by a number of viruses (Michelson, 1999; Vaughan, 1995; Vink *et al.*, 1999). This dysregulation of the immune response may also lead to autoimmunity although the precise mechanisms are still unclear (Scotet *et al.*, 1999; Whitton and Fujinami, 1999).

## THE GUILLAIN-BARRÉ SYNDROME

The most frequent acute immune-mediated neuropathy is the Guillain-Barré syndrome (GBS). It was named after G. Guillain and J.A. Barré who described the syndrome in 1916, together with A. Strohl (Guillain *et al.*, 1916). The clinical presentation usually consists of a rapidly evolving generalized symmetrical weakness in combination with areflexia. The weakness frequently involves respiratory muscles turning patients respirator dependent. In addition to their weakness, patients may suffer from paresthesias and autonomic disturbances (Hughes, 1990). Although weakness is the common denominator of GBS patients, there is a large variability in the severity and distribution of the weakness and the extent of sensory involvement (Ropper, 1992; Van der Meché *et al.*, 1997). The disease is self-limiting and weakness is most severe within two to three weeks. Most patients recover, although residual deficits can be disabling. Even when patients are treated in well-equipped intensive care units, mortality rates still range from 3-7% (Van Koningsveld *et al.*, 2000b).

A recent epidemiological study in the Netherlands revealed an incidence of 1.18/100.000 per year, stable from 1986 until 1995 (Van Koningsveld *et al.*, 2000b). Persons of all ages can be affected but there is a gradual rise in incidence with increasing age, possibly with a minor hump in young adults (Hughes and Rees, 1997; Van Koningsveld *et al.*, 2000b). Males are slightly more affected than females. Most cases of GBS are solitary but rarely small clusters of GBS occur, mostly in association with contaminated drinking water (Khoury, 1978; Roman, 1995; Sliman, 1978). In European countries there is no seasonal variation in the incidence of GBS (Hughes and Rees, 1997; Van Koningsveld *et al.*, 2000b) but an exception is found in Northern China, where summer epidemics of GBS occur annually (Griffin *et al.*, 1995; Ho *et al.*, 1995; McKhann *et al.*, 1993). Recently another area with a seasonal variation has been described on the Caribbean island Curaçao where an increase of the incidence of GBS during the wet season was observed (Van Koningsveld *et al.*, 2000a).

Treatment of GBS patients primarily consists of general medical care and monitoring of basal autonomic functions such as heart rate, blood pressure and ventilatory capacity. Plasma exchange (PE) and intravenous immunoglobulins (IVIg) have been demonstrated to have a beneficial effect on both the pace of recovery and the residual functional deficit (Guillain-Barré syndrome Study Group, 1985; Plasma Exchange/Sandoglobulin Guillain-Barré Syndrome Trial Group, 1997; Van der Meché and Schmitz, 1992). Corticosteroids do not have a beneficial effect but in combination with IVIg they may have a synergistic effect

which is currently investigated in a randomized clinical trial (Dutch Guillain-Barré Study Group, 1994; Guillain-Barré Syndrome Steroid Trial Group, 1993). In the Netherlands, IVIg is the first-choice treatment (Van der Meché and Schmitz, 1992).

**Cranial nerve variants of GBS**

In addition to the variation in clinical symptoms between GBS patients, several cranial nerve variants of GBS can be distinguished of which the Miller Fisher syndrome (MFS) occurs most frequently (Ropper, 1991). In 1956, Miller Fisher described the combination of external ophthalmoplegia, limb ataxia and areflexia in the absence of limb weakness known as MFS (Miller Fisher, 1956). Most MFS patients make a good recovery. Overlap cases between MFS and GBS with a combination of oculomotor and limb weakness also occur. Other variants are the pharyngeo-cervico-brachial form of GBS and the acute oropharyngeal palsy (Koga *et al.*, 1998a; O'Leary *et al.*, 1996). The cranial nerve variants mostly have a limited distribution of weakness (e.g. only some cranial nerves). Sometimes these variants merge into a more generalized pattern of weakness but the symptoms can also remain regionally restricted (Ter Bruggen *et al.*, 1998). Most of them are rather rare and together they comprise 5-10% of GBS patients (Ropper, 1991).

**Demyelinating and axonal forms of GBS**

GBS comprises cases in which the type of nerve fiber injury is predominantly caused by demyelination as well as cases in which the primary pathological process is axonal degeneration (Hughes *et al.*, 1999). In Europe and Northern America the most frequent pattern is demyelinating, electrophysiologically characterized by multifocal slowing of nerve conduction and partial conduction block (Hughes *et al.*, 1999). In severe demyelinating cases, secondary axonal degeneration may occur which can make the distinction with primary axonal degeneration difficult (Berciano *et al.*, 1993, 1997; Hall *et al.*, 1992).

Primary axonal forms of GBS have a reduction in distally evoked muscle and sensory action potentials with relatively spared conduction velocity (Alam *et al.*, 1998; Feasby *et al.*, 1986; Hadden *et al.*, 1998; Van der Meché *et al.*, 1991). This form of GBS occurs frequently in China, Japan and Mexico (Griffin *et al.*, 1995; Ramos-Alvarez *et al.*, 1969; Sobue *et al.*, 1997). Cases with only motor involvement are also termed acute motor axonal neuropathy (AMAN) while cases with both motor and sensory symptoms are called acute motor-sensory axonal neuropathy (AMSAN; Griffin *et al.*, 1995). Distinguishing severe cases of demyelinating GBS from axonal GBS based on electrophysiology is difficult as both forms can produce similar patterns. Both axonal degeneration and very distal demyelination may lead to inexcitable nerves (Berciano *et al.*, 1993, 1997; Feasby *et al.*, 1993).

**Histopathology of GBS**

In demyelinating GBS, a multifocal and segmental inflammatory process with infiltration of macrophages can be observed (Figure 2; Asbury *et al.*, 1969; Honavar *et al.*, 1991; Prineas, 1981, 1994). The presence of a lymphocytic infiltrate was thought to indicate a pri-

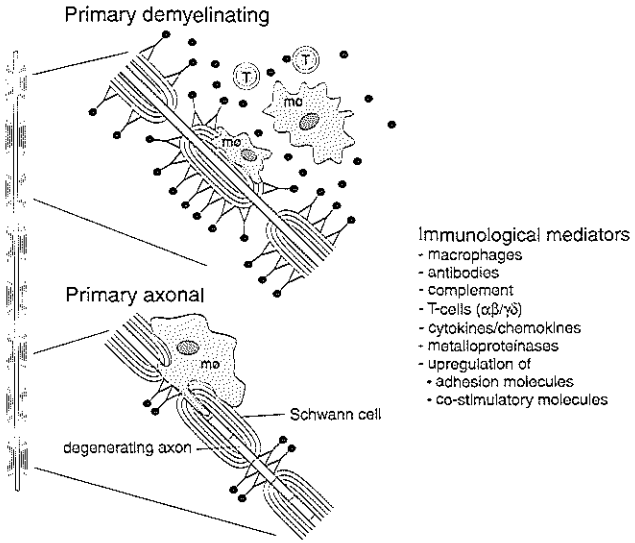


**Figure 2.** Histopathology of the Guillain-Barré syndrome and key immunological players. In primary demyelinating forms of GBS (upper panel) macrophages attack the Schwann cell. Immunoglobulins bind to the outer surface of the Schwann cell and activate complement. T cells may be directly cytotoxic, produce cytokines or be involved in breakdown of the blood-nerve barrier. In primary axonal forms of GBS (lower panel), the attack is initially directed against the nodal axolemma, guided by bound immunoglobulins and complement. Macrophages penetrate the basal lamina of the Schwann cell and enter the periaxonal space. Eventually, the axon degenerates. Lymphocytic infiltration is absent.

mary T cell mediated attack on the Schwann cell. However, recent studies on early (3 days) cases of demyelinating GBS demonstrated the presence of complement activation products (C3d and the membrane attack complex C5b-9) on the outer surface of the Schwann cell and only few infiltrating lymphocytes (Hafer-Macko *et al.*, 1996b). The outer myelin lamellae had vesicular changes. In cases from 7 and 9 days, demyelination was complete and a lymphocytic infiltrate was present (Hafer-Macko *et al.*, 1996b). The sequence of events leading to demyelination is probably as follows. Binding of anti-neural antibodies to the Schwann cell is followed by activation of complement and formation of transmembrane pores. The resulting myelin breakdown products are removed by the macrophages (Hughes *et al.*, 1999).

Due to the limited number of early cases studied so far, it can not be excluded that T cells directly mediate demyelination, perhaps by secretion of directly neurotoxic cytokines such as TNF-α (Putzu *et al.*, 2000; Selmaj and Raine, 1988). Alternatively, T cells may have a role in the opening of the blood-nerve barrier or may not even be associated with tissue damage but with repair or limiting the immunological attack (Pollard *et al.*, 1995; Schwartz and Cohen, 2000).

Autopsy studies of patients who were classified as having axonal GBS with electrophysiological methods confirmed the concept of primary axonal degeneration without significant demyelination (Hafer-Macko *et al.*, 1996a). Lymphocytic infiltration is absent or limit-

ed (Griffin *et al.*, 1995). In early cases, there is lengthening of the nodal gap together with binding of immunoglobulins and activation of complement. Macrophages are present over these nodes of Ranvier and penetrate the basal lamina of the Schwann cell (Griffin *et al.*, 1996; Hafer-Macko *et al.*, 1996a). Macrophage processes can be observed between the myelin sheath and the axon (Figure 2). In the final stage the axon is destroyed from the root to its most distal part. Rapid recovery in some AMAN patients can be explained by selective involvement of only the distal nerve fibers, which lack a blood-nerve barrier. Regeneration of this relatively short distance is supposed to be accomplished in several weeks (Ho *et al.*, 1997).

### Antecedent infections as triggering factors

Although numerous case reports have appeared and a large number of different micro-organisms has been linked to GBS, there have been only a limited number of case-control studies assessing the identity of the precipitating infectious agents. Both a British and a Dutch study identified *C. jejuni* as the most frequent precipitating infectious agent and CMV as the second most common (Table 3; Jacobs *et al.*, 1998; Winer *et al.*, 1988c). Other infectious agents significantly associated with GBS are EBV and *M. pneumoniae* (Jacobs *et al.*, 1998; Winer *et al.*, 1988c).

**Table 3. Antecedent infections in patients with the Guillain-Barré syndrome.**

| Micro-organism | Frequency | References |
|---|---|---|
| *Campylobacter jejuni* | 14-66% | Hao *et al.*, 1998; Ho *et al.* 1995; Jacobs *et al.* 1998; Kaldor and Speed 1984; Kuroki *et al.* 1993; Mishu *et al.* 1993; Rees *et al.* 1995; Speed *et al.* 1987; Winer *et al.* 1988 |
| Cytomegalovirus | 5-15% | Dowling and Cook 1981; Hao *et al.* 1998; Irie *et al.* 1996; Jacobs *et al.* 1998; Schmitz and Enders 1977; Terryberry *et al.* 1995; Winer *et al.* 1988; Yuki and Tagawa 1998 |
| Epstein-Barr virus | 0-10% | Dowling and Cook 1981; Hao *et al.* 1998; Jacobs *et al.* 1998; Terryberry *et al.* 1995; Winer *et al.* 1988 |
| *Mycoplasma pneumoniae* | 1-11% | Goldschmidt *et al.* 1980; Hao *et al.* 1998; Jacobs *et al.* 1998 Kusunoki *et al.* 1995; Terryberry *et al.* 1995; Winer *et al.* 1988 |
| *Hemophilus influenzae* | 1-13% | Jacobs *et al.* 1998; Mori *et al.* 2000 |

The infectious agents that have been linked to GBS are also related to other immune-mediated diseases and autoimmune phenomena. Reactive arthritis is frequently preceded by infection with *C. jejuni* (Sieper *et al.*, 1996). EBV and CMV infections are associated with an increased frequency of auto-antibodies (Hebart *et al.*, 1996; Hutt-Fletcher *et al.*, 1983; Price *et al.*, 1993; Vaughan, 1995). *M. pneumoniae* infections are followed by autoimmune hemolytic anemias, the formation of cold-agglutinins and polyclonal B cell stimulation (Biberfeld and Gronowicz, 1976; Clyde, 1993).

The relative frequencies of antecedent infections may differ between countries. In Northern China and Curaçao, *C. jejuni* infections occur in up to 75% of the cases, depending on the serological criteria used (Ho *et al.*, 1995; Van Koningsveld *et al.*, 2000a). Due to differences in serological assays and criteria for seropositivity it is very difficult to compare the

results from different studies (Blaser and Duncan, 1984; Herbrink *et al.*, 1988; Ho *et al.*, 1995). Population-based comparative geographical studies conducted by a central laboratory are therefore needed to address this issue.

The cranial nerve variants of GBS are also preceded by infectious diseases (Merkx *et al.*, 1994; Takano and Yuki, 1995; Willison and O'Hanlon, 1999). Due to the very limited number of patients, case control studies have not been performed. Based on small series and case reports it can be concluded that *C. jejuni* infections can also induce MFS and other cranial nerve variants but the relative frequency is unknown (Jacobs *et al.*, 1995; Salloway *et al.*, 1996; Yuki *et al.*, 1995c).

### *C. jejuni* and GBS

*C. jejuni* is the most frequently identified infectious agent in GBS (Jacobs *et al.*, 1998; Kuroki *et al.*, 1991; Rees *et al.*, 1995b; Winer *et al.*, 1988c). In humans, *C. jejuni* infections usually cause a bloody or watery diarrhea but the infection may also pass unnoticed (Blaser, 1997). Chickens are considered to be the major reservoir of *C. jejuni* but meat from other animals, contaminated water supplies, pets and raw milk may also be important (Hanninen *et al.*, 2000; Hudson *et al.*, 1999; Nielsen *et al.*, 1997; Sacks *et al.*, 1986). In the period between the acute infection, and the development of neurological symptoms, the organism is rapidly cleared from the gut in most patients. This makes culturing *C. jejuni* from stools of GBS patients rather difficult (Blaser, 1997).

The complete genomic structure of *C. jejuni* has recently been unraveled and the genome includes highly variable regions especially in regions coding for surface structures, indicating a high degree of variability in surface antigens (Parkhill *et al.*, 2000). In Japan and some other areas, there is a predominance of the O:19 serotype among *C. jejuni* isolates from GBS patients indicating an increased potential of this specific serotype to induce GBS (Kuroki *et al.*, 1993; Nachamkin *et al.*, 1999a; Yuki *et al.*, 1997b). In South Africa the O:41 serotype is over-represented (Lastovica *et al.*, 1997). Although *C. jejuni* strains with the O:19 serotype have been isolated from GBS patients from Western European countries, the serotype distribution of GBS and MFS strains isolated from Dutch patients is heterogeneous and resembles the serotype distribution in the Dutch population as a whole (Endtz *et al.*, 2000). Consequently, the observed clonality of GBS-associated strains from Japan and South Africa was absent in Dutch GBS-associated strains (Endtz *et al.*, 2000; Fujimoto *et al.*, 1997; Nishimura *et al.*, 1996a; Wassenaar *et al.*, 2000).

### Anti-glycolipid antibodies

Following the molecular mimicry hypothesis, auto-antibodies or auto-reactive T cells that cross-react with microbial antigens must be present. Antibodies against glycolipids of peripheral nerve can be considered as such cross-reactive antibodies. When patients with an IgM paraproteinaemic neuropathy were found to have IgM antibody reactivity against myelin associated glycoprotein (MAG) and sulfated glycolipids, a search for anti-glycolipid antibodies in other neurological syndromes was started (Ilyas *et al.*, 1984, 1985a). Much later,

these anti-glycolipid antibodies were shown to cross-react with microbial antigens, mainly of *C. jejuni*. The search for anti-ganglioside antibodies has led to a large body of literature with many conflicting results. These inconsistencies may depend on patient selection, geographical differences and/or technical variations. Despite these contradictions, it is clear that the association between anti-glycolipid antibodies and GBS with its regional variants is undisputable. The exact role of these antibodies is still unknown but anti-glycolipid antibodies can bind to peripheral nerve and have been shown to have an effect on neuromuscular transmission, pointing to an important role in the pathogenesis (see below; Goodyear *et al.*, 1999; O'Hanlon *et al.*, 1996, 1998; Plomp *et al.*, 1999).

*Gangliosides*

Gangliosides are a subgroup of membrane glycolipids and are highly enriched in the nervous system (Figure 3; Ledeen, 1985). They are composed of a ceramide tail inserted in the lipid bilayer and a highly variable oligosaccharide moiety protruding externally (Figure 4; Ledeen and Yu, 1982). The presence of sialic acid is necessary by definition, but asialogangliosides also exist. Ganglioside nomenclature according to Svennerholm is based on identity of the hexoses in the backbone, number of sialic acids, migration in thin-layer chromatography and isomer forms. Gangliosides are implicated in cell growth, differentiation and recognition (Hakomori, 1981). They can act as receptors for bacterial toxins and have been also suggested to play a role in signal transduction (Fishman *et al.*, 1993; Hakomori, 1990; Kurzchalia and Parton, 1999). In this thesis the term "glycolipid" will also be used to indicate both gangliosides and asialo-gangliosides. "Glycoconjugate" is used to indicate a carbohydrate structure on either a lipid or a protein.

There is a difference in ganglioside composition between axons and myelin but also between motor and sensory nerves. GM1 and GD1a are the predominant gangliosides in axons compared to LM1, GM3 and GD1b in myelin (Ogawa-Goto *et al.*, 1992, 1993; Svennerholm *et al.*, 1994; Svennerholm and Fredman, 1990). Furthermore, the concentration of GM1 and GD1a is higher in myelin from human motor nerves than from sensory nerves (Ogawa-Goto *et al.*, 1992). GQ1b is enriched in the third, fourth and sixth cranial nerves, which innervate the oculomotor muscles (Chiba *et al.*, 1997). It has been hypothesized that anti-glycolipid antibodies exert their effect via binding to surface gangliosides and that the relative expression of gangliosides determines the level of binding of auto-antibody and therefore also the pattern of clinical features (Van der Meché and Van Doorn, 1995).

It is important to note that the glycosyl moieties of gangliosides can also be present on peripheral nerve glycoproteins (Corbo *et al.*, 1993; Ilyas *et al.*, 1984; Quarles, 1997). Although the antibodies are called "anti-ganglioside antibodies" because our detection systems use purified gangliosides, in vivo these antibodies may not only bind to peripheral nerve glycolipids but also to glycoproteins.

*Specificity and isotype of anti-glycolipid antibodies*

The extensive search for many different anti-glycolipid antibodies has been stimulat-

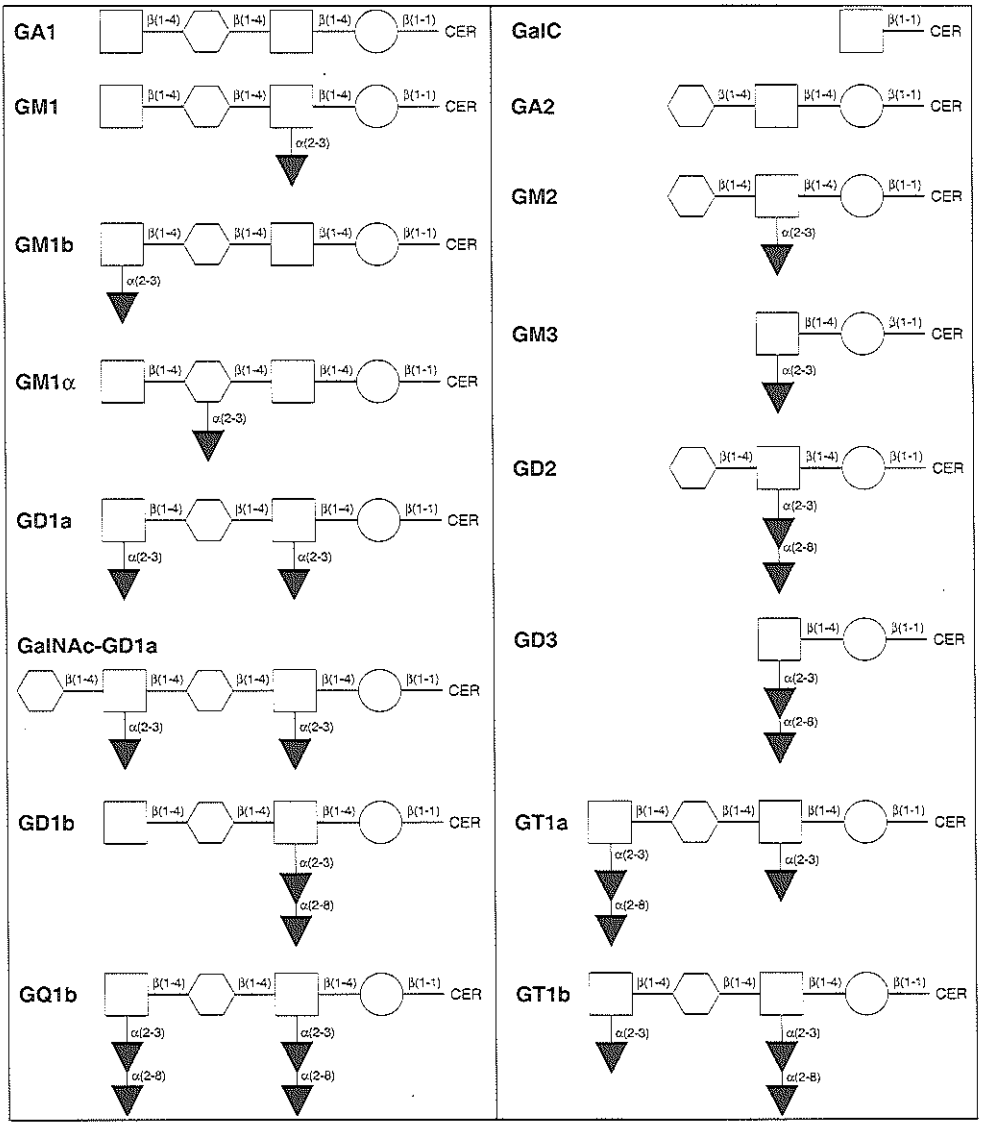


**Figure 3.** Structures of glycolipids relevant in immune-mediated neuropathies. □ galactose, ▼ sialic acid, ○ glucose, ⬡ N-acetyl-galactosamine, CER ceramide.

ed by the finding that antibody reactivity against some glycolipids has been correlated with antecedent clinical infections and clinical and electrophysiological subtype. (Ho *et al.*, 1999; Jacobs *et al.*, 1998; Mizoguchi *et al.*, 1994; Van der Meché and Van Doorn, 1995; Yuki *et al.*, 1992b). Since the first description of anti-ganglioside reactivity in acute phase serum from GBS patients in 1988 (Ilyas *et al.*, 1988), many studies have confirmed and extended these

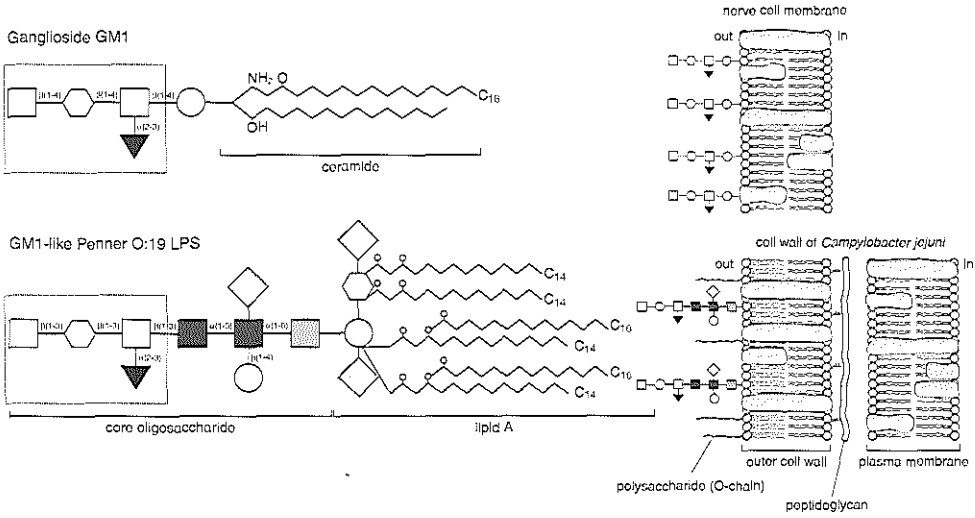


**Figure 4.** Structures of the cell membrane of a nerve cell and the cell wall of the Gram-negative bacterium *Campylobacter jejuni*. Gangliosides are located in the nerve cell membrane, lipopolysaccharides containing ganglioside mimics are located in the outer part of the cell wall of *C. jejuni*.

findings (Quarles and Weiss, 1999). GBS patients have antibody reactivity against numerous glycolipids, summarized in Table 4. It is important to note that the specificity ranges widely between patients and the frequencies of antibody reactivity against a specific ganglioside never reach 100% of patients, further indicating the heterogeneity of GBS. Another important issue is the presence of far more glycolipid species in peripheral nerve than tested so far (Chiba *et al.*, 1997; Ogawa-Goto *et al.*, 1993; Svennerholm *et al.*, 1994). Therefore, it is possible that GBS patients who do not have anti-glycolipid reactivity against one or more of the glycolipids tested, do have antibodies that react with a yet undefined glycolipid.

Ganglioside GM1 has been studied most extensively and antibody reactivity against this ganglioside is not only found in GBS patients but also in patients with chronic inflammatory demyelinating neuropathy (CIDP), multifocal motor neuropathy, paraproteinaemic neuropathy and in patients with lower motor neuron syndromes (Sadiq *et al.*, 1990). Antibodies against ganglioside GQ1b are found in >90% of MFS patients and can also be detected in GBS patients with oculomotor symptoms and patients with acute oropharyngeal palsy (Chiba *et al.*, 1993; Kusunoki *et al.*, 1999a; O'Leary *et al.*, 1996).

Naturally occurring anti-glycolipid antibodies are generally of low titer and of the IgM isotype (Casali and Notkins, 1989). They may have a lower affinity than anti-glycolipid antibodies in neuropathy patients (Mizutamari *et al.*, 1998). In contrast, anti-glycolipid antibodies in GBS patients can reach a high titer and are frequently of the IgA and IgG isotype (Jacobs *et al.*, 1996b; Quarles *et al.*, 1990; Willison and Kennedy, 1993). The titer generally decreases in parallel with clinical recovery but in some patients, anti-ganglioside titers remain high despite disappearance of clinical symptoms (Mizoguchi, 1998; Nobile-Orazio *et al.*,

**Table 4. Anti-glycoconjugate reactivity, antecedent infections and clinical features in immune-mediated neuropathies.**

| Glycoconjugate | Isotype | Antecedent infections in acute neuropathies | Clinical features of neuropathy | Electrophysiological features |
|---|---|---|---|---|
| Galactocerebroside | IgM/IgG | *M. pneumoniae* | - | Demyelinating |
| Sulfatide | IgM/IgG | - | Chronic, predominantly sensory distal | - |
| LM1 | IgM/IgG | - | - | Demyelinating |
| GA1 | IgM/IgG | - | - | - |
| GM1 | IgG>IgM | *C. jejuni* | Acute predominantly motor sparing of cranial nerves | Predominantly axonal |
| GM1 | IgM monoclonal/ polyclonal | - | Multifocal motor neuropathy | Demyelinating or mixed conduction block |
| GM1b | IgG>IgM | *C. jejuni* | Acute predominantly motor sparing of cranial nerves | Predominantly axonal |
| GM2 | IgM>IgG | Cytomegalovirus | Severe motor and sensory deficits | Demyelinating |
| GM3 | IgM/IgG | - | - | - |
| GD1a | IgG | *C. jejuni* | Acute motor axonal neuropathy | Axonal |
| GalNAc-GD1a | IgG>IgM | *C. jejuni* | Acute predominantly motor sparing of cranial nerves | - |
| GD1b | IgG>IgM | *C. jejuni* | - | - |
| GD1b | IgM monoclonal | - | Sensory ataxic neuropathy | Predominantly axonal |
| GD3 | IgG>IgM | - | Miller Fisher syndrome | Predominantly demyelinating |
| GD3 | IgM monoclonal | - | Sensory ataxic neuropathy CANOMAD | - |
| GT1a | IgG>IgM | - | Bulbar signs | - |
| GT1b | IgG>IgM | - | - | - |
| GQ1b | IgG>IgM | - | Miller Fisher syndrome oculomotor weakness in GBS patients | - |
| GQ1b | IgM monoclonal | - | Sensory ataxic neuropathy CANOMAD | - |
| GQ1b-alpha | IgM | - | Sensory ataxic neuropathy | Predominantly axonal |
| MAG | IgM | - | Chronic sensory>motor predominantly distal | Predominantly demyelinating |

1992; Willison, 1996). The presence of IgA antibodies points at a mucosal origin of the antibodies and the IgG1 and/or IgG3 subclass of anti-ganglioside antibodies indicates that CD4+ T cell help is involved, contrary to what is generally described for anti-carbohydrate antibodies (Alfonso *et al.*, 1995; Freimer *et al.*, 1993; Garcia Guijo *et al.*, 1992; Jacobs *et al.*, 1997a; Ogino *et al.*, 1995; Stavnezer, 1996; Willison and Veitch, 1994). Sequencing of the Ig variable region of monoclonal anti-ganglioside antibodies derived from patients with chronic neuropathies show extensive somatic mutation highly suggestive of an antigen driven antibody response (Paterson *et al.*, 1995; Weng *et al.*, 1992; Willison *et al.*, 1996).

*Relation of anti-glycolipid antibody reactivity with antecedent infections*

*C. jejuni* is the infectious agent that has been linked most frequently to anti-ganglioside reactivity in GBS patients (Hughes *et al.*, 1999). In most studies, *C. jejuni* infections have

been linked to anti-GM1 reactivity (Table 4, see Hughes *et al.*, 1999 for review), although this could not be confirmed in some other studies (Vriesendorp *et al.*, 1993). Anti-glycolipid antibodies in *C. jejuni* infected GBS patients are not exclusively directed against GM1. Several groups have documented the presence of antibody reactivity against GM1-like structures, such as GalNAc-GD1a and GM1b (Figure 3; Kusunoki *et al.*, 1996a; Yuki *et al.*, 1996). Furthermore, *C. jejuni* -infected GBS patients have antibodies against a variety of glycolipids and this could explain the lack of an absolute correlation between anti-GM1 antibodies and *C. jejuni* infection (Yuki *et al.*, 1999). In Chinese and Japanese GBS patients there is a correlation between anti-GD1a antibodies and *C. jejuni* infection but this was not found in studies from Europe and Northern America (Ho *et al.*, 1999; Jacobs *et al.*, 1998; Yuki *et al.*, 1992b).

Patients with an uncomplicated *C. jejuni* enteritis and occasionally some healthy individuals display anti-GA1 and anti-GM1 reactivity but the isotype is almost always IgM and the titers of these antibodies are generally much lower (Garcia Guijo *et al.*, 1995; Marcus, 1990; Mizutamari *et al.*, 1994; Willison, 1996). One of the explanations for this finding is that anti-glycolipid antibodies in uncomplicated enteritis patients and in healthy individuals are of lower affinity but this hypothesis awaits experimental confirmation (Deisenhammer *et al.*, 1996; Dippold and Bernhard, 1992; Mizutamari *et al.*, 1998).

The second most frequent antecedent infectious agent, CMV, is associated with antibody reactivity against GM2 (Irie *et al.*, 1996; Jacobs *et al.*, 1997d; Khalili-Shirazi *et al.*, 1999; Yuki and Tagawa, 1998). However, some investigators found that patients with an uncomplicated CMV infection also had anti-GM2 reactivity, questioning the relation between anti-GM2 reactivity and neurological symptoms (Jacobs *et al.*, 1997d; Yuki and Tagawa, 1998).

*M. pneumoniae* infections have been linked to antibodies against galactocerebroside (GalC), a neutral glycolipid (Kusunoki *et al.*, 1995; Nishimura *et al.*, 1996b), although reactivity against other glycolipids has been described (Heckmann *et al.*, 1999; Inuzuka *et al.*, 1988; Yoshino *et al.*, 1992). A complicating factor in the study of this correlation is the small number of cases available for study as *Mycoplasma* infections only account for a small percentage of all GBS cases (Jacobs *et al.*, 1998; Winer *et al.*, 1988c).

*Relation of anti-glycolipid reactivity with electrophysiological subtypes of GBS*

Many groups have tried to identify a correlation between anti-ganglioside reactivity and electrophysiological parameters. In China and Japan, electrophysiological evidence for axonal damage has been linked to GD1a and GM1, but this has not been confirmed in Europe (Hadden *et al.*, 1998; Ho *et al.*, 1999; Hughes *et al.*, 1999; Jacobs *et al.*, 1997c; Kuwabara *et al.*, 1998b). In addition, antibodies against so-called minor gangliosides such as GalNAc-GD1a have been reported to occur frequently in Japanese patients with axonal neuropathy but this could not be demonstrated in Dutch patients (Ang *et al.*, 1999; Hao *et al.*, 1999; Kaida *et al.*, 2000; Yuki *et al.*, 2000). The Dutch patients with anti-GalNAc-GD1a antibodies had a pure motor form of GBS with distally predominant weakness but did not display axonal dam-

age more often than GBS patients without such antibodies (Ang *et al.*, 1999).

*Relation of anti-glycolipid antibodies with clinical features and response to therapy*

The clearest example of an association between clinical features and anti-glycolipid reactivity is the Miller Fisher syndrome. Antibodies against GQ1b can be found in up to 90% of cases of MFS and also in GBS patients with oculomotor symptoms (GBS-MFS overlap; Carpo *et al.*, 1998; Chiba *et al.*, 1993, 1992; Willison and O'Hanlon, 1999; Willison *et al.*, 1993b). This may be explained by the enrichment of GQ1b in oculomotor nerves (Chiba *et al.*, 1997). Anti-GT1a in combination with anti-GQ1b reactivity has been demonstrated in patients with acute oropharyngeal palsy and in GBS patients with bulbar palsy as the initial symptom (Koga *et al.*, 1998a; Mizoguchi *et al.*, 1997).

GBS patients with a pure motor neuropathy with predominant distal weakness frequently have antibodies against GM1, GalNAc-GD1a and/or GM1b (Ang *et al.*, 1999; Hao *et al.*, 1999; Jacobs *et al.*, 1996b; Kaida *et al.*, 2000; Yuki *et al.*, 2000). As these patients with anti-GM1/GalNAc-GD1a/GM1b reactivity also frequently have an antecedent infection with *C. jejuni*, there has been some doubt as to whether pure motor GBS is linked to *C. jejuni* infection or to the anti-ganglioside antibodies (Rees *et al.*, 1995a).

In a series of Dutch studies, some in collaboration with the group of Yuki from Dokkyo University, Japan, a correlation was found between anti-GM1, anti-GalNAc-GD1a and anti-GM1b reactivity and the response to therapy. Although patients with anti-ganglioside antibodies were more severely affected, they responded more favorably to IVIg treatment than patients without anti-ganglioside antibodies (Ang *et al.*, 1999; Jacobs *et al.*, 1996a, 1996b; Yuki *et al.*, 2000). However, these results were not confirmed in another large study (Hadden *et al.*, 1998).

**Antibodies against other nerve antigens**

Long before the search for anti-glycolipid antibodies, researchers have been trying to identify protein components of peripheral nerve as the principal site of attack in GBS (reviewed in Hughes *et al.*, 1999). In most studies, there was no increased incidence of antibody reactivity against homogenates of peripheral nerve in GBS patients compared to controls. Antibodies against P0, P2 and PMP22 are present in serum from GBS patients but their incidence varies widely depending on the detection method and study population (Gabriel *et al.*, 2000; Geczy *et al.*, 1985; Khalili-Shirazi *et al.*, 1993; Ritz *et al.*, 2000; Winer *et al.*, 1988b). However, with the development of new assay systems such as recombinant protein expression, and the awareness that the antibody response in GBS patients is heterogeneous, this issue may be thoroughly addressed in the future (Hughes *et al.*, 1999; Kwa *et al.*, 2000; Ritz *et al.*, 2000; Van der Meché *et al.*, 1997).

**Functional properties of anti-glycolipid antibodies**

It is currently unknown whether the antibodies directed against glycolipids or other nerve antigens described above are responsible for the pathology seen in the nerves of GBS

patients. Many researchers have identified structures in peripheral nerve that bind anti-ganglioside antibodies. A difficulty is posed by the fact that binding of anti-ganglioside antibodies to the cell surface is influenced by relative distribution of gangliosides in the cell membrane and methods used for tissue fixation (Lloyd *et al.*, 1992; Willison and O'Hanlon, 1999). Therefore, no direct inferences can be made from biochemical determinations of the concentration of a ganglioside-species (Chiba *et al.*, 1997; Ogawa-Goto *et al.*, 1992). The possible pathogenic effects of anti-glycolipid antibodies are summarized in Table 5.

**Table 5. Putative functional effects of anti-glycolipid antibodies and T cells in the Guillain-Barré syndrome.**

| Anti-glycolipid antibodies | T cells |
|---|---|
| Binding to nervous tissue | Direct cytotoxicity |
| Complement activation and | Secretion of cytokines |
| assembly of the membrane attack complex | T cell help for antibody production |
| Opsonization and attraction of macrophages | Impairment of the blood-nerve barrier |
| Antibody-dependent cell-mediated cytotoxicity | Regeneration |
| Blocking of ion-receptors leading to conduction failure | |
| Impairment of the blood-nerve barrier | |
| Modulation of cells of the immune system | |

*Binding to nervous tissue and activation of complement*

Although post-mortem studies report the presence of immunoglobulin deposits on the Schwann cell (Hafer-Macko *et al.*, 1996a), to date the specificity of these antibodies is unknown. Based on the assumption that anti-ganglioside antibodies mediate the pattern of clinical symptoms and the ganglioside content of nerve structures, it was expected that anti-GM1 antibodies would preferentially bind to motor nerves and anti-GQ1b antibodies to oculomotor nerves. However, this turned out to be not true. Monoclonal anti-GM1 antibodies from motor neuropathy patients have heterogeneous binding patterns. Anti-GM1 antibodies bind to myelin, axons, nodes of Ranvier and dorsal root ganglion cells but also to extracellular matrix material (O'Hanlon *et al.*, 1996, 1998). For anti-GQ1b antibodies the same holds true. A monoclonal anti-disialosyl antibody was shown to bind to many neural structures, not only including those on oculomotor nerves and dorsal root ganglion cells but also motor end plate regions (Willison *et al.*, 1996). In addition, anti-GM1 antibodies also stained oculomotor nerve (O'Hanlon *et al.*, 1998). This indicates that the distribution of GM1 and GQ1b epitopes does not correspond with the clinical pattern associated with these antibodies (Ganser and Kirschner, 1984; Kusunoki *et al.*, 1993; Paterson *et al.*, 1995; Willison *et al.*, 1997).

An explanation for these observations may be that binding of antibodies represents artefacts caused by processing of the specimens. Another possibility is that in neuropathy patients the blood nerve barrier is selectively impaired, allowing antibody influx only at specific sites. Finally, binding of anti-ganglioside antibodies may not always lead to nerve damage or dysfunction (O'Hanlon *et al.*, 1998; Paparounas *et al.*, 1999; Willison *et al.*, 1997).

Anti-ganglioside antibodies from neuropathy patients have been shown to bind complement in vitro and in vivo (Benatar *et al.*, 1999; Mizutamari *et al.*, 1998; Paparounas *et al.*,

1999; Uetz-Von Allmen *et al.*, 1998). This is in line with the isotype and subclass of the anti-ganglioside antibodies, IgM, IgG1 and IgG3 (Papadea and Check, 1989; Willison and Veitch, 1994). However, despite the activation of complement, cell lysis could not be observed (Benatar *et al.*, 1999; Paparounas *et al.*, 1999). Activation of complement on Schwann cells of affected nerves of GBS patients may have been caused by binding of anti-ganglioside antibodies but this has not been proven (Hafer-Macko *et al.*, 1996b).

*Demyelination*

Effects of GBS serum have been mainly studied in myelinated Schwann cell cultures. Myelin disintegration occurred when GBS sera were applied but this could also be accomplished with serum from patients with other neurological diseases (Birchem *et al.*, 1987; Sawant-Mane *et al.*, 1991; Sawant-Mane *et al.*, 1994). The effect was complement dependent in most studies. In one study, no relation between anti-ganglioside reactivity and cytotoxicity was found (Mithen *et al.*, 1992). However, there have been no studies performed with purified or monoclonal anti-ganglioside antibodies. Serum with anti-GalC antibodies has the ability to cause demyelination in spinal cord cultures (Roth *et al.*, 1985).

Intraneural injection of GBS serum or purified immunoglobulins in rats has yielded variable results (reviewed in Hughes *et al.*, 1999 and Hartung *et al.*, 1995b). Most studies described demyelination and conduction block induced by both GBS and control sera, perhaps resulting from the injection of relatively large amounts of fluid within the perineurial space (Oomes *et al.*, 1991). A limited number of passive transfer studies has yielded rewarding results. Transfer of anti-GD1b antibodies leads to degeneration of axons in the dorsal column (Kusunoki *et al.*, 1999c).

Together, these results indicate that antibodies in serum from GBS patients have the ability to bind to the nerve and cause demyelination. The exact specificity of the demyelinating antibodies is not known but they may well be directed against a glycolipid antigen.

*Electrophysiological effects*

In addition to the secondary effect on conduction following demyelination, antibodies may also have a direct effect on nerve conduction. Various models are currently being developed to study these effects.

Serum samples from a small number of GBS patients cause conduction block when applied to rat sciatic nerve. In one study, the effect was complement dependent and associated with IgM anti-GM1 antibodies but not all sera containing anti-GM1 antibodies produced this blockade (Arasaki *et al.*, 1998). Other studies demonstrated an effect of anti-GM1 antisera on $K^+$ and $Na^+$ channels, depending on the presence of complement (Takigawa *et al.*, 1995; Weber *et al.*, 2000). Due to the limited number of studies, the methodological differences between the studies and the lack of effect of many sera, the role of anti-ganglioside antibodies in this phenomenon is difficult to interpret (Hirota *et al.*, 1997; Hughes *et al.*, 1999; Paparounas *et al.*, 1999). Other groups have attempted to develop in vitro models to study the effect of anti-ganglioside antibodies using motor neuron cell lines but this has not yet been

rewarding (Benatar *et al.*, 1999).

Experiments with the mouse phrenic nerve-diaphragm model have lead to promising results. Intraperitoneal injection of serum from a GBS patient with unknown anti-ganglioside specificity and with a monoclonal antibody against disialogangliosides resulted in paralysis of the diaphragm of mice (Van den Berg *et al.*, 1994; Willison *et al.*, 1996). IgG fractions from MFS patients and monoclonal antibodies with anti-GQ1b reactivity disrupt neuromuscular transmission in a complement dependent manner (Bullens *et al.*, 2000; Goodyear *et al.*, 1999; Plomp *et al.*, 1999). Using another recording method, IgG from GBS patients also had an effect independent of anti-GQ1b reactivity and complement (Buchwald *et al.*, 1995, 1998). Sera from convalescent patients and healthy controls had no effect in both systems. Clearly, more research is needed to resolve these apparent contradictions and to investigate the mechanisms of these electrophysiological effects.

*Impairment of blood-nerve barrier function*

Endoneurial blood vessels contain gangliosides that can be the target of circulating anti-glycolipid antibodies (Kanda *et al.*, 1997, 2000b). Anti-GM1 antibodies can impair the function of a blood-nerve barrier model and may also have an effect on disruption of the blood-nerve barrier in GBS (Kanda *et al.*, 2000a). In addition, anti-glycolipid antibodies and activated T cells may synergistically alter blood-nerve barrier function (Pollard *et al.*, 1995; Spies *et al.*, 1995).

*Modulation of cells of the immune system*

Gangliosides are not only present in the nervous system but are also present on cells of the immune system. GM1 is enriched in functional domains of cells and GA1 is a marker for natural killer (NK) cells (Kurzchalia and Parton, 1999; Slifka *et al.*, 2000). Injection of rodents with anti-GA1 antibodies leads to depletion of NK-cells (Slifka *et al.*, 2000). In GBS patients, the presence of anti-glycolipid antibodies may lead to disturbances in numbers and/or function of cells of the immune system (Yoshii and Shinohara, 1998).

**The role of T cells in GBS**

The role of T cells in the pathogenesis of GBS is inconclusive. Possible roles are summarized in Table 5. An overt problem is caused by the fact that GBS is a post-infectious disease and that the presence of activated circulating T cells may not be related to the presence of neurological disease but to the cellular component of the normal immune response to the preceding infection. The presence of anti-ganglioside antibodies of the IgG1 and IgG3 subclass indicates that T-B cell interactions occur in the induction of the anti-glycolipid response. The activated intraneural T cells may either cause direct T cell mediated damage (Vergelli *et al.*, 1997), perhaps due to the action of the pro-inflammatory cytokines they secrete, or they may impair the function of the blood-nerve barrier (Pollard *et al.*, 1995) allowing entry of anti-neural antibodies. Alternatively, they may be involved in regeneration processes or in terminating the immune response (Cohen and Schwartz, 1999; Hohlfeld *et al.*, 2000; Schwartz

and Cohen, 2000).

A multifocal lymphocytic infiltration of nerves and roots is present in many but not all cases of demyelinating GBS (Asbury *et al.*, 1969; Honavar *et al.*, 1991). Most of the T cells in the infiltrate have an αβ T cell receptor but small numbers of γδ T cells are also present (Ben-Smith *et al.*, 1996; Cornblath *et al.*, 1990; Khalili-Shirazi *et al.*, 1998). Cells are $CD4^+$ or $CD8^+$ relative to their proportion in blood (Cornblath *et al.*, 1990). Many of the infiltrating T cells are activated as indicated by their expression of MHC class II molecules, IFN-γ and TNF-α (Hartung *et al.*, 1995b; Pollard *et al.*, 1987; Putzu *et al.*, 2000; Taylor and Hughes, 1989). In addition to infiltrating activated T cells, GBS patients also have increased numbers of circulating activated T cells. T cells isolated from GBS patients in the acute phase have an enhanced expression of MHC class II, IL-2 receptor and the transferrin receptor (Bansil *et al.*, 1991; Hartung *et al.*, 1995a, 1995b).

Relatively few studies have been performed to investigate the specificity of the activated T cells in GBS patients. In the experimental allergic neuritis model (EAN) in the Lewis rat, passive transfer of P0 or P2 specific T cells leads to neurological disease (Linington *et al.*, 1992; Rostami, 1997; Spies *et al.*, 1995). Some GBS patients show an enhanced proliferation of lymphocytes to P0 or P2 protein but in the same studies, it was also possible to raise P2-specific T cell lines from healthy individuals (Dahle *et al.*, 1997; Khalili-Shirazi *et al.*, 1992, 1993; Luijten *et al.*, 1984; Taylor *et al.*, 1991). T cells which are able to react with glycolipid antigens have been described but as there are no reports about these particular cells in GBS, their presence remains speculative (Benlagha *et al.*, 2000; Brossay *et al.*, 1998; Burdin and Kronenberg, 1999; Kawano *et al.*, 1997; Rosat *et al.*, 1999; Shamshiev *et al.*, 1999).

There is one report about a relatively restricted usage of Vβ chain genes of activated T cells from British GBS patients indicating a T cell response to a common antigen but this antigen has not been characterized (Khalili-Shirazi *et al.*, 1997). A Japanese study could not confirm these results (Ma *et al.*, 1998). Recent reports indicate that *Campylobacter* antigens may selectively stimulate γδ T cells isolated from nerve biopsies and peripheral blood from GBS patients in the acute phase (Ben-Smith *et al.*, 1996, 1997; Borsellino *et al.*, 2000; Cooper *et al.*, 2000).

In summary, none of the numerous putative pathogenic roles of T cells in GBS has been convincingly demonstrated.

**Cytokines and other humoral factors**

Cytokines are involved in the regulation of the immune response and some cytokines can have a demyelinating effect themselves. In vitro and in vivo studies have documented the potential of TNF-α, IL-6 and IL-12 to cause an inflammatory response and demyelination (Deretzi *et al.*, 1999; Hall *et al.*, 2000; Merrill and Scolding, 1999; Pelidou *et al.*, 1999; Redford *et al.*, 1995; Selmaj and Raine, 1988). TNF-α and other pro-inflammatory cytokines have been detected immunohistochemically in sural nerves from GBS patients (Putzu *et al.*, 2000; Zhu *et al.*, 1998).

IL-2 has been shown to be elevated in serum from GBS patients in the acute phase

(Bansil *et al.*, 1991). Other pro-inflammatory cytokines that have been shown to be elevated in GBS and MFS patients in the acute phase are IL-6, IFN-γ and TNF-α (Creange *et al.*, 1996; Exley *et al.*, 1994; Hohnoki *et al.*, 1998; Maimone *et al.*, 1993; Ossege *et al.*, 2000; Sharief *et al.*, 1993, 1997). The levels of most of these substances peak during the acute phase and decrease in parallel with clinical recovery. Levels of TGF-β, an anti-inflammatory cytokine, increase before onset of recovery, suggesting a role in terminating the anti-neural immune response (Creange *et al.*, 1998; Ossege *et al.*, 2000; Sindern *et al.*, 1996). As is the case for T cells, the data on cytokine-levels in serum should be interpreted with caution because control samples from patients with an infectious disease but without neurological complications have not been included in these studies.

Activated macrophages may also secrete enzymes affecting nerve integrity such as proteases and lipases (Hartung and Kieseier, 2000). Matrix metalloproteinases are upregulated in EAN and inhibitors of matrix metalloproteinases show a beneficial effect in EAN (Hughes *et al.*, 1998; Redford *et al.*, 1997).

Thus, the elevated levels of circulating pro-inflammatory cytokines and the presence of potential demyelinating cytokines in nerves from GBS patients indicates an important role for cytokines in the pathogenesis of immune-mediated neuropathies. However, the relationship with antecedent infections and the precise mechanisms of the various cytokines are still unknown.

**Disease susceptibility and disease modifying factors**

The current view is that post-infectious immune mediated diseases occur when a susceptible individual mounts a dysregulated immune response against a micro-organism with specific features (Figure 1). This susceptibility of an individual may have a genetic origin but can also be dependent on the immune status at the time of the antecedent infection. There is a continuing discussion about the relative contribution of host susceptibility factors and infectious agent related factors. The frequency of the infections that precede GBS is so high compared to the relatively low incidence of GBS, that there must be other factors that predispose to the development of neurological symptoms. It has been estimated that only 1 out of 1,000-5,000 individuals that have been infected with *C. jejuni* develops neurological symptoms (Buzby *et al.*, 1997; Havelaar *et al.*, 2000). Infections with CMV and EBV are so common that GBS following infection with these viruses is even more exceptional (Britt and Alford, 1996; Hesse *et al.*, 1983). Another argument in favor of strong influence of host-related factors are the observations that outbreaks of *C. jejuni* infections are common but clusters of post- *C. jejuni* GBS are rare (Ang *et al.*, 2000d; Blaser, 1997; Khoury, 1978; Sliman, 1978; Yuki and Tsujino, 1995).

Alternatively, the low incidence of post-infectious immune-mediated disease may be explained by rare, and as yet undefined, characteristics of infectious agents that precede GBS. For almost all infectious agents that precede GBS, multiple serotypes and/or genotypes have been described. As mentioned before, in Japan and South-Africa, development of GBS is associated with *C. jejuni* strains of the Penner O:19 and O:41 serotypes (Kuroki *et al.*, 1993;

Lastovica *et al.*, 1997), indicating the presence of a serotype specific feature, predisposing for neurological complications. One of these possible features is the presence of ganglioside-mimicry although ganglioside-mimicry of strains that have not been associated with GBS and MFS has been described (Moran, 1997). The presence of ganglioside-mimicry seems therefore necessary but not sufficient for the induction of cross-reactive anti-ganglioside/LPS response and the concomitant induction of neurological symptoms.

*Familial and recurrent GBS*

When determining genetic factors that influence the occurrence of a certain disease, it is obvious to investigate the presence of familial clustering. Few examples of familial GBS have been described (Bar-Joseph *et al.*, 1991; Davidson *et al.*, 1992; MacGregor, 1965; Saunders and Rake, 1965; Wilmshurst *et al.*, 1999; Yuki and Tsujino, 1995). Most of these examples involve cases of GBS that were spaced widely in time but there is one case report in which two sisters developed GBS within the same week. *C. jejuni* was isolated from the stools of both girls (Yuki and Tsujino, 1995). Apart from this exceptional case, the familial associations are so rare that they may be based on chance and hence do not reflect any underlying genetic associations. On the other hand, the combination of a genetic trait and infectious agent with specific features may be so rare that familial clustering will hardly occur. Another argument for host-related factors is the increased chance to develop GBS for the second or the third time, sometimes even triggered by the same kind of infection or vaccination (PA van Doorn, personal communication). This observation indicates that at least some GBS patients are persistenly prone to divert their immune response towards self-antigens.

*HLA-associations*

Quite a number of reports describe the lack of association of both HLA class I en class II alleles with GBS and MFS (Chiba *et al.*, 1995; Hillert *et al.*, 1991; Kaslow *et al.*, 1984; Ma *et al.*, 1998; Winer *et al.*, 1988a). Due to the heterogeneous nature of GBS, immunogenetic analysis of subgroups, defined at different levels, may be much more rewarding (Ma *et al.*, 1998; Rees *et al.*, 1995c; Yuki *et al.*, 1992a). GBS patients may be subgrouped on the basis of antecedent infection, course and severity of the disease, presence of anti-ganglioside antibodies, electrophysiological subtype and response to therapy. An additional complicating factor in immunogenetic analyses are the geographical differences in relative frequencies of alleles.

When subgrouped according to the presence of an antecedent *C. jejuni* infection, a British study identified an association with HLA DQB1*03 and in Japan an association with HLA-B35 was reported (Rees *et al.*, 1995c; Yuki *et al.*, 1992a). Subgrouping based on the presence of anti-ganglioside antibodies has not yet revealed an HLA-association (Koga *et al.*, 1998b). In China there was an association with DRB1*1301 of GBS patients with demyelination but not with axonal involvement (Monos *et al.*, 1997).

Because of the multi-level heterogeneity in the GBS population, the ideal situation would be to study a GBS population where there is only one precipitating factor. Such a sit-

uation appears to be present in rural areas in China and at the Caribbean island of Curaçao (Ho *et al.*, 1995; McKhann *et al.*, 1993; Van Koningsveld *et al.*, 2000a). Another possibility is the detailed study of outbreaks of *C. jejuni* enteritis or other GBS-related infectious diseases, where all individuals have had an infection with an identical micro-organism and investigate the immune response and genetic markers in those individuals (Ang *et al.*, 1998; Yoshikawa *et al.*, 2000).

*Polymorphisms in other immune response genes*

In addition to the HLA-system, more recently polymorphisms in other immune response genes have been identified and some of these polymorphisms have been associated with altered in vitro T cell responses (Feeney *et al.*, 1989; Pociot *et al.*, 1992). Polymorphisms in the TNF-α allele were more frequently present in *C. jejuni* associated GBS patients and polymorphisms in the Fc-γ-receptor have been associated with severity of GBS (Ma *et al.*, 1998; Van der Pol *et al.*, 2000; Vedeler *et al.*, 2000). Both studies have used only a small number of individuals and studies with a large number of patients are required to confirm these results (Pulst, 1999; Sawcer *et al.*, 1997). In addition, there are numerous other immune response genes in which polymorphisms have been described, all potential candidates for a disease-causing and/or modifying factor.

**Animal models for immune-mediated neuropathies**

To study the immunological mechanisms that lead to GBS, an animal model is indispensable. It is possible to induce immune-mediated neuropathies in laboratory animals. In almost all studies, animals were immunized with peripheral nerve homogenates or purified/recombinant glycoproteins or glycolipids in a strong adjuvant.

*Experimental allergic neuritis (EAN)*

Immunization of animals with homogenates of peripheral nerve, which contain a mixture of proteins and glycolipids, leads to a demyelinating disease in a number of animal species, of which the Lewis rat has been studied most extensively (Gold *et al.*, 2000; Vriesendorp, 1997). Purified peripheral nerve glycoproteins that have been demonstrated to be neuritogenic are P0, P2 and PMP22 (Gabriel *et al.*, 1998; Linington *et al.*, 1992; Rostami, 1997; Spies *et al.*, 1995). Although the pathology observed in this model closely resembles the demyelinating form of GBS, the lack of etiological validity defies the use of this model as a model for GBS. Like experimental autoimmune encephalomyelitis, the model can be used to evaluate immunological mechanisms and therapeutic options with the occurrence of peripheral nerve pathology and weakness as outcome measures (Vriesendorp, 1997). However, this model is unsuitable to study the relation between antecedent infections and immune-mediated neuropathy.

*Galactocerebroside-induced neuropathy*

Galactocerebroside (GalC) is a glycolipid and a major constituent of peripheral nerve

(Ogawa-Goto *et al.*, 1993). Repeated immunization of rabbitts with this glycolipid in complete Freund's adjuvant leads to the development of weakness caused by demyelination. Histologically, the nerves of affected rabbits closely resemble those from demyelinating GBS although lymphocytic infiltration is sparse (Saida *et al.*, 1979b, 1981). Serum from these rabbits contain anti-GalC antibodies which have the capacity to cause demyelination and conduction block in vitro and after intraneural injection (Hirota *et al.*, 1997; Saida *et al.*, 1979a; Sumner *et al.*, 1982).

*GM1 induced-neuropathy*

Although many groups have tried to induce a neuropathy by immunization with GM1, there are only two reports describing neurological dysfunction in GM1-immunized animals. In the first study, GM1-immunized rabbits developed a spastic paralysis, while GD1a immunized rabbits and guinea pigs had a flaccid paralysis (Nagai *et al.*, 1976). Histologically, phagocytic cells containing myelin debris could be observed. In the other study, one rabbit developed a subclinical neuropathy which could only be detected with electrophysiological examination. The serum of the rabbit contained IgM and IgG anti-GM1 antibodies. There was mild axonal degeneration in the sciatic nerve and IgM deposits at the nodes of Ranvier (Thomas *et al.*, 1991).

*GD1b-induced sensory ataxic neuropathy*

Patients with a chronic ataxic neuropathy can have a paraprotein that binds to gangliosides with a disialosyl epitope (Herron *et al.*, 1994; Ilyas *et al.*, 1985b). Antiserum that reacts with GD1b also binds to dorsal root ganglions (Kusunoki *et al.*, 1993). Immunization of rabbits with GD1b mixed with keyhole limpet hemocyanin resulted in IgM anti-GD1b antibodies and an ataxic neuropathy and axonal degeneration in the spinal cord and dorsal root, together with degeneration of some nerve cell bodies in the dorsal root ganglion (Kusunoki *et al.*, 1999b, 1996b). Serum IgM antibodies from these rabbits was shown to have a degenerating effect in dorsal root ganglion cultures (Hitoshi *et al.*, 1999; Kusunoki *et al.*, 1999c).

*Experimental Campylobacter neuropathy*

Because there is an obvious link with preceding infections, in particular *C. jejuni*, an animal model based on infection with micro-organisms or immunization with microbial products is of utmost importance. In addition to numerous case reports about idiopathic polyneuropathy in animals (Alford and Satterfield, 1995; Bichsel *et al.*, 1987; Brown *et al.*, 1985; Cummings *et al.*, 1979; Cummings and Haas, 1972; Gerritsen *et al.*, 1996; Northington and Brown, 1982), there is only one as yet unconfirmed report about a GBS-like disease in chickens that lived in the same area as Chinese patients with *C. jejuni* -related GBS (Li *et al.*, 1996).

In this report, a *C. jejuni* strain, isolated from one of the GBS patients, was fed to healthy chickens leading to the development of weakness in a proportion of the animals. Histologically, the sciatic nerves of some of the animals showed axonal degeneration and

macrophages overlying the nodes of Ranvier, identical to axonal GBS. In contrast, some of the nerves from severely paralysed chickens were completely normal. A major drawback of this study was the lack of appropriate controls since the chickens were not screened for previous infection with *C. jejuni*. In addition, no serological studies for anti-ganglioside antibodies were performed so that the role of these antibodies could not be investigated (Li *et al.*, 1996).

Other groups have demonstrated the induction of anti-ganglioside antibodies following immunization of mice, rats and rabbits with purified *C. jejuni* LPS but none of the immunized animals showed any signs of weakness (Ang *et al.*, 2000b; Goodyear *et al.*, 1999; Ritter *et al.*, 1996; Wirguin *et al.*, 1997).

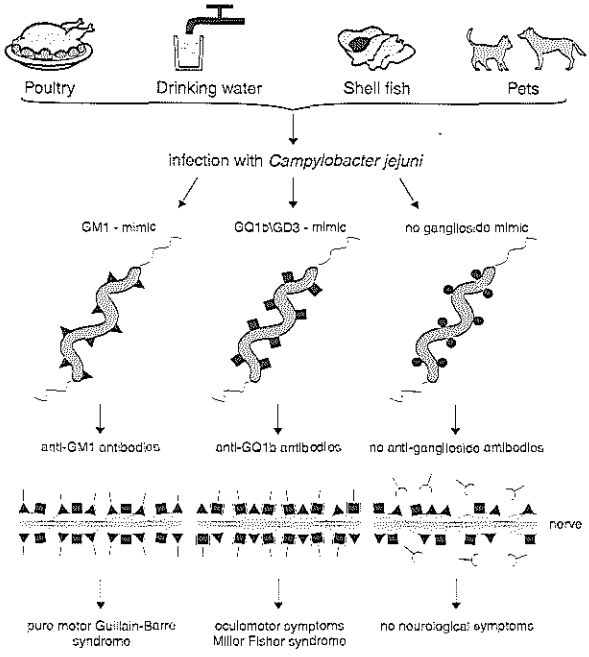


**Figure 5.** Proposed scheme for the role of differences in molecular mimics of *C. jejuni* LPS in the induction of anti-GM1 and anti-GQ1b antibodies and corresponding clinical features in patients with the Guillain-Barré and Miller Fisher syndrome.

## OUTLINE OF THE THESIS

The presence of anti-glycolipid antibodies in serum from patients with acute immune-mediated neuropathies is fundamental to the central hypothesis of the work described here. We hypothesize that acute immune-mediated neuropathies are caused by anti-glycolipid antibodies that are induced by molecular mimicry between microbial and peripheral nerve anti-

gens. The nature of the antecedent infections determines the anti-glycolipid specificity and consequently the pattern of clinical symptoms. A proposed scheme for this series of events following infection with *C. jejuni* is depicted in Figure 5.

In order to test this hypothesis we addressed the following research questions:

1. Can the presence of specific anti-glycolipid antibodies in GBS patients be related to antecedent infections and specific clinical features? (Chapters 2-6, 8)
2. Is there a difference is the frequency of antecedent *C. jejuni* infections and anti-glycolipid reactivity between GBS patients from Japan and The Netherlands? (Chapters 4,5)
3. Do anti-GM2 and anti-GalC antibodies in CMV and *M. pneumoniae* infected GBS patients cross-react with antigens from the corresponding infectious agents? (Chapters 6, 8)
4. Are there genetic and/or serological characteristics that distinguish GBS and MFS-associated *C. jejuni* strains from other *C. jejuni* strains? (Chapters 9, 10)
5. Are there differences in antibody responses to ganglioside-mimics between GBS patients and uncomplicated enteritis patients following infection with *C. jejuni*? (Chapters 7, 10)
6. Can cross-reactive anti-glycolipid/LPS responses and neurological symptoms be induced by immunization of rabbits with *C. jejuni* LPS? (Chapters 11-13)

In the discussion, the relevance of mimicry between microbiological agents and peripheral nerve glycolipids in the pathogenesis of GBS will be discussed. Furthermore, the evidence for a pathogenic role of anti-glycolipid antibodies in the pathogenesis of GBS will be critically reviewed. Finally, directions for future investigations will be indicated, mainly based on the results from the studies presented in this thesis.

# Chapter 1

# RAPIDLY PROGRESSIVE, PREDOMINANTLY MOTOR GUILLAIN-BARRÉ SYNDROME WITH ANTI-GALNAC-GD1A ANTIBODIES

**CW Ang[1,2], N Yuki[3], BC Jacobs[1,2], M Koga[3], PA van Doorn[1], PIM Schmitz[4], FGA van der Meché[1]**

Departments of Neurology[1], Immunology[2] and Trials and Statistics[4], Erasmus University Rotterdam, The Netherlands
Department of Neurology[3], Dokkyo University School of Medicine, Tochigi, Japan

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## ABSTRACT

**Objective**

To investigate the presence of anti-GalNAc-GD1a antibodies in patients with Guillain-Barré syndrome and to determine the relation of anti-ganglioside antibodies with clinical features.

**Background**

The Guillain-Barré syndrome (GBS) is heterogeneous with regard to clinical manifestations, antecedent infections and the presence and specificity of anti-ganglioside antibodies. In recent years, antibodies to minor gangliosides have been identified in serum from GBS patients.

**Methods**

Detection of anti-ganglioside antibodies with ELISA in 132 Guillain-Barré syndrome patients, followed by correlating results to a database with information on antecedent infections and clinical parameters.

**Results**

Anti-GalNAc-GD1a antibodies could be detected in 19 (14%) of GBS patients. The presence of anti-GalNAc-GD1a antibodies was related to antecedent *Campylobacter jejuni* infection ($p<0.001$). GBS patients with anti-GalNAc-GD1a antibodies had a rapidly progressive, more severe, and predominantly distal weakness. Furthermore, they had less sensory loss, paresthesias and cranial nerve involvement. In the majority of the patients, this reactivity was independent of reactivity to GM1. Dividing patients into separate groups based on their reactivity to GalNAc-GD1a and GM1 enabled us to delineate more homogeneous subgroups with regard to clinical features.

**Conclusions**

This study provides further evidence for the hypothesis that antecedent infections and the specificity of subsequent anti-neural antibody responses determine the clinical manifestations in GBS patients.


## INTRODUCTION

The Guillain-Barré syndrome (GBS) is an acute immune-mediated polyneuropathy, characterized by rapidly progressive muscle weakness and variable sensory loss. GBS patients show a large diversity in clinical manifestations, pathological features and outcome (Hughes, 1990) and differences in anti-neural immune responses may determine the observed clinical heterogeneity (Van der Meché and Van Doorn, 1995).

Antibodies that react with various gangliosides have been demonstrated in different proportions of GBS patients (Ilyas *et al.*, 1988; Jacobs *et al.*, 1998). Antibody reactivity to GM1 (Figure 1), one of the major gangliosides in peripheral nerve (Ogawa-Goto *et al.*, 1990),

has been studied most extensively. The presence of anti-GM1 antibodies in GBS patients is associated with a more severe (Rees and Hughes, 1994), pure motor variant (Visser *et al.*, 1995; Yuki *et al.*, 1990) with more extensive axonal degeneration (Gregson *et al.*, 1993; Yuki *et al.*, 1990) and a predominantly distal distribution of weakness (Jacobs *et al.*, 1996b). In concurrence with these clinical features, the concentration of GM1 is higher in the myelin of motor nerves compared to sensory nerves (Ogawa-Goto *et al.*, 1992). Furthermore, human anti-GM1 antibodies preferentially bind to the distal motor terminals at the end plate (Illa *et al.*, 1995). The presence of anti-GQ1b antibodies is highly associated with the Miller Fisher syndrome (MFS) and oculomotor weakness in GBS patients (Chiba *et al.*, 1993; Willison *et al.*, 1993b). This association correlates with the prominent expression of GQ1b in oculomotor nerves (Chiba *et al.*, 1993).

In addition to major gangliosides such as GM1, peripheral nerves contain many other species of gangliosides (Hakomori, 1981; Ledeen and Yu, 1982). Recent reports on antibodies these minor gangliosides such as *N*-acetylgalactosaminyl GD1a (GalNAc-GD1a; Figure 1) in serum of GBS patients indicate that minor gangliosides can be targets for anti-ganglioside antibodies (Kusunoki *et al.*, 1994; Yuki *et al.*, 1996). These studies also suggested a strong correlation of anti-GalNAc-GD1a reactivity with preceding gastro-intestinal infection, in particular with *C. jejuni*. Furthermore, electrophysiological studies in these patients indicate that the presence of anti-GalNAc-GD1a antibodies is associated with axonal damage.

In the present study we determined antibody reactivity to the minor ganglioside GalNAc-GD1a in a group of 132 clinically well-defined GBS patients. We investigated the relation between reactivity to GalNAc-GD1a and a large number of clinical features. In addition, we determined the reactivity of these serum samples with ganglioside GM1 to investigate the relation between clinical manifestations and the fine-specificity of anti-ganglioside antibodies.

## PATIENTS AND METHODS

### Patients

Pre-treatment serum samples were obtained from 132 of 147 GBS patients who participated in the Dutch GBS trial, which compared the therapeutic effects of plasma exchange (PE) and intravenous immunoglobulins (IVIg; Van der Meché and Schmitz, 1992) and reactivity to several gangliosides has already been reported (Jacobs *et al.*, 1998; Jacobs *et al.*, 1997d; Jacobs *et al.*, 1996b). Fifteen cases were excluded because no pre-treatment serum was available. The excluded cases did not differ from the other patients with regard to their clinical manifestations and course of disease.

All patients fulfilled the criteria for GBS (Asbury and Cornblath, 1990), were unable to walk 10 m independently, and were admitted to the Dutch GBS trial within two weeks of onset of weakness. The functional score and the Medical Research Council (MRC) sum score (Kleyweg *et al.*, 1991), ranging from 60 (normal) to 0 (tetraplegic) were determined at study

entry and subsequently at 16 time points during a follow-up period of six months. The rapidity of progression was indicated by the number of days from the onset of weakness to the moment of maximal weakness. The maximum severity of weakness was indicated by the lowest MRC sum score. From 93 patients EMG data were available for analysis.

**Anti-ganglioside serology**

GalNAc-GD1a and GM1 were prepared from bovine brain as described elsewhere (Hirabayashi *et al.*, 1988; Yuki *et al.*, 1996). Serum samples were tested for the presence of IgM and IgG antibodies to the above mentioned gangliosides using ELISA (Yuki *et al.*, 1997a). Five picomoles of each ganglioside were placed in individual wells of microtiter plates. Wells without ganglioside were used as control. Serum samples were diluted serially starting at 1:500, added to each well and incubated at 4°C overnight. Peroxidase-conjugated goat anti-human γ- or μ-chain-specific antibody (Dako, Denmark; diluted 1:1,000) was added and kept at 20°C for 2 hours, and developed. Optical densities (OD) were calculated by subtracting the OD from uncoated wells from the OD from coated wells. Serum was considered positive for anti-ganglioside antibodies when the OD was 0.1 or higher at a dilution of 1:1,000. The assays were done by one of the authors (M.K.), who was unaware of the clinical information. High purity of the antigens was demonstrated with thin-layer chromatography overlay and was reported elsewhere (Yuki *et al.*, 1996).

**Infection serology**

Detection of serum antibodies against *C. jejuni*, cytomegalovirus (CMV), Epstein-Barr virus (EBV) and *Mycoplasma pneumoniae* and description of criteria for positivity were described before (Jacobs *et al.*, 1998).

**Statistical methods**

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

## RESULTS

Results from anti-ganglioside antibody assays and the correlation with antecedent infections and several clinical features are summarized in Table 1. Antibodies against GalNAc-GD1a (anti-GalNAc-GD1a) were found in 19 (14%) of 132 GBS patients. Ten (53%) of these 19 patients had IgM antibodies, 12 (63%) had IgG antibodies and three (16%) had both isotypes. The anti-GalNAc-GD1a titers ranged from 1,000 up to 4,000 for IgM antibodies and from 1,000 up to 128,000 for IgG antibodies. There was no difference in treatment

**Table 1. Clinical characteristics of 132 patients with Guillain-Barré syndrome in relation to anti-GalNAc-GD1a antibodies.**

| | Anti-GalNAc-GD1a positive | Anti-GalNAc-GD1a negative | p-value |
|---|---|---|---|
| n | 19 (14%) | 113 (86%) | |
| *C. jejuni* | 15 (79%) | 30 (27%) | <0.001 |
| Cytomegalovirus | 2 (11%) | 17 (15%) | n.s.[d] |
| Epstein-Barr virus | 1 (5%) | 13 (12%) | n.s. |
| M. pneumoniae | 0 (0%) | 7 (6%) | n.s. |
| sex (male/female) | 13 vs 6 | 57 vs 56 | n.s. |
| age[a] | 54 (5-81) | 47 (13-77) | n.s. |
| diarrhoea | 9 (47%) | 14 (12%) | <0.001 |
| respiratory tract infection | 6 (32%) | 40 (35%) | n.s. |
| sensory deficit at entry | 6 (32%) | 70 (64%) | 0.013 |
| cranial nerve involvement | 7 (37%) | 76 (67%) | 0.011 |
| paresthesias | 7 (37%) | 97 (86%) | <0.001 |
| pure motor variant | 11 (58%) | 12 (11%) | <0.001 |
| MRC[b] sum score[c] at entry[a] | 30 (6-50) | 40 (5-56) | 0.04 |
| days to lowest MRC sum score[a] | 5 (2-18) | 9 (3-21) | 0.002 |
| lowest MRC sum score[a] | 17 (0-50) | 34 (0-55) | 0.003 |
| predominantly distal weakness | 14 (74%) | 32 (29%) | <0.001 |
| time to independent locomotion[a] | 153 (6-181) | 55 (8-181) | 0.02[e] |

a. Median (2.5-97.5) percentile
b. MRC, Medical Research Council
c. MRC sum score ranges from 60 (normal) to 0 (tetraplegic)
d. n.s. = not significant
e. Log-rank test

(PE vs. IVIg) between anti-GalNAc-GD1a positive and negative patients. Serum from eight patients demonstrated both anti-GalNAc-GD1a and anti-GM1 reactivity. Eleven patients had only anti-GalNAc antibodies and 13 patients had only anti-GM1 (Table 2). There were more males in the group with anti-GalNAc-GD1a antibodies but this was not statistically significant (Tables 1 and 2).

Patients with anti-ganglioside antibodies more often had serological evidence of antecedent infection with *C. jejuni* than patients without these antibodies. Fifteen of 19 (79%) GalNAc-GD1a positive patients (Table 1, $p<0.001$) had serological evidence for an infection with *C. jejuni*. These correlations could be found for both IgM and IgG antibodies (data not shown).

There were no correlations with the other antecedent infections but a few patients had serological evidence for other infections than *C. jejuni*. One CMV-infected patient showed IgM reactivity to GalNAc-GD1a but not to GM1. In an earlier study, serum from this patient was also shown to react with GM2 (Jacobs *et al.*, 1997d). Another patient with only anti-GalNAc-GD1a antibodies had a high titer of IgG antibodies against *C. jejuni* and IgM antibodies against CMV and EBV.

The presence of antibodies against GalNAc-GD1a was correlated with several clinical features. These manifestations were predominantly related to the IgG isotype. Patients with

**Table 2. Clinical characteristics of patients with Guillain-Barré syndrome and antibodies to GalNAc-GD1a and/or GM1.**

| | Anti-GalNAc-GD1a-positive Anti-GM1-negative | Anti-GalNAc-GD1a-negative Anti-GM1-positive | Anti-GalNAc-GD1a-positive Anti-GM1-positive |
|---|---|---|---|
| n | 11 | 13 | 8 |
| *C.jejuni* | 9 (81%) | 9 (70%) | 6 (75%) |
| sex (male/female) | 8 vs 3 | 9 vs 4 | 5 vs 3 |
| age[a] | 54 (5-81) | 47 (9-77) | 53 (39-67) |
| diarrhoea | 5 (45%) | 4 (31%) | 4 (50%) |
| respiratory tract infection | 4 (37%) | 3 (23%) | 2 (25%) |
| sensory deficit at entry | 5 (45%) | 8 (62%) | 1 (13%) |
| cranial nerve involvement | 6 (55%) | 7 (54%) | 1 (13%) |
| paresthesias | 8 (62%) | 8 (61%) | 2 (25%) |
| pure motor variant | 5 (45%) | 4 (30%) | 6 (75%) |
| MRC[b] sum score[c] at entry[a] | 35 (8-50) | 32 (0-45) | 29 (6-44) |
| days to lowest MRC sum score[a] | 6 (3-15) | 6 (3-15) | 5 (2-18) |
| lowest MRC sum score[a] | 18 (0-50) | 20 (0-43) | 13 (0-36) |
| predominantly distal weakness | 6 (54%) | 6 (46%) | 8 (100%) |

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

anti-GalNAc-GD1a antibodies more frequently had a history of diarrhoea ($p<0.001$, Table 1). This association was present for both IgM and IgG antibodies (data not shown). GBS patients with anti-GalNAc-GD1a antibodies had a rapidly progressive, more severe and predominantly distal weakness (Table 1). Furthermore, anti-GalNAc-GD1a antibodies were associated with less involvement of cranial nerves, less sensory disturbances at admission and less paresthesias (Table 1).

The presence of anti-ganglioside antibodies was associated with slower recovery. Patients with anti-GalNAc-GD1a antibodies had a significantly longer median time to recov-

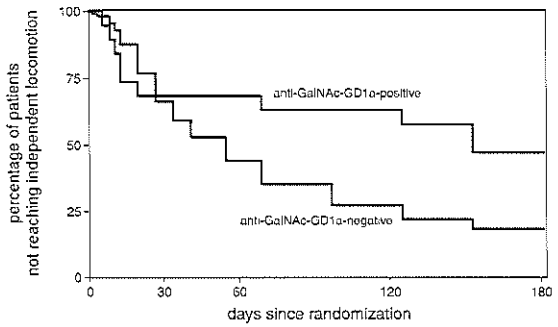


**Figure 2. Kaplan-Meier curve indicating the proportion of patients who were not able to walk independently for 10 m.** Follow-up was 181 days. Patients were grouped according to the presence of antibodies against GalNAc-GD1a (logrank test: p=0.02).

er compared to patients without these antibodies (Figure 2, p=0.02). After 181 days of follow up, 42% of patients with anti-GalNAc-GD1a antibodies were not able to walk independently for 10 meters, compared to 19% in the group without antibodies to GalNAc-GD1a (p=0.03, data not shown).

Analysis of electrophysiological features revealed no statistically significant differences in EMG patterns between patients with and without antibodies against GalNAc-GD1a (data not shown). We divided the patients with anti-ganglioside reactivity into three different groups, based on the fine specificity of reactivity to GalNAc-GD1a, GM1 or both gangliosides (Table 2). We compared the clinical features of the three groups. The three groups closely resembled each other with respect to age, sex and antecedent infection with *C. jejuni* (Table 2). There were however some differences. The eight patients with antibody reactivity against both GalNAc-GD1a and GM1 form a clinically more homogeneous group. All eight patients in this group had predominantly distal weakness and 7 (87%) had no cranial nerve involvement (Table 2). Motor predominance was present in 6 (75%) patients. In the groups with only anti-GalNAc-GD1a or anti-GM1 reactivity these percentages were lower, but the differences did not reach statistical significance. Disease severity, as indicated by the lowest MRC sum score, did not differ between the groups.

## DISCUSSION

This analysis of the presence of antibodies against the minor ganglioside GalNAc-GD1a and clinical features of 132 patients with GBS supports the notion that in addition to antibodies against major ganglioside species such as GM1, antibodies against minor gangliosides are important to distinguish clinical subgroups. The clinical features of the group of patients with antibodies against GalNAc-GD1a strongly resembled that of patients with anti-GM1 antibodies. Although in a quarter of the patients with anti-ganglioside antibodies reactivity against both GalNAc-GD1a and GM1 could be detected, the correlations with clinical features in the group with only anti-GalNAc-GD1a antibodies remained consistent. In addition, by determining anti-GM1 reactivity, and subgrouping patients on differential reactivity to GalNAc-GD1a and/or GM1 more homogeneous groups could be delineated.

The anti-ganglioside reactivity of these serum samples to several gangliosides has already been described (Jacobs *et al.*, 1998). A cut-off titer of 1,000 for the Japanese anti-ganglioside assay resulted in a very high degree of correlation between the Dutch and Japanese assay.

In GBS patients, the presence of anti-GalNAc-GD1a antibodies was significantly associated with antecedent *C. jejuni* infections. This finding supports earlier studies (Kusunoki *et al.*, 1994; Yuki *et al.*, 1996) in which was reported that all patients with anti-GalNAc-GD1a antibodies had suffered from gastro-intestinal infection. However, in our patients, although there was a strong association with both diarrhoea and antecedent *C. jejuni* infection, a considerable proportion of patients had a negative serology for *C. jejuni* and

did not report symptoms of gastrointestinal infection. One patient with IgM anti-GalNAc-GD1a antibodies had serological evidence for a CMV infection, another patient with IgM anti-GalNAc-GD1a antibodies had antibodies against CMV and EBV. Neither of these two patients did report gastrointestinal symptoms prior to the onset of GBS. These findings suggest that anti-GalNAc-GD1a antibodies can not only be induced by ganglioside-like structures on *C. jejuni* but also by viruses. However, in our study we found no virus-infected patients with anti-GalNAc-GD1a antibodies of the IgG isotype.

In eight of 19 patients with anti-GalNAc-GD1a antibodies, antibodies against GM1 could be detected as well. This can be explained by the presence of two or more populations of antibodies, one against GalNAc-GD1a and one against GM1. These two populations presumably are induced by different ganglioside-like structures on LPS molecules from one *C. jejuni* strain (Yuki *et al.*, 1995a). We had no *C. jejuni* isolates from these patients so we could not investigate whether LPS molecules from *C. jejuni* strains, isolated from patients with both anti-GalNAC-GD1a antibodies and anti-GM1 antibodies, possess different ganglioside-like structures. Alternatively, these patients have one population of antibodies recognizing the GalNAc(β1-4)Gal(α2-3)NeuAc trisaccharide that is shared by GalNAc-GD1a and GM1 (Figure 1). Patients with antibodies reacting with GM1 but not with GalNAc-GD1a may recognize the Gal(β1-3)GalNAc moiety, present on GM1 but not on GalNAc-GD1a. Further affinity studies of anti-ganglioside antibodies or absorption studies with GalNAc-GD1a and GM1 could be helpful in addressing these issues.

The clinical features of patients with only anti-GalNAc-GD1a antibodies, only anti-GM1 antibodies or antibodies against both gangliosides showed considerable overlap. However, the group of patients with antibodies against both gangliosides is more homogeneous with respect to distal distribution of weakness and less involvement of sensory and cranial nerves. This indicates that GBS patients with GM1 antibodies form a heterogeneous group that can be subdivided on the basis of reactivity with GalNAc-GD1a.

In Japanese patients, anti-GalNAc-GD1a antibodies were associated with axonal damage. In the current group of Dutch patients this correlation was absent, which is in accordance with data from Dutch patients with anti-GM1 antibodies (Jacobs *et al.*, 1997c).

In the patients with anti-GalNAc-GD1a antibodies that were described previously

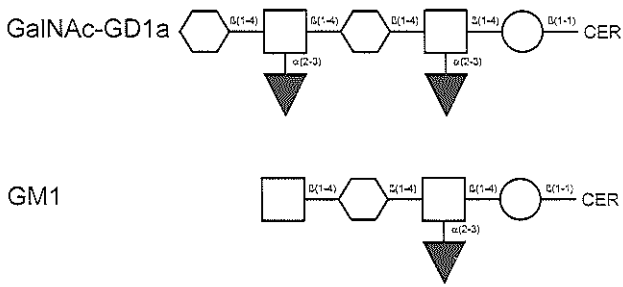


**Figure 1. Structure of gangliosides.** □ galactose, ⬡ N-acetyl galactosamine, ○ glucose ▼ N-acetyl neuraminic acid (sialic acid), CER, ceramide.

(Yuki *et al.*, 1996). IgG antibodies were of the IgG1 and IgG3 subclass. IgG1 and IgG3 were found to be more effective than the other IgG subclasses in complement activation, attachment to cell membranes through Fc-receptors and mediation of antibody-dependent cytotoxicity (Papadea and Check, 1989). In our study, there also was a strong association between clinical features and IgG anti-ganglioside antibodies. Although we did not determine the subclass distribution of anti-GalNAc-GD1a antibodies, we already reported that IgG anti-ganglioside antibodies in a subgroup of these patients belong to the IgG1 and IgG3 subclass (Jacobs *et al.*, 1997a).

At present, it is unknown whether anti-GalNAc-GD1a antibodies have an effect on neuromuscular transmission. Serum containing antibodies to GQ1b has been shown to interfere with neuromuscular transmission in the mouse phrenic nerve/diaphragm preparation (Plomp *et al.*, 1999). The pathogenicity of anti-GM1 antibodies has been demonstrated in various models but the mechanism of action is unknown (Santoro *et al.*, 1992; Takigawa *et al.*, 1995). Based on the large overlap of reactivity to GM1, anti-GalNAc-GD1a antibodies might have an effect similar to that of anti-GM1 antibodies. However, due to the fine-specificity of the anti-ganglioside antibodies, their site of action and thereby clinical manifestations may slightly differ.

This is the first report on the relation between serum antibody reactivity against the minor ganglioside GalNAc-GD1a and clinical features in a large group of patients with GBS. We found that 14% of GBS patients had serum antibodies against GalNAc-GD1a and that in the majority of patients, this reactivity to GalNAc-GD1a was independent of reactivity to GM1. Furthermore, we demonstrated that the presence of these antibodies was related to antecedent *C. jejuni* infection as well as to a rapidly progressive, more severe and predominantly distal weakness and less sensory loss, paresthesias and cranial nerve involvement. On the basis of reactivity against both GalNAc-GD1a and GM1 we could delineate an group of patients that was more homogeneous with respect to the clinical parameters mentioned above. As a large proportion of these patients did not have antibodies against GM1, additional testing for reactivity against GalNAc-GD1a can enlarge the group of GBS patients who are prone to develop a predominantly motor form of GBS with a poor recovery.

# Chapter 3

# CLINICAL FEATURES AND RESPONSE TO TREATMENT IN GUILLAIN-BARRÉ SYNDROME ASSOCIATED WITH ANTIBODIES TO GM1B GANGLIOSIDE

**N Yuki[1], CW Ang[2,3], M Koga[1], BC Jacobs[2,3], PA van Doorn[2], K Hirata[1], FGA van der Meché[2]**

Department of Neurology[1], Dokkyo University School of Medicine, Tochigi, Japan
Departments of Neurology[2] and Immunology[3], Erasmus University Rotterdam, The Netherlands

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## ABSTRACT

GM1b is a minor ganglioside in human peripheral nerves. Serum anti-GM1b antibodies frequently are present in patients with Guillain-Barré syndrome (GBS). In this collaborative study, we investigated the antecedent infections, clinical features, and response to treatment of GBS patients with anti-GM1b antibodies. Of 132 GBS patients who participated in the Dutch GBS trial that compared the effect of intravenous immunoglobulins and plasma exchange, 25 (19%) patients had anti-GM1b antibodies. IgM antibodies were present in 14, IgG antibodies in 15, and both isotypes in 4 patients. The 25 patients with anti-GM1b antibodies had a clinical pattern distinct from that of the other 107 GBS patients. They more often had an episode of gastro-intestinal illness ($p<0.001$) and frequently showed serological evidence of recent infection by *Campylobacter jejuni* ($p<0.001$). The anti-GM1b-positive subgroup was marked by more rapidly progressive ($p=0.004$), more severe ($p<0.001$), and predominantly distal weakness ($p=0.001$). Cranial nerve involvement ($p=0.03$) and sensory deficits ($p=0.003$) were less common in the patients with anti-GM1b antibodies. The presence of anti-GM1b antibodies was associated with slower recovery ($p=0.02$). The clinical manifestations predominantly were associated with anti-GM1b antibodies of the IgG isotype. Fourteen (56%) of the 25 patients with anti-GM1b antibodies also had anti-GM1 antibodies. The group of patients with both antibodies was clinically more homogenous and had a more rapidly progressive, pure motor neuropathy. The subgroup of anti-GM1b-positive GBS patients responded well to treatment with immunoglobulins but not to plasmapheresis. The distinctive clinical features of the patients with anti-GM1b antibodies show that acute motor neuropathy represents a specific subgroup within GBS and that recognizing these patients may have consequences as to the choice of therapy.


## INTRODUCTION

Gangliosides, a family of sialic acid-containing glycosphigolipids, are highly concentrated in nervous tissues. The ganglioside composition of the peripheral nervous system differs from that of the central nervous system both quantitatively and qualitatively (reviewed in Ogawa-Goto and Abe, 1998). Sialosyl paragloboside, GM1, GM3, GM2, and sialosyl lactosaminyl paragloboside are the major monosialoganglioides in human peripheral nerve, in which GM1b (Figure 1) has not been identified chemically. Kusunoki et al. reported that thin-layer chromatogram-immunostaining of a monosialoganglioside fraction with anti-GM1b-positive sera produced a reactive band with the mobility of the standard GM1b, indicative that GM1b is present in human peripheral nerve in relatively low concentration (Kusunoki *et al.*, 1996a). This minor ganglioside is recognized specifically by sera from patients with Guillain-Barré syndrome (GBS; Kusunoki *et al.*, 1996a; Yuki *et al.*, 1997a). Whether a relation exists between antecedent events, clinical manifestations, and the presence of anti-GM1b antibod-

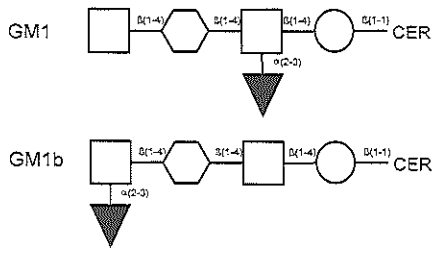


**Figure 1. Structures of minor monosialoganglioside GM1b and major monosialoganglioside GM1.**
□ Galactose, ⬡ *N*-acetylgalactosamine, ○ glucose ▼ *N*-acetylneuraminic acid; Cer, ceramide.

ies, however, has yet to be clarified.

There is a correlation between particular clinical features in GBS and several species of anti-ganglioside antibodies. Anti-GQ1b IgG antibodies, which cross-react with GT1a, are associated with acute paresis of the extraocular muscles in patients with Miller Fisher syndrome, GBS, Bickerstaff's brainstem encephalitis, and acute ophthalmoparesis without ataxia (Chiba *et al.*, 1993; Yuki, 1996). Anti-GT1a IgG antibodies, which do not cross-react with GQ1b, are present in some patients with the pharyngeal-cervical-brachial variant of GBS (Koga *et al.*, 1998a; Mizoguchi *et al.*, 1994). The presence of anti-GM1 antibodies has been reported, to be associated with a very severe, pure motor variant of GBS, and a worse recovery (Hadden *et al.*, 1998; Rees *et al.*, 1995a; Visser *et al.*, 1995), but other studies have not found these associations (Enders *et al.*, 1993; Vriesendorp *et al.*, 1993). In this collaborative study, we determined the presence of anti-GM1b antibodies in patients who participated in the Dutch GBS trial that compared the effect of intravenous immunoglobulins (IVIg) and plasma exchange (PE; Van der Meché and Schmitz, 1992). We analyzed whether the presence of the autoantibodies is related to distinct clinical manifestations, as well as the response to each form of treatment. In addition, we determined the reactivity of serum samples with the major monosialoganglioside GM1 in order to investigate the relationship between clinical manifestations and anti-ganglioside reactivity.

## PATIENTS AND METHODS

### Patients

Pre-treatment serum samples were obtained from 132 of 147 GBS patients who participated in the Dutch GBS trial (Van der Meché and Schmitz, 1992). Fifteen patients were excluded because no suitable serum was available. They did not differ from the other patients with regard to clinical manifestations and the course of the disease. All the patients fulfilled the criteria for GBS (Asbury *et al.*, 1978), were unable to walk 10 meters independently, and were admitted to the Dutch GBS trial within 2 weeks of the onset of muscle weakness. The functional and Medical Research Council (MRC) sum scores (Kleyweg *et al.*, 1991), ranging

from 60 (normal) to 0 (tetraplegic), were determined at entry to the study and 16 times during the 6-month follow-up period. The rapidity of progression was indicated by the number of days after the onset of weakness to the moment of maximal weakness. Maximum severity of weakness was indicated by the lowest MRC sum score.

**Electrodiagnostic examination**

The patients were divided into 5 categories based on EMG findings, as described elsewhere (Jacobs *et al.*, 1997c): (i) distal demyelination, (ii) randomly distributed demyelination, (iii) both demyelination and axonal degeneration, (iv) distal axonal degeneration, and (v) proximal axonal degeneration. Residual patients were subgrouped as unclassifiable. Thirty-seven patients were excluded from the analysis because their EMG data were incomplete. These patients did not differ from the others with respect to clinical manifestation and the course of the disease.

**Anti-ganglioside serology**

GM1b and GM1 were purified from bovine brain, and the high purity of each ganglioside was confirmed by thin-layer chromatography, as reported elsewhere (Hirabayashi *et al.*, 1990). Five picomoles of each monosialoganglioside was placed in separate wells of microtiter plates (Yuki *et al.*, 1997a). Serum samples diluted serially starting at 1:500 were added to each well, and the whole incubated at 4°C overnight. Peroxidase-conjugated anti-human μ-chain or γ-chain-specific antibody (Dako, Denmark; diluted 1:1,000) was added, after which the plates were kept at 20°C for 2 h then developed. Serum was considered positive for anti-ganglioside antibodies when the absorbance value was 0.1 or higher at the dilution of 1:1,000. The anti-ganglioside reactivities of these serum samples to several other gangliosides has been reported by Jacobs et al. (Jacobs *et al.*, 1998). The assays were done by one of the authors (M.K.), who was unaware of the clinical information. A cut-off titer of 1,000 for the Japanese anti-ganglioside assay gave a very high degree of correlation between the Dutch and Japanese assays.

**Infection serology**

In a previous study, we reported that antecedent infections by *C. jejuni*, cytomegalovirus (CMV), Epstein-Barr virus (EBV), or *Mycoplasma pneumoniae* was more frequent in GBS patients than in patients with other neurological diseases (Jacobs *et al.*, 1998). Detection of serum antibodies against *C. jejuni* and CMV as well as the criteria for positivity have been reported elsewhere (Jacobs *et al.*, 1996b; Visser *et al.*, 1996). Assays to determine if there had been recent infection by EBV or *M. pneumoniae* used routine techniques (Jacobs *et al.*, 1998). The presence of IgM antibodies against EBV or *M. pneumoniae* was considered to be evidence of recent infection.

**Statistical analysis**

Differences in proportions were tested by the $chi^2$ test without continuity correction or

by Fisher's exact test. Differences in medians were tested by the Wilcoxon-Mann-Whitney-U test. The time needed for patients to require independent locomotion was analyzed by the Kaplan-Meier method and the log-rank test. A p-value of less than 0.05 was considered to be significant.

## RESULTS

### Antecedent infection

High anti-GM1b antibody titers, 1,000 or more, were found in 25 (19%) of the 132 GBS patients. Fifteen (60%) of these 25 patients had IgG anti-GM1b antibodies, 14 (56%) had IgM antibodies, and 4 (21%) had both isotypes. GBS patients with anti-GM1b antibodies more frequently had had preceding diarrhea and showed serological evidence of recent *C. jejuni* infection as compared to the patients without these antibodies ($p<0.001$) (Table 1). In contrast, positive anti-GM1b serology was not correlated with CMV, EBV, or *M. pneumoniae* infection.

### Clinical features

Anti-GM1b-positive patients more frequently had distal-dominant weakness than did the anti-GM1b-negative patients ($p=0.001$) (Table 1). Cranial nerve involvement ($p=0.03$), paresthesias ($p<0.001$), and sensory deficits at entry ($p=0.003$) were less common in the patients with anti-GM1b antibodies. At entry, absence of cranial nerve involvement and sensory deficit was associated with the presence of IgG anti-GM1b (respective p values, 0.002 and 0.01), but not with IgM anti-GM1b antibodies.

The GBS patients with anti-GM1b antibodies had a significantly more rapid onset, the nadir being reached earlier than in the other 107 patients ($p=0.004$). Limb weakness was more severe both at entry and at nadir in the anti-GM1b-positive patients than in the patients without these antibodies (respective p values, 0.02 and <0.001). The severity of weakness at entry and the rapidity of progression were associated with the presence of IgG anti-GM1b (respective p values, 0.007 and <0.001) but not with that of IgM anti-GM1b antibodies. The patients with anti-GM1b antibodies had a significantly longer time to recover compared to the patients without these antibodies ($p=0.02$, Figure 2).

Analysis of electrophysiological features showed no statistically significant differences in the EMG patterns of the patients with or without anti-GM1b antibodies (Table 2). None of the 10 patients with IgG anti-GM1b antibodies, however, had distal demyelination, whereas 17 (22%) of those without these antibodies did. In contrast, there was more proximal axonal degeneration in 2 (20%) of the IgG anti-GM1b-positive patients and in 5 (6%) of the IgG anti-GM1b-negative patients.

We divided the patients into 4 groups based on antibody reactivity to GM1b alone, GM1 alone, or both monosialogangliosides (Table 3) and compared the clinical features of the groups. The groups with anti-ganglioside antibodies closely resembled each other, espe-

**Table 1. Anti-GM1b antibodies and clinical features.**

| | IgG and/or IgM anti-GM1b | | | IgG anti-GM1b | | | IgM anti-GM1b | | |
|---|---|---|---|---|---|---|---|---|---|
| | positive | negative | *p* value | positive | negative | *p* value | positive | negative | *p* value |
| number | 25 | 107 | | 15 | 117 | | 14 | 118 | |
| antecedent infectious agent | | | | | | | | | |
| *C. jejuni* | 19 (76%) | 26 (24%) | <0.001 | 12 (80%) | 33 (28%) | <0.001 | 11 (79%) | 34 (29%) | <0.001 |
| cytomegalovirus | 2 (8%) | 17 (16%) | n.s. | 0 | 19 (16%) | n.s. | 2 (14%) | 17 (14%) | n.s. |
| Epstein-Barr virus | 2 (8%) | 12 (11%) | n.s. | 0 | 14 (12%) | n.s. | 2 (14%) | 12 (10%) | n.s. |
| *M. pneumoniae* | 1 (4%) | 6 (6%) | n.s. | 0 | 7 (6%) | n.s. | 1 (7%) | 6 (5%) | n.s. |
| sex (male/female) | 17 vs 8 | 53 vs 54 | n.s. | 9 vs 6 | 61 vs 56 | n.s. | 10 vs 4 | 60 vs 58 | n.s. |
| age[a] | 47 (5-81) | 50 (13-77) | n.s. | 53 (12-81) | 47 (10-77) | n.s. | 38 (5-72) | 51 (14-78) | n.s. |
| diarrhea | 11 (44%) | 12 (11%) | <0.001 | 7 (47%) | 16 (14%) | 0.002 | 6 (43%) | 17 (14%) | 0.008 |
| respiratory tract infection | 8 (32%) | 38 (36%) | n.s. | 5 (33%) | 41 (35%) | n.s. | 5 (35%) | 41 (34%) | n.s. |
| cranial nerve involvement | 11 (44%) | 72 (67%) | 0.03 | 4 (27%) | 79 (68%) | 0.002 | 8 (57%) | 75 (64%) | n.s. |
| sensory deficit at entry | 8 (33%) | 68 (66%) | 0.003 | 4 (29%) | 72 (64%) | 0.01 | 5 (36%) | 71 (63%) | n.s. |
| paresthesias | 11 (44%) | 93 (87%) | <0.001 | 7 (47%) | 97 (83%) | 0.001 | 5 (36%) | 99 (84%) | <0.001 |
| pure motor variant | 13 (52%) | 10 (10%) | <0.001 | 10 (67%) | 13 (11%) | <0.001 | 6 (43%) | 17 (14%) | 0.004 |
| MRC[b] sum score[c] at entry[a] | 32 (0-51) | 40 (7-56) | 0.02 | 28 (0-50) | 40 (7-56) | 0.007 | 41 (8-51) | 40 (6-56) | n.s. |
| days to lowest MRC sum score[a] | 6 (2-18) | 9 (3-21) | 0.004 | 5 (2-7) | 9 (3-21) | <0.001 | 7 (2-18) | 8 (3-21) | n.s. |
| lowest MRC sum score[a] | 14 (0-50) | 35 (0-55) | <0.001 | 17 (0-50) | 34 (0-55) | 0.03 | 18 (0-50) | 32 (0-55) | 0.03 |
| predominantly distal weakness | 16 (64%) | 30 (29%) | 0.001 | 11 (73%) | 35 (31%) | 0.001 | 9 (64%) | 37 (32%) | 0.02 |
| time to independent locomotion | 153 (6-181) | 55 (8-181) | 0.02 (logrank) | 69 (6-181) | 55 (9-181) | n.s. | 139 (6-181) | 55 (9-181) | n.s. |

a. Median (2.5-97.5) percentile

b. MRC, Medical Research Council

c. MRC sum score ranges from 60 (normal) to 0 (tetraplegic)

n.s., not significant

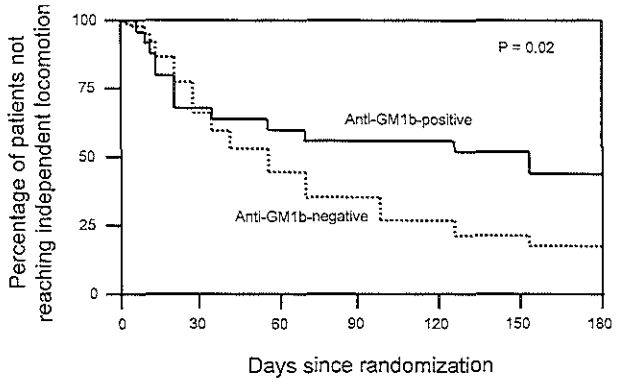


**Figure 2.** Kaplan-Meier curves showing the percentage of patients who did not regain independent locomotion by the end of the 181-day follow up. Continuous line = anti-GM1b-positive Guillain-Barré syndrome; broken line = anti-GM1b-negative Guillain-Barré syndrome (P(logrank)=0.02).

cially with respect to a high frequency of serologic evidence of *C. jejuni* infection, but there were slight differences. Fourteen patients had both anti-GM1b and anti-GM1 antibodies, and those who belonged to this group more often had rapidly progressive, predominantly motor form of GBS.

**Table 2. EMG-categories in GBS patients in relation to anti-GM1b antibodies.**

| EMG categories | Anti-GM1b IgM-positive | Anti-GM1b IgG-positive | Anti-GM1b-positive IgM and/or IgG | Anti-GM1b negative |
|---|---|---|---|---|
| Distal demyelination | 1(11%) | 0 | 16(6%) | 17(22%) |
| More random demyelination | 3(33%) | 4(40%) | 6(38%) | 27(34%) |
| Demyelination and axonal degeneration | 1(11%) | 1(10%) | 2(13%) | 13(16%) |
| Distal axonal degeneration | 0 | 0 | 0 | 1(1%) |
| More proximal axonal degeneration | 1(11%) | 2(20%) | 2(13%) | 5(6%) |
| Unclassifiable | 3(33%) | 3(30%) | 5(31%) | 16(20%) |
| Total 95 patients | 9 | 10 | 16 | 79 |

Values are numbers of patients; percentages are in parentheses.

## Effect of treatment

Of the patients with anti-GM1b antibodies, 15 were treated with PE and 10 with IVIg. Of those treated with PE, the subgroup with anti-GM1b antibodies showed slower recovery compared to the subgroup without these antibodies ($p<0.001$, Figure 3A). In the group treated with IVIg, there was no difference between the patients with and without anti-GM1b (Figure 3B).

GBS patients with anti-GM1b antibodies responded differently with respect to the therapy used. The subgroup with anti-GM1b antibodies treated with IVIg had significantly faster recoveries than the patients treated with PE ($p<0.001$, Figure 4). There was no differ-

**Table 3.Clinical characteristics of patients with Guillain-Barre syndrome and autoantibodies to GM1b and/or to GM1.**

| | anti-GM1b-positive anti-GM1-positive | anti-GM1b-positive anti-GM1-negative | anti-GM1b-negative anti-GM1-positive | anti-GM1b-negative anti-GM1-negative |
|---|---|---|---|---|
| number | 14 | 11 | 7 | 100 |
| positive *C. jejuni* serology | 10 (71%) | 9 (82%) | 5 (71%) | 21 (21%) |
| sex (male/female) | 10 vs 4 | 7 vs 4 | 4 vs 3 | 49 vs 51 |
| age[a] | 47 (15-74) | 39 (5-81) | 52 (9-77) | 43 (10-73) |
| diarrhea | 7 (50%) | 4 (36%) | 1 (14%) | 11 (11%) |
| respiratory tract infection | 3 (21%) | 5 (45%) | 2 (29%) | 36 (36%) |
| sensory deficit at entry | 3 (21%) | 5 (45%) | 6 (86%) | 62 (62%) |
| cranial nerve involvement | 4 (29%) | 7 (64%) | 4 (57%) | 68 (68%) |
| paresthesias | 5 (36%) | 6 (55%) | 5 (71%) | 88 (88%) |
| pure motor variant | 9 (64%) | 4 (36%) | 1 (14%) | 9 (9%) |
| MRC[b] sum score[c] at entry[a] | 29 (0-44) | 36 (8-51) | 35 (1-45) | 38 (6-52) |
| days to lowest MRC sum score[a] | 5 (2-18) | 6 (3-15) | 6 (3-12) | 8 (3-17) |
| lowest MRC sum score[a] | 9 (0-36) | 18 (0-50) | 20 (0-43) | 28 (0-50) |
| predominantly distal weakness | 11 (79%) | 6 (55%) | 3 (43%) | 27 (27%) |

a. Median (2.5-97.5) percentile

b. MRC, Medical Research Council

c. MRC sum score ranges from 60 (normal) to 0 (tetraplegic)

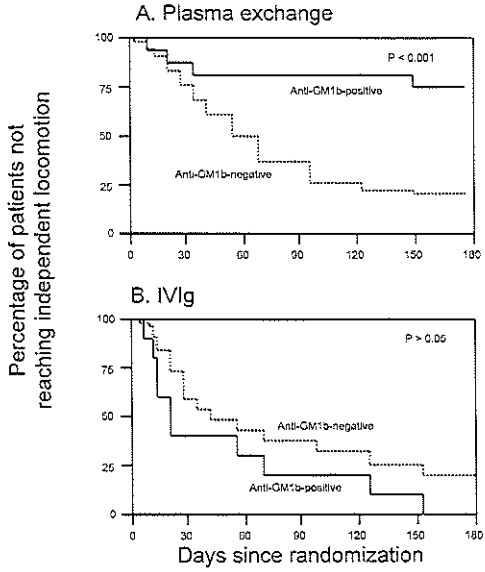


**Figure 3** (**A**) Kaplan-Meier curves showing the percentage of patients who could not walk independently by the end of the 181-day follow up period after plasma exchange treatment. (p(logrank)<0.001) (**B**) Kaplan-Meier curves showing the proportion of patients who could not walk independently by the end of the 181-day follow up period after intravenous immunoglobulin treatment. Continuous line = anti-GM1b-positive Guillain-Barré syndrome; broken line = anti-GM1b-negative Guillain-Barré syndrome (p(logrank)>0.05).

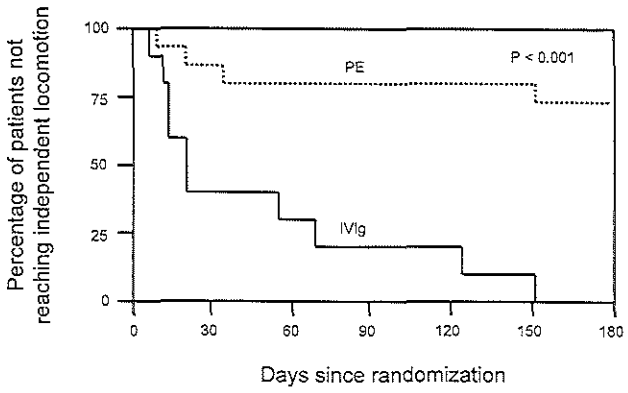


**Figure 4. Kaplan-Meier curves showing the proportion of patients with anti-GM1b antibodies by treatment group who did not regain independent locomotion by the end of the 181-day follow up.** Continuous line = intravenous immunoglobulins (IVIg); broken line = plasma exchange (PE) (p(logrank)<0.001).

ence between the treatment modalities in the patients without anti-GM1b antibodies.

## DISCUSSION

We have confirmed and extended the findings on anti-GM1b reactivity in GBS patients. Anti-GM1b antibodies were found in 25 (19%) of the 132 Dutch GBS patients, results comparable to those reported by Kusunoki et al.[2] (21% of the Japanese GBS patients) but slightly lower than those reported by Yuki et al. (Yuki *et al.*, 1999; Yuki *et al.*, 1997a). (35% of the Japanese GBS patients and 25% of the Chinese GBS patients). In the latter study, a lower cut-off value was used than this study. Kusunoki et al. reported that 5 (56%) of 9 Japanese GBS patients with anti-GM1b antibodies had positive *C. jejuni* serology (Kusunoki *et al.*, 1996a). We reported that 12 (60%) of 20 Japanese GBS patients who had high IgG anti-GM1b antibody titers had had a gastrointestinal infection and that *C. jejuni* was isolated from 3 of them (Yuki *et al.*, 1997a). Immunostaining results for monoclonal anti-GM1b antibody indicate that lipopolysaccharide from *C. jejuni* has the GM1b epitope, but no statistical association was shown between antecedent *C. jejuni* infection and the presence of anti-GM1b antibodies in these Japanese studies. Eleven (79%) of 14 Chinese patients with IgG anti-GM1b antibody had serologic evidence of recent *C. jejuni* infection, and positive *C. jejuni* serology was correlated with the presence of IgG anti-GM1b antibody. Also in the Dutch population, we found a significant association between the presence of both IgG and IgM anti-GM1b antibodies and positive *C. jejuni* serology. None of the IgG anti-GM1b-positive patients had serologic evidence of CMV, EBV, or *M. pneumoniae* infection.

No relation between clinical manifestation and the presence of anti-GM1b antibodies was identified in both Japanese studies (Kusunoki *et al.*, 1996a; Yuki *et al.*, 1997a) and

Chinese study (Yuki *et al.*, 1999). In the Dutch population, the GBS patients with anti-GM1b antibodies experienced a more rapid onset of weakness, reached the nadir earlier, more frequently had predominantly distal weakness, and less frequently had a sensory deficit or cranial nerve involvement. Involvement of the cranial nerves is reported to be related to the severity of limb weakness, but in our study the GBS patients with anti-GM1b antibodies did not have a mild form of the disease (Winer *et al.*, 1988d). The clinical manifestations associated with the presence of anti-GM1b antibodies predominantly were of the IgG class. GBS patients with anti-GM1b IgM did not differ significantly from the patients without anti-GM1b IgM with respect to severity at entry, days to peak severity, and cranial and sensory nerve impairment.

The clinical features of the group of patients with anti-GM1b antibodies strongly resembled those of patients with anti-GM1 antibodies reported earlier (Jacobs *et al.*, 1996b; Visser *et al.*, 1995). Tables 1 and 4 shows that anti-GM1b antibodies as well as anti-GM1antibodies are associated with pure motor variant of GBS. In 56% of the anti-GM1b-positive patients, reactivity against GM1 was present. Clinical features of the patients with only anti-GM1b antibodies, only anti-GM1 antibodies, or with both antibodies overlapped considerably, but the group of patients with both antibodies was more homogeneous. Patients who belonged to this group tended to have a more rapidly progressive, pure motor neuropathy. These findings indicate that GBS patients with anti-GM1 antibodies constitute a heterogeneous group that can be subdivided on the basis of the presence of anti-GM1b antibodies. Although both anti-GM1 and anti-GM1b antibodies are associated with the same clinical phenotype, we could not conclude which antibodies are more important because of the small numbers in each group (Table 3). As molecular mimicry between GM1 and *C. jejuni* LPS is implicated in the induction of anti-GM1 antibodies, anti-GM1b antibodies may also be induced by a preceding infection with *C. jejuni* (Yuki *et al.*, 1995a; Yuki *et al.*, 1997a). A single strain of *C. jejuni* isolated from a patient with GBS may display microheterogenity in the core oligosaccharide, expressing various epitopes GM1, GD1a, GD3, and GT1a (Aspinall *et al.*, 1994c; Yuki *et al.*, 1995a). Infection by a particular strain of *C. jejuni* may induce anti-GM1b antibodies in some patients, anti-GM1 antibodies in others, or both. Patients with reactivity against both GM1 and GM1b may have 2 populations of antibodies or have cross-reactive antibodies to their common structure in GM1 and GM1b.

Lack of cranial nerve involvement and distal onset of weakness suggest selective involvement of the long nerve fibers. Distal predilection may be associated with the random distribution of demyelination or be the result of a dying-back mechanism after primary or secondary axonal involvement (Van der Meché and Meulstee, 1988; Van der Meché *et al.*, 1988). Kusunoki et al. speculated that anti-GM1b antibodies are associated with primary demyelination, to which axonal damage frequently is added (Kusunoki *et al.*, 1996a). In contrast, we reported that most patients who had high IgG anti-GM1b antibody titers had primary axonal degeneration (Yuki *et al.*, 1997a). In this Dutch population, there was no evidence for distal demyelination in any of the patients with IgG anti-GM1b antibody, whereas 22% of the IgG anti-GM1b-negative patients had it. There were more patients with axonal damage in the

**Table 4. Anti-GM1 antibodies and clinical features.**

| | IgG and/or IgM anti-GM1 | | | IgG anti-GM1 | | | IgM anti-GM1 | | |
|---|---|---|---|---|---|---|---|---|---|
| | positive | negative | *p* value | positive | negative | *p* value | positive | negative | *p* value |
| number | 21 | 111 | | 17 | 115 | | 9 | 123 | |
| antecedent infectious agent | | | | | | | | | |
| *C. jejuni* | 15 (71%) | 30 (27%) | <0.001 | 13 (76%) | 32 (28%) | <0.001 | 7 (78%) | 38 (31%) | 0.004 |
| cytomegalovirus | 0 (0%) | 19 (17%) | 0.04 | 0 (0%) | 19 (17%) | n.s. | 0 90%) | 19 (17%) | n.s. |
| Epstein-Barr virus | 2 (10%) | 12 (10%) | n.s. | 0 (0%) | 14 (12%) | n.s. | 0 (0%) | 19 (15%) | n.s. |
| *M. pneumoniae* | 1 (5%) | 6 (5%) | n.s. | 0 (0%) | 7 (6%) | n.s. | 1 (11%) | 6 (5%) | n.s. |
| sex (male/female) | 14 vs 7 | 56 vs 55 | n.s. | 11 vs 6 | 59 vs 56 | n.s. | 7 vs 2 | 63 vs 60 | n.s. |
| age[a] | 47 (9-77) | 48 (10-78) | n.s. | 53 (9-77) | 47 (10-78) | n.s. | 47 (15-61) | 50 (10-78) | n.s. |
| diarrhea | 8 (38%) | 15 (14%) | 0.006 | 8 (47%) | 15 (13%) | 0.001 | 3 (33%) | 20 (16%) | n.s. |
| respiratory tract infection | 5 (23%) | 41 (37%) | n.s. | 5 (29%) | 41 (35%) | n.s. | 1 (11%) | 45 (37%) | n.s. |
| cranial nerve involvement | 9 (45%) | 67 (63%) | n.s. | 6 (38%) | 70 (63%) | n.s. | 4 (44%) | 72 (61%) | n.s. |
| sensory deficit at entry | 8 (38%) | 75 (67%) | 0.01 | 6 (35%) | 77 (67%) | n.s. | 4 (44%) | 79 (64%) | n.s. |
| paresthesias | 10 (48%) | 94 (85%) | <0.001 | 7 (41%) | 97 (84%) | <0.001 | 4 (44%) | 100 (81%) | 0.009 |
| pure motor variant | 10 (48%) | 13 (12%) | <0.001 | 10 (59%) | 13 (11%) | <0.001 | 3 (33%) | 20 (16%) | n.s. |
| MRC[b] sum score[c] at entry[a] | 32 (0-45) | 40 (8-56) | 0.002 | 30 (0-44) | 40 (7-56) | 0.003 | 36 (1-45) | 40 (6-56) | n.s. |
| days to lowest MRC sum score[a] | 6 (2-18) | 9 (3-21) | 0.003 | 5 (2-18) | 9 (3-21) | 0.002 | 6 (2-15) | 8 (3-21) | n.s. |
| lowest MRC sum score[a] | 17 (0-43) | 34 (0-55) | 0.002 | 17 (0-43) | 34 (0-55) | 0.004 | 17 (0-38) | 31 (0-55) | n.s. |
| predominantly distal weakness | 14 (67%) | 32 (30%) | 0.001 | 14 (82%) | 32 (29%) | <0.001 | 5 (56%) | 41 (34%) | n.s. |
| time to independent locomotion | 69 (4-181) | 55 (8-181) | n.s. | 125 (4-181) | 55 (9-181) | n.s. | 55 (13-181) | 55 (6-181) | n.s. |

a. Median (2.5-97.5) percentile

b. MRC, Medical Research Council

c. MRC sum score ranges from 60 (normal) to 0 (tetraplegic)

n.s., not significant

group with IgG anti-GM1b antibody, but this did not reach statistical significance.

Ho et al. showed that anti-GD1a antibody is associated with axonal but not demyelinating form of GBS (Ho *et al.*, 1999). We investigated the relationship between anti-GM1b reactivity (Japanese test) and anti-GD1a reactivity (Dutch test) and found that there were only 2 patients with reactivity against both gangliosides. Both patients had also anti-GalNAc-GD1a reactivity and serologic evidence of recent *C. jejuni* infection. The first patient had predominantly distal weakness, no cranial nerve involvement but his EMG characteristics were unclassifiable. The second patient had a different clinical pattern. This patient also had anti-GQ1b reactivity and oculomotor symptoms. His EMG showed demyelination. These suggested that anti-GD1a reactivity was not related to anti-GM1b reactivity. In addition, the relation between anti-GD1a reactivity and axonal involvement was not confirmed in the Dutch GBS patients (Ang *et al.*, unpublished observation). Hao *et al.* showed that anti-GalNAc-GD1a antibody is associated with pure motor variant of GBS, which was confirmed in the Dutch GBS patients (Ang *et al.*, 1999; Hao *et al.*, 1999). Eighteen (72%) of the 25 patients with anti-GM1b antibodies had anti-GalNAc-GD1a reactivity as reported earlier (Kusunoki *et al.*, 1996a; Yuki *et al.*, 1999). Patients with reactivity against both GM1b and GalNAc-GD1a may have cross-reactive antibodies to their common structure in GM1b and GalNAc-GD1a.

The anti-GM1b-positive patients treated with PE had a significantly slower recovery than the anti-GM1b-negative patients treated with PE, but this association was absent in anti-GM1b-positive patients treated with IVIg. Interestingly, the anti-GM1b-positive patients treated with IVIg made a significantly faster recovery than the patients treated with PE, as did the patients with anti-GM1 antibodies reported by Jacobs et al. (Jacobs *et al.*, 1996b). Ours is the first study to indicate a difference in the effects of PE and IVIg on GBS patients with anti-GM1b antibodies. Subgrouping GBS patients by their patterns of anti-ganglioside antibodies, as well as antecedent infection, may help to optimize treatment for individual patients. Because this analysis is retrospective and covers relatively small subgroups of patients, the results with respect to the prognostic relevance of anti-GM1b antibodies need to be confirmed by larger prospective studies.

# Chapter 4

## COMPARATIVE STUDY OF PRECEDING *CAMPYLOBACTER JEJUNI* INFECTION IN GUILLAIN-BARRÉ SYNDROME IN JAPAN AND THE NETHERLANDS

**M Koga[1], CW Ang[2,3], N Yuki[1], BC Jacobs[2,3], P Herbrink[4], FGA van der Meché[2], K Hirata[1], PA van Doorn[2]**

Department of Neurology[1], Dokkyo University School of Medicine, Tochigi, Japan
Departments of Neurology[2] and Immunology[3], Erasmus University Rotterdam, The Netherlands
Department of Immunology & Infectious Diseases[4], Diagnostic Centre SSDZ, Delft, The Netherlands

*Submitted*

## ABSTRACT

We made a comparative study in Japan and The Netherlands of the presence of preceding *Campylobacter jejuni* infections in Guillain-Barré syndrome (GBS). It was conducted in two laboratories using different serological criteria. The Japanese results showed no significant difference in the frequency of *C. jejuni* infection in the Japanese (17/88, 19%) and Dutch (21/132, 16%) GBS patients. The Dutch investigation showed a higher frequency in Dutch GBS (45/132; 34%) than in Japanese GBS (20/88; 23%) patients, but the difference did not reach significance. The findings of our collaborative study show that the incidence of antecedent *C. jejuni* infection in GBS in Japan is not higher than in The Netherlands and that serological assays vary considerably with the laboratory.


## INTRODUCTION

The gram-negative bacterium *Campylobacter jejuni* has emerged as the most common antecedent infectious agent in Guillain-Barré syndrome (GBS) (Jacobs *et al.*, 1998). Serological studies showed that the frequency of prior *C. jejuni* infection in GBS ranges from 17 to 66%, the frequencies in northern China (66%) and Japan (45%) being higher than those in western countries such as the United States (17%, 36%) the United Kingdom (26%) The Netherlands (32%) Germany (39%) and Australia (38%) (Enders *et al.*, 1993; Gruenewald *et al.*, 1991; Hao *et al.*, 1998; Ho *et al.*, 1995; Jacobs *et al.*, 1996b; Kaldor and Speed, 1984; Mishu *et al.*, 1993; Rees *et al.*, 1995b). The reports suggest that in Asian countries GBS is more closely associated with *C. jejuni* than it is in western countries. These studies, however, used different assay systems, making it difficult to compare the incidence of preceding *C. jejuni* infection in GBS. A cooperative project by Dokkyo University and Erasmus University was planned to compare the frequencies of *C. jejuni*-associated GBS in Japan and The Netherlands. Serological examinations of the incidence of antecedent *C. jejuni* infection in the same serum samples from Japanese and Dutch GBS patients were made in two laboratories, after which the frequency of *C. jejuni* infection and the correlation between the two different serological assays were analyzed.

## METHODS

### Patients

Pretreatment serum samples were obtained from GBS patients in Japan and The Netherlands, all of whom met the established criteria for GBS (Asbury *et al.*, 1978). The samples were stored at -80°C until used. The Japanese GBS group consisted of 88 consecutive patients who had been referred to the Neuroimmunological Laboratory at Dokkyo University

in 1998, from university hospitals and district general hospitals throughout Japan. None of them had been included in our previous study (Koga *et al.*, 1998c). The Dutch patients consisted of 132 of 147 GBS patients who had participated in the Dutch GBS trial (Van der Meché and Schmitz, 1992), 15 patients being excluded because no serum was available. These patients did not differ from the others with regard to clinical manifestations and the course of the disease. Control serum samples were available from patients with other neurological diseases (OND) and from healthy volunteers (Table 1). There were no significant differences in the ages and sex of the patients with GBS, OND, or of the healthy controls (HC) in Japan and The Netherlands.

**Table 1. Characteristics of Guillain-Barré syndrome (GBS) patients, patients with other neurological diseases (OND) and healthy controls (HC) from Japan and The Netherlands.**

| | GBS | | OND | | HC | |
|---|---|---|---|---|---|---|
| | Japan | The Netherlands | Japan | The Netherlands | Japan | The Netherlands |
| Number | 88 | 132 | 27 | 42 | 56 | 30 |
| Age (years) | | | | | | |
| median | 40 | 48 | 49 | 47 | 49 | 47 |
| range | 14-83 | 5-81 | 21-60 | 12-93 | 16-85 | 20-69 |
| Sex (male/female) | 52/36 | 70/62 | 14/13 | 24/18 | 28/28 | 20/10 |

### *C. jejuni* serology

Antecedent *C. jejuni* infection was examined serologically by enzyme-linked immunosorbent assays (ELISA) done in the two laboratories. The antigen protein for ELISA used at Dokkyo University (Japanese ELISA) was prepared from a *C. jejuni* Penner O:19 strain, which had been isolated from a Japanese GBS patient, and used 100 ng/well (Koga *et al.*, 1998c). Serum was considered positive when anti-*C. jejuni* IgG antibody titers were 2000 or more in the Japanese ELISA. The cut-off was defined based on the findings for 17 GBS patients, from whom *C. jejuni* had been isolated, and findings for 46 HC subjects. This system was proved 82% sensitive and 88% specific, and the presence of anti-*C. jejuni* IgG antibody alone provided sufficient evidence of recent *C. jejuni* infection (Koga *et al.*, 1998c). Serum samples from the Japanese subjects first were used for the Japanese ELISA then immediately frozen at -80°C and with ample dry ice air-lifted to The Netherlands for testing by The Netherlands ELISA (Jacobs *et al.*, 1996b). Serum samples from Dutch individuals were treated similarly. In the Netherlands ELISA, the antigen protein was prepared from a *C. jejuni* Lau 48 strain isolated from an enteritis patient and used 300 ng/well. Serological evidence of recent *C. jejuni* infection was defined as the presence of anti-*C. jejuni* IgM and/or IgA antibodies (Jacobs *et al.*, 1996b). The Japanese and Dutch investigators were blinded to the diagnoses for each other's patients and serological results during the testing.

### Statistical analysis

The Spearman rank correlation test was used to test the association between the

Japanese and Netherlands' ELISAs. The difference in the frequency of positive *C. jejuni* serology between these ELISAs was tested by the Mantel-Haenszel procedure. Other differences between groups were examined by the chi2 or Fisher's exact test where appropriate. Differences in medians were examined by the Mann-Whitney U test. A difference was considered significant when the p-value was less than 0.05. All statistical analyses were made with Statcel® software (OMS, Saitama, Japan).

## RESULTS

### Japanese ELISA

Serological evidence of recent *C. jejuni* infection was found in 17 (19%) of the 88 Japanese GBS patients and 21 (16%) of the 132 Dutch GBS patients (Fig 1A). There was no significant difference between the Japanese and Dutch GBS and OND patients, or HCs. In both the Japanese and Dutch populations, the frequency of positive *C. jejuni* serology was higher in the GBS than in the OND patients or in the HCs, but this difference was significant only for Japanese GBS and the HCs (p=0.0001).

### Netherlands ELISA

The incidence of positive *C. jejuni* serology in the Dutch GBS patients (45/132; 34%) was higher than in the Japanese GBS patients (20/88; 23%) (p=0.07) (Fig 1B). No significant differences were found between the Japanese (15%) and Dutch (5%) OND or between the Japanese (18%) and Dutch (10%) HCs. Dutch GBS patients had positive *C. jejuni* serology significantly more often than did the HC (p=0.009) and those who had OND (p=0.0002), whereas in the Japanese population, there was only a slight, insignificant difference in *C. jeju-*

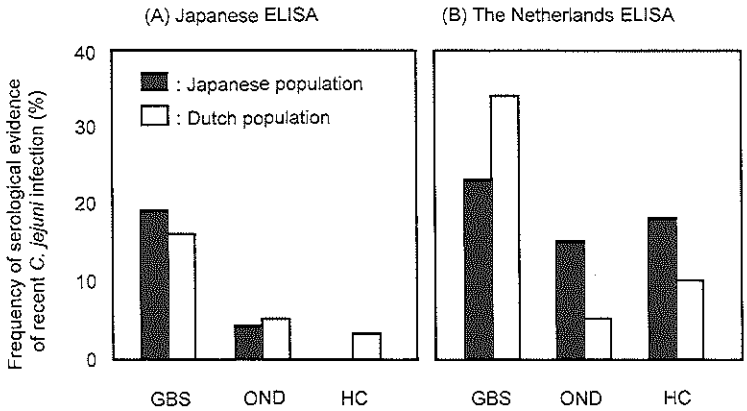


**Figure 1. Frequency of serological evidence of recent *Campylobacter jejuni* infection in two laboratories. (A)** Japan and **(B)** The Netherlands. Solid bar, Japanese population; striped bar, Dutch population; ELISA, enzyme-linked immunosorbent assay; GBS, Guillain-Barré syndrome; OND, other neurological diseases; HC, healthy controls.

*ni* serology among the GBS (23%) and OND (15%) patients, and the HCs (18%).

**Comparison of ELISA systems**

*C. jejuni* serology results showed good correlations in the Japanese ($p<0.0001$, $rs=0.77$) and Dutch ($p<0.0001$, $rs=0.76$) populations. Opposite results for both ELISAs were seen in 64 (17%) of 375 sera included in this study (Table 2). Most of these sera were positive in The Netherlands ELISA but negative in the Japanese assay. The frequency of positive results was higher in The Netherlands ELISA (22%) than in the Japanese ELISA (11%) ($p=0.00003$).

The group with positive serology in the Japanese ELISA and negative serology in The Netherlands assay consisted of 8 patients with GBS (5 Japanese and 3 Dutch), 2 with OND (1 Japanese and 1 Dutch), and 1 HC (Dutch). The group with negative serology in the Japanese ELISA and positive serology in The Netherlands assay consisted of 35 patients with GBS (8 Japanese and 27 Dutch), 5 with OND (4 Japanese and 1 Dutch), and 13 HCs (10 Japanese and 3 Dutch).

**Table 2. Test characteristics of the *Campylobacter* ELISA systems in Japan and The Netherlands.**

| | | Japanese ELISA | |
|---|---|---|---|
| | | positive | negative |
| The Netherlands ELISA | positive | 31 (8%) | 53 (14%) |
| | negative | 11 (3%) | 280 (74%) |

## DISCUSSION

Isolation of *C. jejuni* from the stools of a patient is the most specific method for determining *C. jejuni* enteritis. Because GBS develops only 1-3 weeks after diarrhea and the excretion period of the bacteria is limited, by this criterion a large proportion of *C. jejuni*-related GBS patients would be negative. The incidence of antecedent gastrointestinal symptoms does not always reflect the frequency of preceding *C. jejuni* infection in GBS because asymptomatic *C. jejuni* infections are frequent and probably differ by country (Blaser and Reller, 1981). In contrast, serodiagnostic methods are suitable for investigating preceding *C. jejuni* infection between countries.

We performed serological assays on the same serum samples in two widely separated laboratories. Both ELISA systems showed that the incidence of *C. jejuni* serology does not differ in Japanese and Dutch GBS patients, but the true incidence of preceding *C. jejuni* infection in GBS is still not clear. Based on Japanese ELISA results, we elsewhere reported a 31% frequency of antecedent *C. jejuni* infection in Japanese GBS patients (Koga *et al.*, 1998c), higher than that found in the present study. This may be due to the difference in patient populations surveyed in the previous and present studies. Because the population in the present

study consisted of consecutive patients, whereas that in the previous study was not, we think that the Japanese patients in the present study are representative for the total group of Japanese GBS patients.

Results of the assay systems routinely used in the two countries' laboratories were well correlated. The Netherlands ELISA, however, showed a higher frequency of positive results. In the Dutch patients with GBS, in particular, there was a great difference in seropositive frequency (34% in The Netherlands' ELISA, 16% in the Japanese ELISA). This indicates that the incidence of preceding *C. jejuni* infection in GBS varies markedly with the assay's sensitivity and that it is difficult to compare the incidences of *C. jejuni*-related GBS that were obtained by serological methods in previous studies. It is noteworthy that a high frequency of positive serology was found for Japanese patients with OND (15%) and the HCs (18%) by The Netherlands ELISA, indicative that specificity of an assay used to determine *C. jejuni* serology may depend not only on the antigen preparation used but on the origin of the test population. The Japanese ELISA seems more suitable than The Netherlands' ELISA for comparative examination for frequency of GBS subsequent to *C. jejuni* infection.

In conclusion, our comparative study showed that there is no significant difference in the incidence of preceding *C. jejuni* infection in GBS patients in Japan and in The Netherlands. Interlaboratory differences indicate that any worldwide comparative investigation of *C. jejuni* serology should be done in a single laboratory.

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# Chapter 3

# DIFFERENTIAL IMMUNE RESPONSE TO GANGLIOSIDES IN GUILLAIN-BARRÉ SYNDROME PATIENTS FROM JAPAN AND THE NETHERLANDS

**CW Ang[1,2], M Koga[3], BC Jacobs[1,2], N Yuki[3], K Hirata[3], FGA van der Meché[1], PA van Doorn[1]**

Departments of Neurology[1] and Immunology[2], Erasmus University Rotterdam, The Netherlands
Department of Neurology[3], Dokkyo University School of Medicine, Tochigi, Japan

*Submitted*

## ABSTRACT

Anti-ganglioside antibodies are consistently found in Guillain-Barré syndrome (GBS) patients from different geographical parts of the world. Some studies have indicated that there are differences in relative frequencies of anti-ganglioside reactivity and isotype distribution between GBS patients from Asia and from Europe. Asian patients were reported to have a higher incidence of anti-ganglioside reactivity with a predominance of the IgG isotype. Alternatively, the observed differences could be caused by differences in test systems. We investigated antibody reactivity against the gangliosides GM1, GM1b and GalNAc-GD1a in GBS patients from Japan and The Netherlands in two different laboratories. The total number of GBS patients with anti-ganglioside antibodies did not differ between the two countries. GBS patients from the Netherlands more frequently had anti-GalNAc-GD1a antibodies and an IgM anti-ganglioside response. There were slight differences in the ELISA systems but these could be explained by differences in cut-off values. Our results indicate that geographical determined factors, dependent on either the host or the inciting pathogen, determine the isotype distribution and fine-specificity of anti-ganglioside antibodies in GBS patients.


## INTRODUCTION

Patients with the Guillain-Barré syndrome (GBS) frequently have antibody reactivity against neural gangliosides such as GM1, GM2, GD1a, GalNAc-GD1a and GM1b (Figure 1) (Ang *et al.*, 1999; Hao *et al.*, 1998; Hao *et al.*, 1999; Ho *et al.*, 1999; Jacobs *et al.*, 1996b;

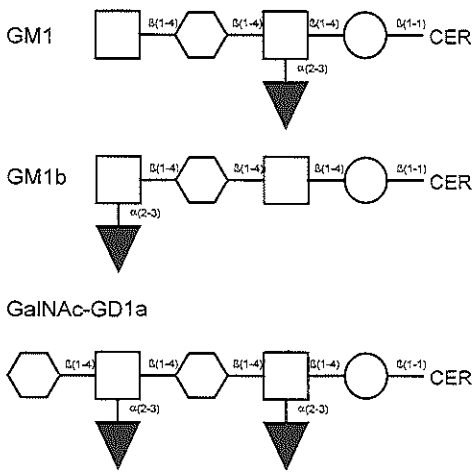


**Figure 1.** Structure of gangliosides ○ N-acetyl-galactosamine, □ galactose, ▼ sialic acid, ○ glucose, CER ceramide.

Kaida *et al.*, 2000; Yuki *et al.*, 2000). The specificity of the anti-ganglioside response in GBS patients can be related to specific antecedent infections and patterns of clinical and electrophysiological features. Antibody reactivity against GM1, GM1b and GalNAc-GD1a is associated with preceding infections with *Campylobacter jejuni* and a motor neuropathy with predominant distal weakness (Ang *et al.*, 1999; Jacobs *et al.*, 1996b; Kaida *et al.*, 2000; Yuki *et al.*, 2000). Anti-GM2 reactivity is associated with cytomegalovirus (CMV) infections and anti-GD1a antibodies occur frequently in patients with electrophysiological evidence for axonal degeneration (Ho *et al.*, 1999; Jacobs *et al.*, 1997d; Khalili-Shirazi *et al.*, 1999; Yuki *et al.*, 1993b).

The frequencies of antibody reactivity against gangliosides in patients with GBS differs between geographical locations. GBS-populations from Japan and China were reported to have anti-ganglioside reactivity in up to 51%, compared to 32% in GBS patients from Europe (Hao *et al.*, 1998; Jacobs *et al.*, 1996b; Kuwabara *et al.*, 1998a; Rees *et al.*, 1995a). In addition, most patients from Asia have a selective IgG response against gangliosides compared to the equal presence of IgM and IgG anti-ganglioside antibodies in patients from Europe (Ho *et al.*, 1995; Jacobs *et al.*, 1996b). There are several explanations for these observed differences. A higher frequency of antecedent infections, with e.g. *C. jejuni*, may lead to an increase in the frequency of antibodies that are associated with this infection. Another explanation may be a difference in host-related immunogenetic factors, leading to a preferential IgM or IgG response. Alternatively, the observed differences are caused by differences in anti-ganglioside assay systems (Willison *et al.*, 1999).

In the present study we have investigated the frequency of antibody reactivity against gangliosides GM1, GM1b, GalNAc-GD1a in serum from GBS patients, neurological control patients and healthy individuals from Japan and The Netherlands. We used both a Japanese and Dutch anti-ganglioside assay. Using both assay systems, we found that the total number of GBS patients with anti-ganglioside antibodies did not differ between Japan and the Netherlands. However, Dutch GBS patients more frequently have an IgM response and anti-GalNAc-GD1a reactivity compared to Japanese GBS patients.

## PATIENTS AND METHODS

Pretreatment serum samples were obtained from GBS patients in Japan and The Netherlands, all of whom met the established criteria for GBS (Asbury *et al.*, 1978) The samples were stored at -80°C until used. The Japanese GBS group consisted of 88 consecutive patients who had been referred to the Neuroimmunological Laboratory at Dokkyo University in 1998, from university hospitals and district general hospitals throughout Japan. None of them had been included in previous studies. The Dutch patients consisted of 132 of 147 GBS patients who had participated in the Dutch GBS trial comparing plasma exchange and intravenous immunoglobulins (IVIg). The 15 excluded patients did not differ from the others with regard to clinical manifestations and the course of the disease and were not tested because no

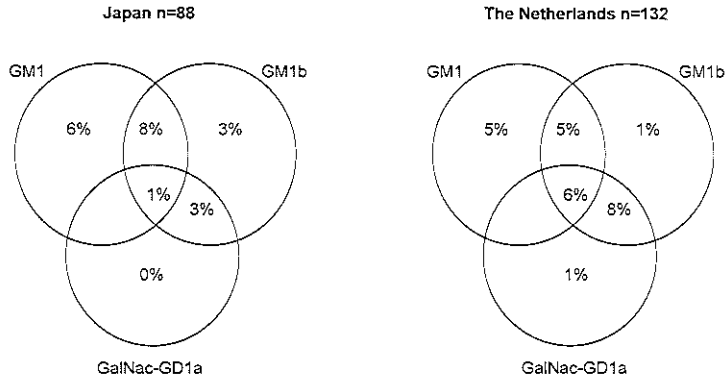


**Figure 2.** Fine specificity of antibody reactivity against GM1, GM1b and/or GalNAc-GD1a in Guillain-Barré syndrome patients from Japan (n=88) and The Netherlands (n=132).

serum was available. Control serum samples were available from patients with other neurological diseases (OND) and from healthy volunteers (Table 1). There were no significant differences in age and sex between the patients with GBS, OND, or of the healthy controls (HC) from Japan and The Netherlands.

IgA, IgG and IgM anti-ganglioside reactivity was determined as described previously (Jacobs *et al.*, 1995; Yuki *et al.*, 1997a). Assays for anti-GM1b and anti-GalNAc-GD1a reactivity were only performed in Japan. In the Japanese system, the criteria for positivity were a corrected optical density (OD)-value of >0.1 at a serum dilution of 1:1000. In the Dutch system, criteria for positivity were a corrected OD-value of 0.1 for IgA, 0.2 for IgG and 0.3 for IgM at a serum dilution of 1:200. Differences in proportions were tested with the chi-square test. A p-value <0.05 was considered to be significant.

**Table 1. Characteristics of Guillain-Barré syndrome (GBS) patients, patients with other neurological diseases (OND) and healthy controls (HC) from Japan and The Netherlands.**

| | GBS | | OND | | HC | |
|---|---|---|---|---|---|---|
| | Japan | The Netherlands | Japan | The Netherlands | Japan | The Netherlands |
| Number | 88 | 132 | 27 | 42 | 56 | 30 |
| Age (years) | | | | | | |
| median | 40 | 48 | 49 | 47 | 49 | 47 |
| range | 14-83 | 5-81 | 21-60 | 12-93 | 16-85 | 20-69 |
| Sex (male/female) | 52/36 | 70/62 | 14/13 | 24/18 | 28/28 | 20/10 |

# RESULTS

The combined anti-ganglioside reactivity against GM1, GM1b and GalNAc-GD1a did

not differ between GBS patients from Japan and the Netherlands (22 vs 25%, Table 2). However, analysis of the response to the three gangliosides seperately, resulted in a difference between GBS patients from Japan and The Netherlands. Anti-GalNAc-GD1a reactivity was present more frequently in Dutch patients. Such a difference was not observed for anti-GM1 or anti-GM1b reactivity.

Fine-specificity of the anti-ganglioside antibodies is depicted in Figure 2. Reactivity against more than 1 ganglioside occurs often in GBS patients from both countries. In Japanese GBS patients this shared reactivity is mostly between GM1 and GM1b. Patients from the Netherlands have a different pattern of shared reactivity. 14% has reactivity against both GM1b and GalNAc-GD1a and 6% has reactivity against all three gangliosides. It is important to note that reactivity against GM1b is the common denominator in most GBS patients from both Japan and The Netherlands. Thus, Japanese and Dutch GBS patients have a different fine-specificity of reactivity against GM1, GM1b and GalNAc-GD1a.

The isotype-distribution of the anti-ganglioside antibodies showed a relative lack of IgM anti-ganglioside reactivity in Japanese patients. This was observed for reactivity against all three gangliosides and was significant (Table 2, p=0.008). The difference between Japanese and Dutch patients was not due to an exclusive but to an additional IgM anti-ganglioside response of the Dutch GBS patients. 11/33 (33%) of the GBS patients from The Netherlands had both IgG and IgM anti-GM1, GM1b or GalNAc-GD1a reactivity. In contrast, only 1/20 (5%) Japanese GBS patients had both IgG and IgM anti-ganglioside antibodies (p=0.02). IgA anti-GM1 antibody reactivity did not occur without additional IgG and/or IgM reactivity. There was no difference in IgA anti-GM1 antibody reactivity between Japan

**Table 2. Specificity and isotype distribution of anti-ganglioside antibodies in Guillain-Barré patients from Japan and The Netherlands (cut-off 1000).**

| | Japanese patients (n=88) | Netherlands patients (n=132) | p-value |
|---|---|---|---|
| GM1 | | | |
| IgA | 3 (3%) | 3 (2%) | n.s. |
| IgG | 13 (15%) | 17 (13%) | n.s. |
| IgM | 1 (1%) | 10 (8%) | 0.03 |
| Total | 14 (16%) | 22 (17%) | n.s. |
| GM1b | | | |
| IgG | 12 (14%) | 15 (11%) | n.s. |
| IgM | 2 (2%) | 15 (11%) | 0.01 |
| Total | 14 (16%) | 26 (20%) | n.s. |
| GalNAc-GD1a | | | |
| IgG | 2 (2%) | 12 (9%) | 0.04 |
| IgM | 2 (2%) | 10 (8%) | n.s. |
| Total | 4 (5%) | 19 (14%) | 0.02 |
| GM1/GM1b/GalNAc-GD1a | | | |
| IgG | 17 (19%) | 24 (18%) | n.s. |
| IgM | 3 (3%) | 18 (14%) | 0.005 |
| Total | 19 (22%) | 33 (25%) | n.s. |

and The Netherlands.

Depending on the cut-off value for positivity, results from both the Japanese and Dutch ELISA system reached the same conclusion. With both assays, there was a significant difference in anti-ganglioside reactivity between GBS patients and controls from Japan and the Netherlands (data not shown). With a cut-off titer of 1000 in the Japanese system and 200 in the Dutch system, both systems indicated that 16-17% of GBS patients, from either Japan or the Netherlands had anti-GM1 antibodies. Testing at lower dilutions decreased the correlation between the ELISA systems. At a cut-off level of 500 in the Japanese test, the percentage of GBS patients with IgM antibodies against any of the three gangliosides increased from 3% to 14% for the Japanese patients and from 15% to 24% for the Dutch patients. When lowering the cut-off value to 100 in the Dutch ELISA system, the same trend could be observed for the Japanese patients (from 3% to 13%) but not for the Dutch patients (10% to 11%). These differences are caused by the presence of lower titer IgM anti-ganglioside antibodies in a considerable proportion of Japanese GBS patients that are detected when testing serum from these patients at a lower dilution.

## DISCUSSION

We have demonstrated a difference in fine-specificity and isotype distribution of serum anti-ganglioside antibody reactivity between GBS patients from Japan and The Netherlands. By testing the serum samples in two seperate anti-ganglioside assay systems we excluded the possibility that these differences were assay-dependent.

The preferential expression of IgG anti-ganglioside antibodies in Asian GBS patients has been described before (Ho *et al.*, 1995; Kusunoki *et al.*, 1996a; Yuki *et al.*, 1999). The differences in isotype distribution may depend on the infectious agents that trigger the anti-ganglioside response. *C. jejuni* strains with the Penner O:19 serotype are frequently isolated from Japanese GBS patients (Kuroki *et al.*, 1993; Yuki *et al.*, 1997b). In the Netherlands, this strains with this serotype only constitute a minority of GBS-related strains (Endtz *et al.*, 2000). It is possible that strains with the O:19 serotype preferentially induce an IgG response. Alternatively, host-factors such as differences in immunogenetic polymorphisms may contribute to a differential isotype response against glycolipid antigens. To our knowledge, no such geographical determined differences in isotype responses have been reported so this explanation remains speculative.

We have previously shown that antibody reactivity against GM1, GM1b and GalNAc-GD1a is associated with antecendent *C. jejuni* infections and a motor neuropathy with predominantly distal weakness (Ang *et al.*, 1999; Jacobs *et al.*, 1996b; Yuki *et al.*, 2000). The overlap between GM1, GM1b and GalNAc-GD1a positive GBS patients indicates that *Campylobacter* infections are not specifically related to anti-GM1 reactivity but to antibody reactivity against GM1-like gangliosides (Figure 1). In addition, antibody reactivity against GM1-like gangliosides instead of GM1 itself seems to be related to a distally dominant motor

neuropathy. Therefore, the anti-GM1, GM1b and GalNac-GD1a antibodies may all bind to the same target in the nerve.

The comparison of our ELISA systems indicates that serum samples with high-titer anti-ganglioside antibodies are recognized in both systems. The situation is more complicated for samples with lower titer anti-ganglioside reactivity. Our studies indicate that for comparative studies, either a detailed comparison of assay systems before determining the cut-off values or a central laboratory with a conservative cut-off value is obligatory (Willison *et al.*, 1999).

In conclusion, we have demonstrated that GBS patients in Japan and The Netherlands have differences in specificity and isotype distribution of anti-ganglioside antibodies. Extension of these type of studies to other geographical regions, in combination with a survey of antecedent infections, is desirable.

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# Chapter 6

## CROSS-REACTIVE ANTI-GALACTOCEREBROSIDE ANTIBODIES ARE ASSOCIATED WITH *MYCOPLASMA PNEUMONIAE* INFECTIONS IN THE GUILLAIN-BARRÉ SYNDROME

**CW Ang[1,2], AP Tio-Gillen[1,2], J Groen[3], P Herbrink[4], BC Jacobs[1,2], R van Koningsveld[1], ADME Osterhaus[3], FGA van der Meché[1], PA van Doorn[1]**

Departments of Neurology[1], Immunology[2] and Virology[3], Erasmus University Rotterdam, The Netherlands
Department of Immunology & Infectious Diseases[4], Diagnostic Centre SSDZ, Delft, The Netherlands

*Submitted*

## ABSTRACT

*Mycoplasma pneumoniae* infections induce a variety of serious neurological complications including the Guillain-Barré syndrome (GBS). We investigated the presence of antibody reactivity in serum from patients with GBS against the glycolipid galactocerebroside (GalC) in relation to a preceding *M. pneumoniae* infection. We detected that anti-GalC antibody reactivity was present in GBS patients. Furthermore, anti-GalC-reactivity was associated with *M. pneumoniae* infections and that the anti-GalC antibodies cross-reacted with *M. pneumoniae* antigens. We conclude that anti-GalC antibodies in GBS patients may be induced by molecular mimicry with *M. pneumoniae*. The role of anti-GalC antibodies in the pathogenesis of *M. pneumoniae*-associated GBS remains to be determined.


## INTRODUCTION

The Guillain-Barré syndrome is frequently preceded by infections. The most frequent antecedent infectious agent is *Campylobacter jejuni*, followed by cytomegalovirus (CMV), Epstein-Barr virus and *Mycoplasma pneumoniae* (Dowling and Cook, 1981; Goldschmidt *et al.*, 1980; Hao *et al.*, 1998; Jacobs *et al.*, 1998; Winer *et al.*, 1988c). The heterogeneity with respect to the triggering infection is parallelled in the specificity of the anti-glycolipid reactivity. *C. jejuni* infections have been linked to anti-GM1 and anti-GD1b reactivity whereas CMV infections are associated with anti-GM2 reactivity (Irie *et al.*, 1996; Jacobs *et al.*, 1998; Jacobs *et al.*, 1997d; Rees *et al.*, 1995a). In *C. jejuni* and CMV-associated GBS, the anti-glycolipid antibodies cross-react with bacterial and viral antigens, indicating that they have been induced by molecular mimicry (Ang *et al.*, 2000c; Irie *et al.*, 1996; Jacobs *et al.*, 1997b; Yuki, 1997).

Several authors have described anti-glycolipid antibodies, mainly against galactocerebroside (GalC), in small numbers of neuropathy patients with a preceding *M. pneumoniae* infection (Hao *et al.*, 1998; Kusunoki *et al.*, 1995; Kusunoki *et al.*, 1996c). However, there is controversy about the presence of anti-GalC reactivity in GBS patients and the relation with antecedent *M. pneumoniae* infections. One of the contributing factors is the small number of GBS patients with antecedent *M. pneumoniae* infection (Hao *et al.*, 1998). GalC is one of the major glycolipids of peripheral nerve and has been implicated in both structure and function of myelinated nerve fibers (Coetzee *et al.*, 1998). Strong evidence for a potential role of anti-GalC antibodies in the pathogenesis of immune-mediated neuropathies comes from immunization studies in the rabbit. Repeated immunization of rabbits with GalC in a strong adjuvant leads to the induction of anti-GalC antibodies and a demyelinating neuropathy (Saida *et al.*, 1979b). Furthermore, anti-GalC antibodies were shown to have a demyelinating effect in vitro (Hirota *et al.*, 1997; Sumner *et al.*, 1982).

The aim of the present study was to determine anti-GalC antibody reactivity in a large

group of GBS patients and to correlate the antibody reactivity to antecedent infections and clinical and electrophysiological characteristics. We demonstrate that anti-GalC antibodies in GBS patients are associated with antecedent *M. pneumoniae* infections and that they cross-react with *M. pneumoniae* antigens.

## PATIENTS AND METHODS

Serum samples, taken before treatment with intravenous immunoglobulins (IVIg), were obtained from 130 of 147 GBS patients who participated in the Dutch GBS trial, which compared the therapeutic effects of plasma exchange (PE) and IVIg (Van der Meché and Schmitz, 1992). Seventeen cases were excluded because no serum was available. The excluded cases did not differ from the other patients with respect to their clinical manifestations and course of disease.

Antecedent *M. pneumoniae* infections were defined as an elevated IgM titer against with ELISA (Virion-Serion, Würzburg, Germany). Serology for other antecedent infections and other glycolipids has been described before (Jacobs *et al.*, 1998). Because only 7/130 patients from the Dutch PE/IVIg GBS trial had serological evidence for a *M. pneumoniae* infection, we identified 8 additional *M. pneumoniae* infected patients from the ongoing IVIg vs IVIg + methylprednisolone trial, a prospective epidemiological study of GBS in the Netherlands and from patients whose serum had been sent to the Neuroimmunological Diagnostic Unit of the Erasmus University Rotterdam. As controls we took serum samples from 26 healthy controls, 30 patients with other neurological diseases, 19 patients with a *M. pneumoniae* infection without neurological complications and 30 patients with an uncomplicated *C. jejuni* enteritis.

IgM and IgG anti-GalC reactivity was determined with ELISA as described before but with 900 pmol GalC/well (Ang *et al.*, 2000c). In *M. pneumoniae*-infected GBS patients, GalC reactivity was confirmed with thin-layer chromatography (TLC). Cut-off level for GalC-reactivity was based on the mean plus 3 standard deviations corrected optical density-values of the 26 healthy controls. All *M. pneumoniae* positive GBS patients and controls were tested for additional antibody reactivity against GM1, GM2, GD1a, GD1b, GQ1b and GA1 as described previously (Ang *et al.*, 2000c). To investigate whether the anti-GalC antibodies cross-reacted with *M. pneumoniae* antigens, we used an inhibition ELISA system. *M. pneumoniae* antigen (strain NCTC#10119, grown in cell-free medium, Virion-Serion) was added to diluted serum samples containing IgG anti-GalC reactivity and incubated for 3.5 hours at 4°C. After centrifugation, serum samples were tested in ELISA for anti-glycolipid reactivity as described above. Serum from a Miller Fisher syndrome (MFS) patient with IgG anti-GQ1b reactivity was taken as control.

Correlation of anti-GalC reactivity in GBS patients was done as described before (Ang *et al.*, 1999). We used the chi-square or Fischer exact to test equality of proportions and the

Mann-Whitney U test for continuous variables. A p-value of <0.05 was considered to be significant.

## RESULTS

Anti-GalC reactivity was found in 16 (12%) of the 130 GBS patients from the Dutch GBS trial. 12 patients had only IgM antibodies, 3 had only IgG antibodies and one patient had both IgG and IgM anti-GalC reactivity. We did not detect any GalC reactivity in healthy controls or neurological controls. *M. pneumoniae* infections were correlated with the presence of anti-GalC antibodies (p=0.011). Although six *C. jejuni* infected patients had anti-GalC antibody reactivity, this relation was not significant. We could not identify any significant correlations of anti-GalC reactivity with clinical parameters although GalC-positive patients tended to have more respiratory tract infections, sensory deficits and paresthesias and a relatively mild form of the disease when compared to patients without anti-GalC reactivity. 11/13 GalC-positive patients from whom electrophysiological data were available had predominantly demyelinating features on the EMG (data not shown).

In the additional group of *M. pneumoniae*-associated GBS patients, we confirmed the frequent presence of anti-GalC antibodies (Figure 1). IgM and/or IgG anti-GalC reactivity

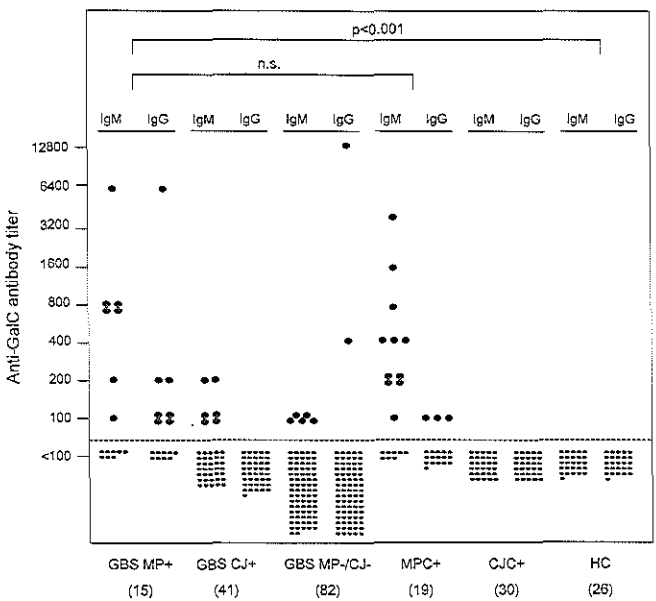


**Figure 1.** Titers of anti-galactocerebroside (GalC) antibodies in Guillain-Barré patients and control subjects. GBS Guillain-Barré syndrome, MP *M. pneumoniae*, CJ *C. jejuni*, MPC *M. pneumoniae* control, CJC *C. jejuni* control, HC Healthy control.

was present in 11/15 (73%) of *M. pneumoniae*-infected GBS patients. Anti-GalC reactivity was not specifically associated with the occurrence of neurological symptoms following *M. pneumoniae* infection, because patients with an antecedent infection with *M. pneumoniae* but without neurological symptoms also had anti-GalC reactivity (11/19, 58%). IgG anti-GalC reactivity was more frequent in the GBS-*M. pneumoniae* group than in the *M. pneumoniae* - control patients although this was not significant. Additional antibody reactivity against Gal and was present in some anti-GalC positive GBS patients and *M. pneumoniae* control patients. Additional anti-GM1 reactivity was only present in some GBS patients. The anti-GalC reactivity decreased during follow-up, indicating that the antibodies do not result from the nerve damage (data not shown).

To assess cross-reactivity of the anti-GalC antibodies, we incubated anti-GalC reactive serum samples with *M. pneumoniae* antigen. Following incubation of anti-GalC reactive serum with *M. pneumoniae* antigen, the anti-GalC reactivity decreased in a dose-dependent manner while anti-GQ1b reactivity was unaffected (Figure 2), demonstrating cross-reactivity of anti-GalC antibodies with *M. pneumoniae* .

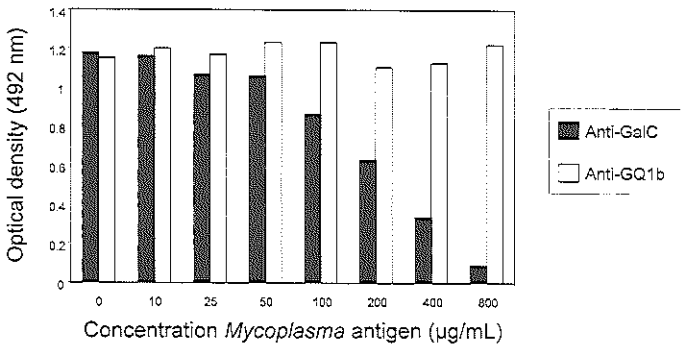


**Figure 2.** Inhibition of IgG anti-GalC but not GQ1b reactivity by *M. pneumoniae* antigens. Diluted sera were incubated with increasing doses of *M. pneumoniae* antigen. Anti-GalC reactivity is dose-dependently decreased, whereas anti-GQ1b reactivity remains unaffected.

## DISCUSSION

We have demonstrated that anti-GalC antibodies occur in patients with GBS, that they are associated with *M. pneumoniae* infections and that they are cross-reactive with *M. pneumoniae* antigens. This study confirms and extends earlier reports about anti-GalC reactivity in GBS (Hao *et al.*, 1998; Ilyas *et al.*, 1991; Kusunoki *et al.*, 1995). In several other studies no reactivity against GalC could be demonstrated, but these differences may depend on the methods used (Rostami *et al.*, 1987; Winer *et al.*, 1988b). *M. pneumoniae* infections can cause various neurological complications including GBS and meningo-encephalitis (Clyde, 1993; Komatsu *et al.*, 1998). In some cases of post-*M. pneumoniae* encephalitis antibodies against

GalC and other brain antigens are present (Nishimura *et al.*, 1996b). This selective involvement of the central or peripheral nervous system despite the presence of anti-GalC antibodies in both types of neurological complications and the presence of GalC in both the central and peripheral nervous system indicates that other factors such as a selective impairment of the blood-brain barrier or the blood-nerve barrier or a difference in fine-specificity of the anti-GalC antibodies (Coetzee *et al.*, 1998). Anti-GalC antibodies were not specific for GBS patients with an antecedent *M. pneumoniae* infection as has been reported before (Nishimura *et al.*, 1996b).

Anti-GalC antibody reactivity was not related to a specific pattern of clinical features, as has been demonstrated for anti-GM1, anti-GM1b, anti-GalNAc-GD1a and anti-GD1a antibodies (Ang *et al.*, 1999; Ho *et al.*, 1999; Jacobs *et al.*, 1996b; Kaida *et al.*, 2000; Yuki *et al.*, 2000). However, we found the presence of demyelinating features in the majority of our GalC positive GBS patients.This observation is in accordance with the reports on GalC-induced neuropathy in rabbits. These rabbits develop an immune-mediated neuropathy histologically characterized by demyelination (Saida *et al.*, 1979b). Furthermore, rabbit anti-GalC antibodies have a demyelinating effect on Schwann-cell cultures and optic nerve (Roth *et al.*, 1985; Saida *et al.*, 1979b; Sergott *et al.*, 1984). It would therefore be interesting to investigate the demyelinating potential of human anti-GalC antibodies in these systems.

The anti-GalC antibodies may have been induced by molecular mimicry because only the anti-GalC reactivity was inhibited by *M. pneumoniae* antigens. In many *M. pneumoniae* infected patients, cold agglutinins develop (Clyde, 1993). Cold agglutinins are mostly IgM antibodies and are directed against the I-antigen of erythrocytes. This I-antigen serves as a receptor for *M. pneumoniae* (Feizi and Loveless, 1996). The exact mechanism of cold agglutinin development is unknown but anti-GalC antibodies may have been induced by a similar mechanism. The absence of reactivity against a number of other glycolipid antigens indicates that the anti-GalC antibodies are not induced by polyclonal B cell stimulation (Biberfeld and Gronowicz, 1976).

Recently, GalC has been identified as an antigen that can be processed via the CD1-pathway of antigen presentation (Park and Bendelac, 2000). Furthermore, techniques have become available to detect GalC-reactive T cells in vitro using glycolipid containing tetramere CD1 molecules (Benlagha *et al.*, 2000). It is tempting to speculate on the presence of GalC and other glycolipid-reactive T cells in patients with GBS and their role in the pathogenesis.

In conclusion, we have provided further evidence for the hypothesis that in GBS patients, antecedent infections determine the specificity of the resulting cross-reactive anti-glycolipid antibodies, presumably due to molecular mimicry. The role of anti-GalC antibodies in the development of nerve damage and dysfunction remains to be determined.

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# Chapter 7

## A CASE OF GUILLAIN-BARRÉ SYNDROME FOLLOWING A FAMILY OUTBREAK OF *CAMPYLOBACTER JEJUNI* ENTERITIS

**CW Ang[1,2], PA van Doorn[1], HPh Endtz[3], ISJ Merkies[1], BC Jacobs[1,2], MA de Klerk[1,2], R van Koningsveld[1], FGA van der Meché[1]**

Departments of Neurology[1], Immunology[2], Medical Microbiology & Infectious Diseases[3], Erasmus University Rotterdam, The Netherlands

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## ABSTRACT

We describe an outbreak of *Campylobacter jejuni* enteritis involving three family members of whom one developed Guillain-Barré syndrome (GBS). The patients' serum reacted strongly with several gangliosides and with the lipopolysaccharide (LPS) fractions from the *C. jejuni* strains isolated from his family members. Only low titer anti-ganglioside antibodies were found in his siblings. HLA-typing did not indicate a locus associated with auto-antibody production. Comparing the immune response in GBS patients and *C. jejuni* enteritis patients can be of great value in determining the additional factors that lead to post-*Campylobacter* GBS. Ganglioside mimicry alone is necessary but not sufficient for the induction of anti-ganglioside antibodies. Other susceptibility factors are required to induce an anti-neural immune-response.


## INTRODUCTION

The Guillain-Barré syndrome (GBS) is preceded by an infection with the enteric pathogen *Campylobacter jejuni* in about one third of the cases (Hughes *et al.*, 1999). The exact pathogenic mechanism by which an infection with *C. jejuni* leads to GBS still remains to be elucidated. The induction of anti-ganglioside antibodies by molecular mimicry between lipopolysaccharides (LPS) from *C. jejuni* and glycolipids is thought to be very important in the pathogenesis of GBS (Yuki *et al.*, 1993a). Controversy remains to what extent microbial and/or host factors contribute to the development of an anti-glycolipid immune response and neurological symptoms. Several observations point to microorganism-related factors that contribute to neurological sequelae. First, in a large proportion of GBS cases in Japan, *C. jejuni* with Penner serotype 19 were isolated while the prevalence of this serotype in the normal Japanese population is relatively low (Kuroki *et al.*, 1993). Second, several groups have demonstrated a clear difference in ganglioside-like structures between *C. jejuni* strains isolated from patients with GBS and patients with the Miller Fisher syndrome (Jacobs *et al.*, 1995; Yuki *et al.*, 1994b). The Miller Fisher syndrome is considered to be a variant of GBS and is associated with the presence of antibodies against ganglioside GQ1b (Chiba *et al.*, 1993). *C. jejuni* strains from GBS patients were shown to react with anti-GM1 antibodies and cholera toxin while strains from MFS patients recognised anti-GQ1b antibodies (Jacobs *et al.*, 1997a; Sheikh *et al.*, 1998; Yuki *et al.*, 1994b). In addition to these microorganism-related factors, other observations indicate a role for host-related factors. Although *C. jejuni* enteritis occurs in epidemics, epidemics of GBS have never been described and only one out of 1,000 *C. jejuni* enteritis patients will develop GBS (Mishu Allos, 1997). Furthermore, the presence of ganglioside-like epitopes in *C. jejuni* LPS is not restricted to GBS or MFS related *C. jejuni* strains (Gregson *et al.*, 1997; Jacobs *et al.*, 1997a; Sheikh *et al.*, 1998). These findings suggest that in addition to several microorganism-related factors, host factors that

influence the immune response against *C. jejuni* may determine whether an individual will develop GBS following an infection with *C. jejuni*. Analysis of differences in the immune response between *C. jejuni* associated GBS patients and patients with an uncomplicated *C. jejuni* enteritis may be helpful in exploring the mechanisms that lead to an anti-neural immune response. In the present paper, we describe a family in which three members had *C. jejuni* enteritis. Only one member of the family developed GBS. The finding that one *C. jejuni* strain infected several genetically related individuals, leading to different clinical syndromes, is particularly interesting and prompted us to initiate further detailed studies. Analysis of the humoral immune response to *Campylobacter* antigens and gangliosides in all four members of this family and an analysis of immunogenetic factors gives insight into the mechanism by which *C. jejuni* infections can lead to GBS.

## FAMILY REPORT

In July 1995, five days after a short episode of diarrhoea, a previously healthy ten-year-old boy developed generalized muscle weakness and was admitted to our hospital. He had no sensory symptoms, no cranial nerve involvement and had no complaints of pain. Electrophysiological studies revealed very low compound muscle action potentials in the lower limbs and slightly diminished conduction velocity of motor nerves in the upper limbs. Compound sensory nerve action potentials were normal. He fulfilled the diagnostic criteria for GBS (Asbury and Cornblath, 1990) and made a complete recovery. The patient's father and brother also had symptoms of acute diarrhoea, eight and three days before the onset of the patient's complaints. The duration and severity of the diarrhoea did not differ from the patient. All members of the family had attended a garden barbecue with the consumption of chicken, two days before the start of diarrhoeal symptoms. The patient's mother and none of the other guests had experienced gastro-intestinal symptoms. Father, mother and brother of the patient did not have any neurological complaints and electrophysiological studies in these individuals did not reveal any abnormalities.

## METHODS

### Culture studies

Three stool samples were received from all four members of the family. They were inoculated on a selective *Campylobacter* enrichment broth, two different selective agar media and on blood agar with a filter method. Identification was done using routine methods. Recovered *C. jejuni* strains were further characterized with the O (Penner) and HL(Lior) serotyping methods. LPS fractions were analyzed for their reactivity with a panel of sera containing anti-GM1 and anti-GQ1b antibodies as described before (Jacobs *et al.*, 1997a).

**Serological studies**

Serological assays to determine a recent infection included *Borrelia*, *C. jejuni*, cytomegalovirus, Epstein-Barr virus, herpes simplex virus, measles, mumps, *Mycoplasma pneumoniae*, varicella zoster virus. Antibody reactivity against glycolipids GM1, GM2, GD1a, GD1b, GT1a, GT1b, GQ1b and GA1 were determined with ELISA and confirmed with thin-layer chromatography as described previously (Jacobs *et al.*, 1997a). Anti-LPS reactivity was determined with ELISA and confirmed with Western blot using a chemiluminescence detection system. Subclass distribution of IgG anti-LPS antibodies was analyzed with ELISA (Jacobs *et al.*, 1997a).

**HLA-typing**

HLA typing for class I and II antigens was performed with a lymphocytotoxicity assay.

## RESULTS AND DISCUSSION

All three members of the family with diarrhoea had elevated IgM and/or IgA antibodies and IgG antibodies against a protein extract of *C. jejuni*, indicating a recent infection(Herbrink *et al.*, 1988). *C. jejuni* was isolated from the stools of father and brother but we were unable to culture *C. jejuni* from the stools of the patient. The two strains that were isolated from the father and brother both had Penner serotype O:2 and Lior serotype HL:4. Stool cultures from the mother were negative and she had no serological evidence for recent infection with *C. jejuni*. The history of diarrhoea and the presence of IgM anti-*C. jejuni* antibodies in the serum of the patient strongly suggest that the GBS patient had also been infected with *C. jejuni* (Table 1). No evidence for a recent infection with other microbial agents was present. The identical serotype of both *C. jejuni* strains and the temporal relationship between the gastro-intestinal complaints of the family members are strong evidence for the fact that the GBS patient was infected with the same strain as his brother and father.There is a striking difference in the immune response towards different *Campylobacter* antigens. All three patients with diarrhoea had antibodies against protein antigens that are used in the diagnostic serological assay (Herbrink *et al.*, 1988). In contrast, only the GBS patient's IgG strongly reacted with the LPS fraction, including the LPS from the *C. jejuni* strains that were isolated from his father and brother (Table 1, Figure 1). The subclass of these antibodies was IgG1 and IgG3, in concordance with findings in other *C. jejuni*-related GBS cases (Jacobs *et al.*, 1997a). This pattern of anti-bacterial reactivity is accompanied with the presence of neurological symptoms. There may be several reasons for the different antibody response to *C. jejuni* antigens. First, although the three individuals were probably infected with the same *C. jejuni* strain, differential expression of ganglioside-like structures on the same strain in the three enteritis patients might account for differences in immune-response. Alternatively, or in addition, genetic host susceptibility factors are responsible for an anti-LPS response. Only the

**Table 1. Diarrhoeal symptoms, culture results and antibody reactivity to *Campylobacter jejuni* antigens and glycolipids.**

| | | Patient | Father | Brother | Mother |
|---|---|---|---|---|---|
| Diarrhoea | | + | + | + | - |
| *Campylobacter jejuni* | | | | | |
| Serology[a] | | + | + | + | - |
| Culture[b] | | - | + | + | - |
| Anti-lipopolysaccharide antibodies[c] | | | | | |
| | *C. jejuni*-Father | IgG1, IgG3 | - | - | - |
| | *C. jejuni*-Brother | IgG1, IgG3 | - | - | - |
| Anti-ganglioside antibodies[d] | | | | | |
| IgM | GA1 | >1.600 | 100 | - | - |
| | GM1 | 800 | - | - | - |
| | GM2 | 100 | - | 200 | - |
| | GD1a | - | - | - | - |
| | GD1b | 200 | - | - | - |
| | GT1a | - | - | - | - |
| | GT1b | - | - | - | - |
| | GQ1b | - | - | - | - |
| IgG | GA1 | >1.600 | - | - | - |
| | GM1 | 400 | - | - | - |
| | GM2 | - | - | - | - |
| | GD1a | - | - | - | - |
| | GD1b | - | - | - | - |
| | GT1a | - | - | - | - |
| | GT1b | - | - | - | - |
| | GQ1b | - | - | - | - |

a. Antigen for serology is a protein extract.
b. *Campylobacter jejuni* strains from father and brother had both Penner serotype O:2 and Lior serotype HL:4.
c. Results are for IgG antibodies. When reactivity was present, IgG subclass was determined. LPS from strains isolated from the father and brother was used.
d. Results are expressed as titer, - = titer <100.

GBS patient had high titer anti-glycolipid antibodies of both IgM and IgG isotype (Table 1). The father only had low titer IgM antibodies against GA1 and the brother had low titer IgM antibodies against GM2 (Table 1). Serum from the mother did not display any anti-ganglioside reactivity. These anti-ganglioside antibodies could not be demonstrated in follow-up serum samples (data not shown) which suggests that they result from *C. jejuni* infection. The possibility that both father and brother had subclinical nerve dysfunction was ruled out with electrophysiological examination. However, it would be interesting to know whether low titer anti-ganglioside antibodies would be part of the normal immune response following *C. jejuni* enteritis. Several investigators have addressed this issue but results are conflicting (Sheikh *et al.*, 1998; Von Wulffen *et al.*, 1994). *C. jejuni* strains with the Penner O:2 serotype have been isolated from GBS patients before but it is unclear whether strains with this serotype contain GM1-like epitopes (Aspinall *et al.*, 1993a). The LPS fractions from the strains that

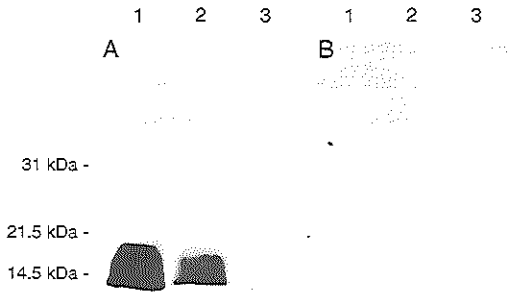


**Figure 1.** Antibody response to *C. jejuni* lipopolysaccharides (LPS). Immunoblots of *C. jejuni* LPS overlaid with (**A**) serum from the Guillain-Barré patient (**B**) serum from his brother. Lanes 1, LPS from non-Guillain-Barré syndrome related *C. jejuni* strain CCUG 6824 (0.5 µg); lanes 2 , LPS from the *C. jejuni* strain isolated from the brother (1.0 µg) and lanes 3, LPS from a *C. jejuni* strain isolated from an unrelated GBS patient (1.0 µg). The Guillain-Barré patient has strong IgG reactivity to *C. jejuni* LPS while no anti-LPS reactivity can be detected in serum from his brother.

were isolated from the father and from the brother reacted with a panel of GM1-positive sera but not with a panel of GQ1b-positive sera (data not shown). This reaction pattern indicates that the LPS fraction from these Penner O:2 strains contains a ganglioside-like epitope that can be recognized by anti-GM1 antibodies.The fact that one infectious agent infected three genetically related individuals with a very different immunological response and outcome points into the direction of host-susceptibility factors that determine the immune-response towards *C. jejuni* and thereby determine the development of neurological symptoms. Previous studies have reported the predominance of the HLA-B35, HLA-B54, HLA-Cw1 and HLA-DQB1*03 haplotypes in GBS patients with an antecedent infection with *C. jejuni* (Koga *et al.*, 1998b; Rees *et al.*, 1995c; Yuki *et al.*, 1992a). The patient did not have any of the HLA molecules mentioned above (Table 2). The brother and father were both typed HLA-A2/DR4/DQ8 (Table 2), which might protect from neurological sequelae. As we did not investigate polymorphisms in other immune-response genes, the three enteritis patients may differ with respect to other host susceptibility genes.In conclusion, we have shown that induc-

**Table 2. HLA haplotypes of all individuals of the family.**

| | | |
|---|---|---|
| Patient: | A3, B7, Cw7, DR17, DQ2 | |
| | A28, B62, Cw3, DR13, DQ6 | |
| Brother: | A2, B7, Cw7, DR4, DQ8 | |
| | A28, B62, Cw3, DR13, DQ6 | |
| Father: | A2, B7, Cw7, DR4, DQ8 | |
| | A3, B7, Cw7, DR17, DQ2 | |
| Mother: | A2, B39, Cw--, DR--, DQ7 | |
| | A28, B62, Cw7, DR13, DQ6 | |

tion of anti-LPS and anti-ganglioside antibodies coincides with the development of neurological symptoms following *C. jejuni* infection. The presence of ganglioside mimicry appears to be a necessary but not a sufficient determinant for the induction of anti-ganglioside antibodies. Other factors which may be either host or microorganism-related are needed to induce an anti-neural immune-response.

**Acknowledgements**

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# Chapter 8

## CROSS-REACTIVE ANTIBODIES AGAINST GM2 AND CMV-INFECTED FIBROBLASTS IN GUILLAIN-BARRÉ SYNDROME

**CW Ang[1,2], BC Jacobs[1,2], AH Brandenburg[3], JD Laman[2], FGA van der Meché[1], ADME Osterhaus[3], PA van Doorn[1]**

Departments of Neurology[1], Immunology[2] and Virology[3], Erasmus University Rotterdam, The Netherlands

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## ABSTRACT

**Objective**

To investigate whether anti-GM2 antibodies in patients with Guillain-Barré syndrome (GBS) are induced by molecular mimicry with cytomegalovirus (CMV).

**Background**

Antibodies against ganglioside GM2 are frequently present in the serum from GBS patients with an antecedent infection with CMV.

**Methods**

Detection of inhibition of anti-GM2 reactivity following incubation of GM2 reactive serum samples with fibroblasts infected with a Guillain-Barré associated CMV-strain. Control sera consisted of GQ1b-reactive samples, control antigens included uninfected fibroblasts and fibroblasts that were infected with other herpesviruses.

**Results**

Serum IgM reactivity with GM2 was decreased in a dose dependent manner after incubation with CMV-infected fibroblasts. Incubation of anti-GM2 positive serum samples with uninfected fibroblasts and fibroblasts infected with varicella-zoster virus did not inhibit anti-GM2-reactivity while this reactivity was slightly decreased after incubation with herpes simplex virus type 1 in one patient. Antibodies against ganglioside GQ1b did not react with CMV-infected fibroblasts.

**Conclusions**

CMV-infected fibroblasts express ganglioside-like epitopes that specifically recognize anti-GM2 antibodies. These results support the hypotheses that anti-ganglioside antibodies in CMV-infected GBS patients are induced by molecular mimicry between GM2 and antigens that are induced by a CMV infection.


## INTRODUCTION

Although the pathogenesis of the Guillain-Barré syndrome (GBS) is not clearly understood, the close relationship with preceding infections indicates that various infectious agents are able to trigger an immune-mediated attack on peripheral nerves (Hafer-Macko *et al.*, 1996b; Hartung *et al.*, 1995c). Cytomegalovirus (CMV) infections have frequently been reported to be associated with GBS (Dowling and Cook, 1981; Jacobs *et al.*, 1998; Winer *et al.*, 1988c). Serological studies indicate that up to 13% of GBS patients have experienced a recent infection with CMV (Jacobs *et al.*, 1998; Schmitz and Enders, 1977; Winer *et al.*, 1988c). The first reports on isolation of CMV in patients with GBS date from 1967 (Klemola *et al.*, 1967). It is presently unknown whether CMV strains that are associated with neurological deficits possess specific properties that distinguish them from other CMV strains.

In *Campylobacter jejuni* associated GBS, cross-reactive antibodies between *C. jejuni*

lipopolysaccharides (LPS) and several gangliosides (e.g. GM1, GD1b and GQ1b) are present (Chiba *et al.*, 1993; Jacobs *et al.*, 1997b; Rees *et al.*, 1995a; Sheikh *et al.*, 1998; Yuki *et al.*, 1997a). In addition, it has been demonstrated that LPS from *C. jejuni* strains isolated from GBS-patients display ganglioside mimicry (Aspinall *et al.*, 1994a; Moran and O'Malley, 1995; Yuki *et al.*, 1993a). Anti-ganglioside antibodies can bind to peripheral nerves and may interfere with neuromuscular transmission (O'Hanlon *et al.*, 1996; Plomp *et al.*, 1999). These findings support the hypothesis that anti-ganglioside antibodies, induced by molecular mimicry, have a role in the development of GBS.

In GBS, anti-GM2 antibodies have been detected in 22-67% of CMV-infected patients (Jacobs *et al.*, 1997d; Khalili-Shirazi *et al.*, 1999; Yuki and Tagawa, 1998). The mechanism by which these antibodies are formed is unknown. Viral infections may lead to auto-antibody production by a variety of mechanisms. Release of cytokines and chemokines by infected cells may lead to B cell activation and enhanced expression of MHC determinants; cryptic antigens may be exposed or shedded, or anti-idiotypic antibodies may be formed. In addition, molecular mimicry, through the induction of a cross-reactive immune response, may also play a role in virus-induced autoimmunity (Irie *et al.*, 1996; Oldstone, 1998).

We investigated whether serum anti-GM2 antibodies from patients with GBS with an antecedent CMV infection react with a GBS-associated CMV strain. We used an inhibition ELISA system to detect a decrease in anti-GM2 reactivity in serum incubated with fibroblasts infected with CMV.

## METHODS

### Patients

Serum from three GBS patients with serologically proven CMV infection and anti-GM2 IgM antibodies was used. As a control we used serum from two GBS patients without CMV infection with anti-GQ1b antibodies. Serum samples were drawn within one month of the onset of neurological symptoms in the actue phase of the disease. All patients required assisted ventilation during the course of the disease. None of the patients had the acute motor axonal neuropathy (AMAN) variant of GBS. Patient characteristics are listed in the Table. Serum from a paraproteinaemic neuropathy patient that reacted with both GM2 (IgM titer 1600) and GM1 (IgM titer 3200) was taken as an additional source of antibodies.

### Cells and viruses

Human embryonic lung fibroblasts were maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Verviers, Belgium), supplemented with 2% fetal bovine serum (BioWhittaker), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures were passaged weekly at a ratio of 1:2 and used at passages 13-18. To infect fibroblasts, monolayers in 162 $cm^2$ bottles were incubated with 10 mL virus suspension in medium for one hour at 37°C, after which 50 ml medium was added. Cells were incubated at 37°C

**Table. Characteristics and ganglioside serology of Guillain-Barré patients used in this study.**

| Patient | Age | Sex[a] | Antecedent infection | Titer of serum anti-ganglioside antibodies[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | GA1 | | GM1 | | GM2 | | GQ1b | |
| | | | | IgM | IgG | IgM | IgG | IgM | IgG | IgM | IgG |
| 1 | 34 | F | Cytomegalovirus (CMV 96-3286) | 400 | - | 1.600 | - | 100 | - | - | - |
| 2 | 20 | F | Cytomegalovirus | - | - | - | - | 400 | - | - | - |
| 3 | 24 | F | Cytomegalovirus | - | - | - | - | 200 | - | - | - |
| 4 | 78 | F | *Campylobacter jejuni* | >800 | 400 | - | - | - | - | 1.600 | 6.400 |
| 5 | 68 | F | Upper respiratory tract infection | - | - | - | - | - | - | 400 | 1.600 |

a F = female.
b - = titer<100.

until the majority of the cells were infected, as evidenced by virus-specific immunofluorescence. After harvesting with trypsinisation and washes in phosphate-buffered saline (PBS), pH 7.4, cells were counted, aliquoted and frozen at -80°C until use.

We were able to isolate a CMV strain (designated 96-3286) from the blood of GBS patient 1 during the acute phase of the disease. Reference CMV strain AD169 was used as a control CMV strain. Varicella-zoster virus (VZV, strain ATCC VR586) and a clinical isolate of herpes simplex virus type 1 (HSV type 1), as evidenced by virus-specific immunofluorescence, served as controls in these experiments.

**Anti-glycolipid antibody and inhibition assays**

The serum of the five patients was tested with ELISA and thin-layer chromatography (TLC) for reactivity with GA1, GM1, GM2 and GQ1b as described previously with minor modifications (Jacobs *et al.*, 1995). In short, 300 pmol of glycolipid was coated in 100 µL ethanol and wells were evaporated to dryness. Wells containing only ethanol were taken as a control. After blocking with PBS containing 1% bovine serum albumin (PBS-BSA, Sigma, St. Louis, MO), the plates were incubated with serum samples diluted in PBS-BSA overnight at 4°C. Following washing with PBS, the plates were incubated for 1.5 hrs with peroxidase-conjugated secondary step antibodies (Jackson ImmunoResearch Labs., West Grove, PA) diluted 1:2,500. After another washing step with PBS, the plates were developed with o-phenylenediamine and hydrogen peroxide. The reaction was terminated with hydrochloric acid and the optical densities (ODs) were read at 492 nm. All samples were tested in duplicate. The specific OD was calculated by subtracting the blank-well OD from the coated-well OD. A serum was considered positive if the specific OD was >0.2 for IgG and >0.3 for IgM antibodies. The reciprocal of the highest dilution that still resulted in a specific OD larger than the cut-off value was taken to be the titer.

To demonstrate cross-reactivity between CMV-antigens and GM2, serum samples containing anti-ganglioside antibodies were incubated with virus infected fibroblasts and uninfected fibroblasts. Serum samples with increasing dilutions from 1:100 up to 1:800 were incubated for five hours at 4°C with $2.0x10^3$ to $2.0x10^6$ infected or uninfected fibroblasts in a total volume of 600 µL PBS-BSA. Serum samples to which only PBS had been added were also taken as a control. After incubation, samples were centrifuged for 5 minutes at 10.000g

and supernatants were tested for anti-ganglioside reactivity using the ELISA assay described above. Percentage inhibition was calculated as follows: {specific OD(incubated with uninfected fibroblasts)-specific OD(incubated with infected fibroblasts)/specific OD(incubated with uninfected fibroblasts)} x 100%. Experiments were repeated at least three times for any combination of patient, serum dilution, amount of viral antigen, and viral strain.

To exclude that binding of CMV-antigens to gangliosides was responsible for an inhibitory effect, GM2-coated ELISA plates were incubated for 4 hours at 4°C with 100 µL of $2.0x10^3$ to $2.0x10^6$ infected or uninfected fibroblasts, washed with PBS, incubated with GM2-positive serum samples and developed as described above.

To study cross-reactivity between GM1 and GM2, diluted serum samples were incubated with gangliosides conjugated to Octyl-Sepharose CL4B beads (Hirabayashi *et al.*, 1983)(Pharmacia, Uppsala, Sweden) and processed in a similar way as in experiments with virus-infected fibroblasts. Serum incubated with unconjugated beads was taken as a control.

**Statistical methods**

Differences in inhibition percentages between patients were tested in a linear regression model using indicator variables coded in an appropriate way in relation to the hypotheses. Differences in inhibition percentages between strains were tested with a one-way analysis of variance with multiple comparisons based on Bonferroni's method. A p-value of $<0.05$ was considered to be significant.

## RESULTS

To assess the fine-specificity of the anti-glycolipid serum antibodies in the five patients, we tested them on a small panel of glycolipids. One of the three GBS patients with CMV infection and IgM anti-GM2 reactivity had additional reactivity to the other glycolipids tested. However, none of these three patients displayed reactivity to GQ1b (Table). One of the two control patients with anti-GQ1b antibodies had an antecedent *C. jejuni* infection and IgM anti-GQ1b and GA1 reactivity, but the serum of this patient did not display any reactivity with GM2 (Table). In all patients, reactivity to all gangliosides decreased with clinical improvement.

To investigate whether the anti-GM2 antibodies in the GBS patients cross-reacted with CMV-antigens, we used an inhibition ELISA system. Absorption of anti-GM2 antibodies with CMV-antigens would lead to a decrease in anti-GM2 reactivity. When we incubated anti-GM2 IgM containing serum samples with increasing quantities of CMV-infected fibroblasts we found a dose-dependent inhibition of anti-GM2 reactivity. The CMV-strain that was isolated from GBS patient 1, was recognized by anti-GM2 antibodies in serum samples from patient 1 as well as in serum samples from the other two GBS patients with an antecedent CMV infection (Figure 1A). In addition, fibroblasts infected with reference CMV-strain AD169 also had an inhibitory effect on anti-GM2 reactivity (Figure 1A). This inhibitory

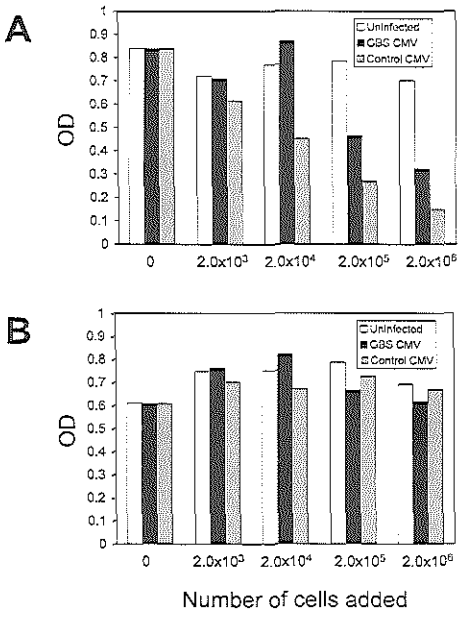


**Figure 1.** Dose dependent inhibition of anti-GM2 but not of anti-GQ1b reactivity following incubation by cytomegalovirus (CMV) infected fibroblasts. Anti-ganglioside reactivity following incubation with increasing numbers of fibroblasts infected with CMV-strain 96-3286 (isolated from GBS patient 1, solid bars), reference strain AD169 (striped bars) but not following incubation with uninfected fibroblasts (open bars). **A**: Anti-GM2 reactivity in serum from patient 2 is dose-dependently decreased with both CMV strains but not with uninfected cells; **B**: Anti-GQ1b reactivity in patient 4 is unaffected. Serum dilution 1:100. OD, optical density.

effect was specific for virus-infected cells as incubation with uninfected fibroblasts had no effect on anti-GM2 reactivity (Figure 1A). This dose dependent inhibitory effect could be demonstrated in all three patients although we were unable to inhibit the anti-GM2 reactivity completely. The possibility that CMV blocks access of anti-GM2 antibodies in the ELISA plate was ruled out by incubating ELISA plates with CMV prior to incubation with serum. Pre-incubation of GM2-coated ELISA plates with CMV-infected fibroblasts did not affect anti-GM2 reactivity (data not shown). When we incubated serially diluted anti-GM2 positive serum samples with different quantities of uninfected and virus-infected cells we also observed inhibition of anti-GM2 reactivity with both CMV strains (Figure 2A).

CMV-strain 96-3286 that was isolated from patient 1 and control CMV strain AD169 slightly differed in their inhibitory effect but this was not significant. At a serum dilution of 1:100, the addition of $2.0x10^6$ fibroblasts infected with CMV-strain 96-3286 resulted in an inhibition of anti-GM2 reactivity ranging from 47% in patient 3 to 67% in patient 2. With an equal number of infected cells, the inhibitory effect on anti-GM2 reactivity was more pronounced with CMV-strain AD169, ranging from 63% in patient 3 to 79% in patient 2.

To exclude the possibility that inhibition of anti-GM2 reactivity after incubation with

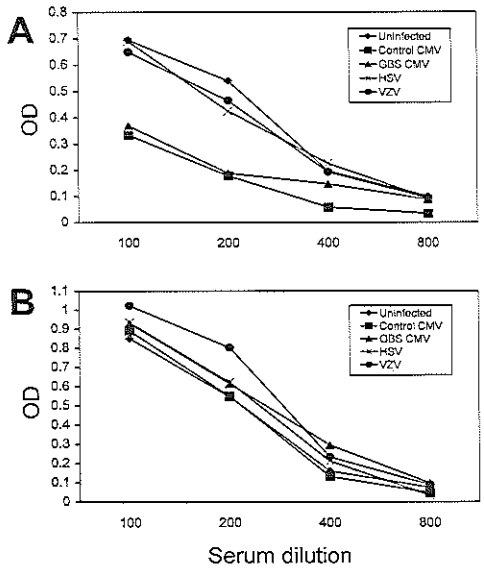


**Figure 2.** Specific inhibition of anti-GM2 reactivity by cytomegalovirus (CMV) infected fibroblasts. **A**: Anti-GM2 reactivity in patient 3 is decreased by pre-incubation with CMV 96-3286 (GBS CMV, isolated from patient 1) and CMV reference strain AD169 (control CMV) but not by uninfected cells, herpes simplex virus type 1 (HSV) or varicella-zoster virus (VZV); **B**: No effect on anti-GQ1b reactivity in patient 5. Serum dilution 1:100-1:800, $2.0x10^6$ cells were added.

CMV-infected fibroblasts was a non-specific effect of CMV-infected fibroblasts on antibody reactivity, we also included serum samples with IgM reactivity to GQ1b. Even after incubation with $2.0x10^6$ CMV-infected fibroblasts or uninfected fibroblasts, we could not detect any effect on anti-GQ1b reactivity (Figure 1B, 2B). The two CMV strains had the same specific inhibitory effect on anti-GM2 but not anti-GQ1b reactivity ($p<0.001$ for both strains).

To investigate whether the inhibitory effect on anti-GM2 reactivity was specific for fibroblasts infected with CMV, we carried out similar experiments with fibroblasts infected with other herpesviruses. Incubation of serially diluted anti-GM2 containing serum samples with $2.0x10^6$ fibroblasts infected with VZV or HSV type 1 had no or only a slight effect on anti-GM2 reactivity (Figure 2A, 3A-C). Incubation of anti-GM2 positive serum samples at a dilution of 1:100 with $2.0x10^6$ HSV type 1 infected fibroblast resulted in a mean inhibitory effect of 3% in patient 1 to 20% in patient 2 (Figure 3A,B). For patients 1 and 3, the inhibition of anti-GM2 reactivity by the two CMV strains differed significantly from the inhibition by HSV type 1 and VZV. In patient 2, both CMV strains only differed from VZV. Although not statistically signifcant, anti-GQ1b reactivity in the anti-GQ1b-positive patients was decreased after incubation with 2.0x106 HSV type1-infected fibroblasts (12-38%). There was a limited reduction of anti-GQ1b reactivity following incubation with VZV-infected fibroblasts (Figure 3 D,E).

To investigate whether both anti-GM2 and anti-GM1 antibodies in patient 1 were able

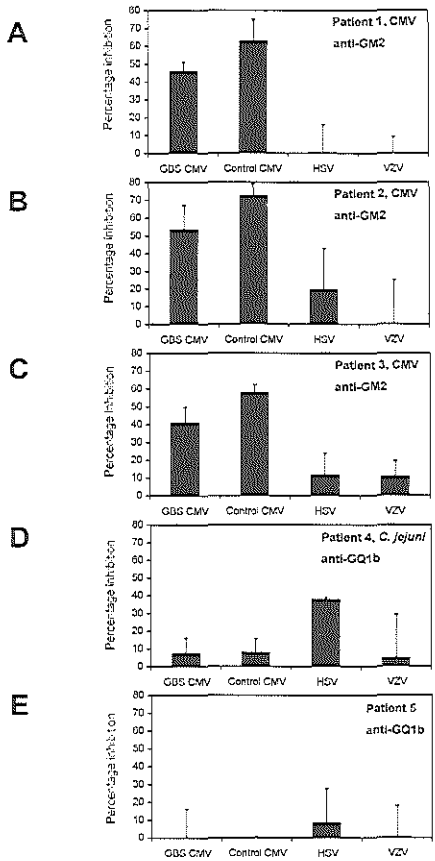


**Figure 3.** Inhibition of anti-GM2 and anti-GQ1b reactivity by preincubation with virus-infected fibroblasts. Inhibition was calculated as indicated in the text. Strains used: cytomegalovirus (CMV) 96-3286 (GBS CMV, isolated from patient 1), reference strain AD169 (control CMV), herpes simplex virus type 1 (HSV) and varicella-zoster virus (VZV). **A**: Patient 1, anti-GM2 reactivity; **B**: Patient 2, anti-GM2 reactivity; **C**: Patient 3, anti-GM2 reactivity; **D**: Patient 4, anti-GQ1b reactivity; **E**: Patient 5, anti-GQ1b reactivity. Bars represent the mean percentage inhibition of three identical experiments. Error bars are shown, p-values of statistical tests are given in the text. Serum dilution 1:100, $2.0 \times 10^6$ cells were added.

to bind to CMV-infected fibroblasts, serum from this patient was incubated with CMV-infected fibroblasts and tested for residual reactivity against GM2 and GM1. Anti-GM2 and anti-GM1 reactivity were both inhibited in serum from patient 1 as well as serum from the paraproteinaemic neuropathy patient (Figure 4A). Following incubation with Sepharose beads conjugated to gangliosides, the anti-GM2 reactivity in patient 1 could be inhibited by GM2 coated beads, but not GD1b coated beads (Figure 4B). GM1-coated beads also had an inhibitory effect on anti-GM2 reactivity, though smaller (Figure 4B). Anti-GM1 reactivity in patient 1 could be inhibited by GM1 coated beads but hardly by GM2 or GD1b coated beads

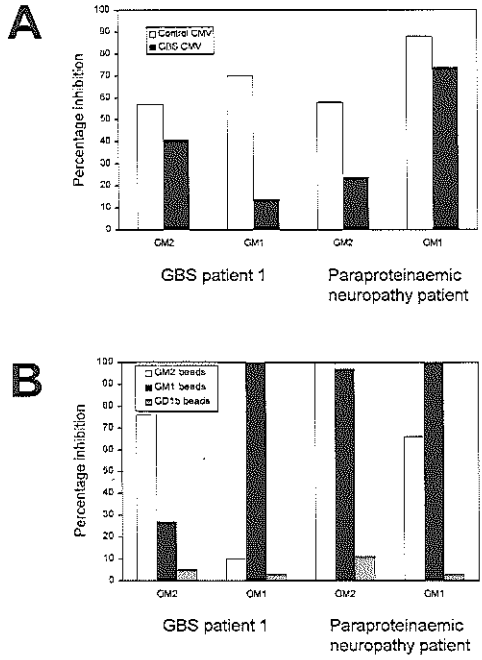


**Figure 4.** Inhibition of anti-GM2 and anti-GM1 reactivity by preincubation with virus-infected fibroblasts and ganglioside conjugated Sepharose beads. Inhibition was calculated as indicated in the text. Strains used: cytomegalovirus (CMV) 96-3286 (GBS CMV, isolated from patient 1), reference strain AD169 (control CMV). Octyl-Sepharose CL4B beads were conjugated to GM2, GM1 and GD1b. **A**: Inhibition of anti-GM1 and anti-GM2 reactivity in patient 1 and a paraproteinaemic neuropathy patient following incubation with CMV-infected fibroblasts. **B**: Differential inhibition of anti-GM2 and anti-GM1 reactivity by GM2 and GM1 beads in patient 1, similar inhibition in the paraproteinaemic neuropathy patient. No inhibition by GD1b beads in both patients.

(Figure 4B). In contrast, in the paraproteinaemic neuropathy patient, both anti-GM1 and anti-GM2 reactivity could be inhibited by GM1 and GM2 coated beads (Figure 4B).

## DISCUSSION

We have demonstrated a dose-dependent inhibition of anti-GM2 reactivity in serum from GBS patients with an antecedent CMV-infection and from a patient with a paraproteinaemic neuropathy, following incubation with CMV-infected fibroblasts. Furthermore, we have shown that this inhibition is specific for CMV-infected fibroblasts and for anti-GM2 antibodies. The decreased anti-GM2 reactivity indicates that anti-GM2 antibodies can recognize antigens expressed on fibroblasts infected with CMV.

In our CMV-infected GBS patients, we could only detect IgM anti-GM2 antibodies.(Jacobs *et al.*, 1997d) but others have found both IgM and IgG anti-GM2 antibodies (Irie

*et al.*, 1996; Khalili-Shirazi *et al.*, 1999; Yuki and Tagawa, 1998). Therefore, it can be inferred that IgG anti-GM2 antibodies will also be able to recognize CMV-infected fibroblasts.

Our results are in accordance with those published by Irie et al (Irie *et al.*, 1996) who demonstrated that incubation of one anti-GM2 containing serum sample with CMV-antigens resulted in a decrease of anti-GM2 reactivity. However, in the study of Irie et al. it is unclear whether the CMV-antigen that was used was a whole cell antigen or purified CMV (Irie *et al.*, 1996). In addition to the above-mentioned study, we investigated whether the inhibitory effect on GM2-reactivity was specific for CMV-infected fibroblasts. We detected no or only a slight decrease in anti-GM2 reactivity when anti-GM2-positive serum samples were incubated with fibroblasts infected with VZV or HSV type 1, two other members of the herpesvirus family. Fibroblasts from the same passage were used in each experiment, to rule out that differences not related to the use of different viruses would influence the outcome of the experiments. These findings indicated that the inhibitory effect on anti-GM2 reactivity was limited to CMV-infected fibroblasts.

We used both a CMV-strain that was isolated from a GBS patient with anti-GM2 antibodies and reference CMV-strain AD169. Both CMV-strains were able to inhibit anti-GM2 reactivity. As we did not test a large panel of CMV-strains, it remains unknown whether all CMV strains are able to inhibit anti-GM2 reactivity or that strain specific properties exist as is the case with ganglioside mimicry in *C. jejuni* lipopolysaccharides (Jacobs *et al.*, 1997b; Moran and O'Malley, 1995). The presence of GM2-like structures on fibroblasts infected with CMV-strains that were isolated from individuals without GBS, may explain the finding that anti-GM2 antibodies are also detected in serum from patients with a CMV-infection without neurological symptoms (Jacobs *et al.*, 1997d; Yuki and Tagawa, 1998).

CMV-infected fibroblasts did not affect anti-GQ1b reactivity. This indicates that the effect of CMV-infected fibroblasts is not mediated by the Fc-receptor and is ganglioside-specific. Remarkably, in one GQ1b-positive patient, GQ1b reactivity was decreased after incubation with HSV type 1-infected fibroblasts although this was not significant. This indicates that anti-GQ1b antibodies may recognize HSV type 1-infected fibroblasts and anti-GQ1b antibodies may be induced by HSV type 1. However, patient 4 did not have serological evidence for a recent infection with HSV type 1 (not shown). In the other GQ1b-positive patient, GQ1b reactivity following incubation with HSV type 1-infected fibroblasts was only slightly affected, indicating different fine-specificity of the anti-GQ1b antibodies in the two patients.

The GBS patient from whom CMV-strain 96-3286 was isolated did not only have antibody reactivity to GM2 but also to GM1. Although anti-GM1 antibodies are infrequently found in CMV-associated GBS, anti-ganglioside antibodies that react with other gangliosides, e.g. GalNAc-GD1a, have been described (Tsukaguchi *et al.*, 1998). In the patient described in this study, the anti-GM1 reactivity decreased in parallel with the anti-GM2 reactivity following incubation with CMV infected fibroblasts. Differential inhibition patterns were observed when this serum was incubated with gangliosides conjugated to Octyl-Sepharose-CL4B beads which indicates that this serum contains different populations of antibodies that

react with GM2 and GM1, but both anti-GM2 and anti-GM1 antibodies can recognize CMV-infected fibroblasts.

There are several mechanisms by which anti-GM2 antibodies can be induced in GBS patients following CMV infection. CMV is known to be able to polyclonally activate B cells (Hutt-Fletcher *et al.*, 1983). However, in the three CM-associated GBS patients that were described in the present study, the reactivity to glycolipids is restricted to a limited number. This points in the direction that anti-GM2 antibodies in CM-infected patients are induced by an antigen-driven mechanism although polyclonal B cell activation can not completely be ruled out. Alternatively, the anti-GM2 antibodies may have been induced by a GM2-like epitope that is expressed on the infected fibroblasts. Several mechanisms may be responsible for the expression of an immunogenic GM2-like epitope in CMV-infected GBS patients. The first possibility is that the CMV virion itself has a GM2-like epitope, e.g. a glycoprotein bearing a GM2-like structure or GM2 itself as part of the the virion membrane (Evans and Webb, 1986). Furthermore, viral infection of fibroblasts may lead to an altered ganglioside pattern of cells (Bai *et al.*, 1992). Fetal brain cells infected with SV-40 virus contained elevated levels of GM2 (Hoffman *et al.*, 1991). It is possible that infection of human fibroblasts with CMV leads to upregulation of GM2 in the cell membrane which makes GM2 immunogenic. Another possibility is that CMV-infection of a cell leads to the exposure of cryptic antigens (Di Rosa and Barnaba, 1998). The accesibility of antibodies that recognize gangliosides depends on the adjacent structures, and the relative amount of gangliosides in the cell membrane (Lloyd *et al.*, 1992). CMV-infection may be able to induce an altered ganglioside composition of the cell-membrane, rendering GM2 immunogenic, even without changing the absolute amount of GM2 in infected fibroblasts.

Our results indicate that in CMV-infected GBS patients, anti-ganglioside antibodies can be induced through molecular mimicry.

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# Chapter 9

# MOLECULAR CHARACTERIZATION OF *CAMPYLOBACTER JEJUNI* FROM PATIENTS WITH GUILLAIN-BARRÉ AND MILLER FISHER SYNDROMES

**HP Endtz[1], CW Ang[2], N van den Braak[1] , B Duim[3], A Rigter[3] , LJ Price[4], DL Woodward[4], FG Rodgers[4], WM Johnson[4], JA Wagenaar[3], BC Jacobs[2], HA Verbrugh[1] and A van Belkum[1]**

Departments of Medical Microbiology & Infectious Diseases[1], Neurology and Immunology[2], Erasmus University Rotterdam, The Netherlands
Department of Bacteriology[3], DLO-Institute for Animal Science and Health, Lelystad, The Netherlands
National Laboratory for Enteric Pathogens[4], Canadian Science Centre for Human and Animal Health, LCDC, Winnipeg, Canada

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## ABSTRACT

*Campylobacter jejuni* has been identified as the predominant cause of antecedent infection in Guillain-Barré Syndrome (GBS) and Miller Fisher Syndrome (MFS). The risk of developing GBS/MFS may be higher after infection with specific *C. jejuni* types. To investigate the putative clonality, 18 GBS/MFS-related *C. jejuni* from The Netherlands and Belgium and 17 control strains were analyzed by serotyping (Penner and Lior), restriction fragment length polymorphism (RFLP) analysis of PCR products of the flaA gene, amplified fragment length polymorphism analysis (AFLP) analysis, pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) analysis. Serotyping revealed 10 different O serotypes and 7 different Lior serotypes, thereby indicating a lack of serotype clustering. Two new O serotypes, O:35 and O:13/65, not previously associated with GBS/MFS were found. Serotype O:19 was encountered only in 2/18 strains and none was of serotype O:41. The results of all genotypic methods also demonstrated substantial heterogeneity. No clustering of GBS- or MFS-related strains occurred and no molecular marker capable of separating pathogenic GBS/MFS from non-GBS/MFS-related enteritis strains could be identified in this study. Sialic acid containing lipopolysaccharides (LPS) are thought to be involved in the triggering of GBS/MFS through molecular mimicry with gangliosides in human peripheral nerves. Therefore, further characterization of GBS/MFS-related *C. jejuni* should target to the genes involved in the synthesis of LPS and the incorporation of sialic acid.


## INTRODUCTION

The Guillain-Barré syndrome (GBS) is the most frequent form of acute inflammatory polyneuropathy. The Miller Fisher syndrome (MFS) is considered as a rare variant of GBS. GBS/MFS patients demonstrate a heterogenous clinical presentation and outcome (Van der Meché and Van Doorn, 1995). *C. jejuni* infections in GBS are associated with the presence of antibodies to GM1 and other peripheral nerve gangliosides (Jacobs *et al.*, 1998; Jacobs *et al.*, 1996b). These antibodies presumably are induced by the infectious agent since *C. jejuni* lipopolysaccharides (LPS) from GBS and MFS patients show molecular mimicry with several gangliosides (Moran, 1997).

*C. jejuni* is the most frequent cause of bacterial diarrhea. Approximately 1 in every 1000 *C. jejuni* infections will be followed by GBS (Mishu Allos, 1997). Several authors have hypothesized that GBS-related *C. jejuni* strains share specific features by which they induce antibodies crossreactive with peripheral nerve tissue. GBS-related *C. jejuni* have been reported to be associated with the specific Penner serotypes O:19 and O:41 and these appeared to be clonally related (Fujimoto *et al.*, 1997; Lastovica *et al.*, 1997; Nishimura *et al.*, 1997). The risk of developing GBS may be higher after infection with serotype O:19 (Mishu Allos, 1997). Unfortunately, the time between the preceding intestinal infection and the onset of

GBS often exceeds the duration of excretion of viable *C. jejuni* in stools. Thus, the number of GBS-related *C. jejuni* isolates available for further study is limited.

The aim of the study was to investigate the genetic variation among these strains using serotyping and various genotyping methods, including a flagellin typing method that determines polymorphisms in the flaA gene (Ayling *et al.*, 1996); amplified fragment length polymorphism (AFLP) analysis , that has recently been adapted for genotyping of *Campylobacter* spp. (Duim *et al.*, 1999); pulsed-field gel electrophoresis (PFGE); and randomly amplified polymorphic DNA (RAPD) analysis (Ayling *et al.*, 1996; Duim *et al.*, 1999; Endtz *et al.*, 1997; Hilton *et al.*, 1997).

## MATERIALS AND METHODS

### Bacterial strains

In order to maximize the number of isolates, we prospectively cultured stools specimens of patients presenting with GBS or MFS, starting in 1994 and using a variety of sensitive and selective culture techniques, including broth enrichment and mechanical filtration. We collected 18 GBS or MFS-related *C. jejuni* strains from patients in The Netherlands and Belgium. The 18 clinical *C. jejuni* strains analyzed in this study were isolated in the acute phase of the disease, from the stools of 17 Dutch patients and one Belgian patient between 1991 and 1998. All patients had a history of diarrhea prior to onset of GBS/MFS and/or anti-*Campylobacter* antibodies, suggestive of a recent infection (Ang *et al.*, 2000d). Four *C. jejuni* isolates were isolated from patients with MFS and 12 from patients with GBS. Two isolates came from the diarheal stools of two family members of a GBS patient, who remained culture- negative throughout, but showed a serological response highly suggestive of a recent *Campylobacter* infection (Ang *et al.*, 2000d). In addition, 9 *C. jejuni* isolates from unrelated enteritis patients, without neurological symptoms, and 8 reference *C. jejuni* O serotypes were included. All GBS patients fulfilled the diagnostic criteria (Asbury and Cornblath, 1990). All MFS patients suffered from ophthalmoplegia, ataxia and areflexia (Miller Fisher, 1956).

### Serotyping

All strains were serotyped with the heat-stable (HS or O) and heat-labile (HL) serotyping schemes of Penner and Lior, respectively. The serotyping was performed at the National Laboratory for Enteric Pathogens, Canadian Science Centre for Human and Animal Health, Winnipeg, Canada, as described previously (Lior *et al.*, 1982; Penner *et al.*, 1983).

### Bacterial DNA isolation

Chromosomal DNA was isolated with the Wizard Genomic DNA purification kit according to the manufacturer's instructions (Promega, Madison, WI).

**PFGE**

PFGE was performed as previously described (Van Belkum *et al.*, 1997). In short, samples of genomic DNA extracted from overnight cultures of the strains were digested with SmaI (Boehringer Mannheim, Germany). Electrophoresis was performed in 1% SeaKam agarose in 0.5x TBE using a BioRad CHEF mapper, programed in the auto-algorithm mode (run time 19 h, switch time 6.75-25 sec). Gels were stained with ethidium bromide for 15 min, destained in distilled water for 1h, and photographed under UV radiation. The gels were inspected visually by two different investigators. The patterns were interpreted according to the criteria decribed by Tenover et al. (Tenover *et al.*, 1995). Isolates that differed by 1-3 bands, consistent with one single differentiating genetic event, were assigned the same capital letter, but with a numbered subtype. Four or more band-differences between two strains were defined as distinct genotypes and were designated with different capital letters.

**RAPD**

RAPD was done in volumes of 50 µL containing 200 µM of each deoxyribonucleotide triphosphate, 50 ng of template DNA, 2.5 U Taq DNA polymerase (Promega, Southhampton, UK) 1.5 mM of $MgCl_2$ and 5 µL of reaction buffer (Promega). The primers used were the enterobacterial repetitive intergenic consensus sequences ERIC-1(5'-ATGTAAGCTC-CTGGGGATTCAC-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGTGAGCG-3' (Nachamkin *et al.*, 1998). Amplification was performed in a DNA thermal cycler (Perkin-Elmer 9600, Norwalk, CT). The PCR program consisted of 4 min at 94°C, followed by 40 cycles of 45 sec at 94°C, 45 sec at 25°C and 1 min at 74°C. The PCR products were analyzed by electrophoresis in 1% agarose gels. After staining with ethidium bromide and destaining, photographs were made using UV transillumination. Banding patterns were analyzed visually by two independent examiners and the profiles were designated by a different capital letter whenever a single band difference was observed.

**AFLP**

AFLP has recently been adapted for genotyping of *Campylobacter* spp., and generated fingerprints with 50 - 80 bands with sizes ranging from 50 to 500 bp. The AFLP reactions were performed as described previously (Duim *et al.*, 1999). The restriction enzymes used were HindIII and HhaI. For DNA amplification the HhaI primer (5'-GATGAGTCCT-GATCGCA-3'), and the fluorescently labelled HindIII primer (5'-GACTGCGTACCAGCT-TA-3') were used. For selective PCR amplification both primers contained a single additional A nucleotide at their 3' ends. AFLP fingerprints were analyzed on a 373A ABI DNA sequencer, followed by numerical analysis of patterns. Strains with similarity levels of >90% were defined as genetically related and assigned with the same capital letter (Duim *et al.*, 1999). Strains that belonged to the same cluster with > 80% similarity were defined as genetic subtypes and assigned with the same capital letter, but with a numbered subtype. Distinct AFLP fingerprints that showed <80% of similarity were designated with different capital letters.

### FlaA PCR/RFLP

For DNA amplification the flaA-primer (5'-CGTATTAACACAAATGTTGCAGC-3') and flaR-primer (5'-GATTTGTTATAGCAGTTTCTGCTATATCC-3') as described by Ayling et al. were used (Ayling *et al.*, 1996). Reaction mixtures consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.01 % w/v gelatin, 2 mM $MgCl_2$, 0.2 µM dNTP's, 50 pmol of each primer, 50 pg genomic DNA and 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer, Gouda, The Netherlands), with a total reaction volume of 50 µl. Reaction conditions were 60 s 94°C followed by 45 cycles of 45 s 94°C, 45 s 55°C, 2 min 72°C, and a 5 min extension at 72°C. After verification of the PCR reaction, 12.5 µl of the amplicon was digested for two hours at 37°C using 10U of DdeI (Boehringer Mannheim, Germany) in a total volume of 15 µl. Restriction fragments were separated on a 2 % (w/v) NuSieve (FMC, Rockland, MD) and 0.5 % (w/v) MP (Boehringer Mannheim, Germany) ethidium bromide stained agarose gel in 1 x TAE. Gels were electrophoresed for 4 hours at 80V, digitalized and saved as TIFF files. Distinct flaA fingerprints, showing a single band difference were designated with different capital letters.

### Data processing

Levels of similarities between banding patterns were determined with the GelCompar v4.1 software (Applied Maths, Kortrijk, Belgium). For analysis of AFLP fingerprints the Pearson product-moment correlation coefficient was used. The flaA, PFGE and RAPD banding patters were analyzed with the Dice band-based coefficient. Cluster analysis was performed with the UPGMA method (Vauterin and Vauterin, 1992).

## RESULTS

### Serotyping

Serotyping of 18 GBS/MFS-related strains revealed 10 different O serotypes and 7 different HL serotypes (Table 1). *C. jejuni* O:19, Lior 77 was encountered in 2/18 (11%) patients. *C. jejuni* O:2 was found in one GBS patient, in two family members of another GBS patient, and in one MFS patient. *C. jejuni* O:4/64 was encountered in one GBS and a MFS patient; the related *C. jejuni* O:4/13/64 was found in one GBS patient. *C. jejuni* O:13/65 and O:35 were two new serotypes not earlier described in association with GBS/MFS (Table 1). Four patients with MFS had *C. jejuni* of different O serotypes (Table 1). All other strains had unique O-serotypes.

### FlaA PCR/RFLP

flaA PCR/RFLP analysis of 18 GBS/MFS-associated *C. jejuni* strains identified 12 distinct patterns (Table 1, Fig.1). Analysis of GB13 (O:2) and GB14 (O:2), both strains isolated from family members of a GBS patient, produced flaA patterns indistinguishable from GB17 (O:4/13/64) and MF 6 (O:4/64). The two O:19 strains (GB3 and GB18) showed iden-

**Table 1. Survey of serotyping and genotyping data for *Campylobacter jejuni* strains associated with Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS).**

| Strain code | O-serotype | HL-serotype | PFGE | RAPD | | flaA | AFLP |
|---|---|---|---|---|---|---|---|
| | | | | ERIC-1 | ERIC-2 | | |
| GBS/MFS | | | | | | | |
| GB1 | 1 | UT | A | A | A | A | A |
| GB2 | UT | 36 | B | B | B | B | B |
| GB3 | 19 | 77 | C | A | C | B | B |
| GB4 | 37 | 28 | D | C | D | C | C |
| GB5 | 4/64 | 1 | E | D | E | D | D |
| MF6 | 4/64 | 1 | E1 | E | E1 | E | E |
| MF7 | 35 | UI | F | F | F | F | F |
| MF8 | 23/36 | 5 | G | G | G | G | G |
| GB11 | 2 | 4 | H | H | E1 | H | H1 |
| GB13 | 2 | 4 | H1 | H1 | H | E | H |
| GB14 | 2 | 4 | H1 | H1 | E1 | E | H |
| GB15 | 5/34 | UT | I | I | I | I | I |
| GB16 | 13/65 | UT | J | J | J | B | J |
| GB17 | 4/13/64 | UT | K | K | E | E | E |
| GB18 | 19 | 77 | L | L | G | B | B |
| GB19 | 4/50 | 7 | M | M | K | J | J |
| MF20 | 2 | 4 | N | N | L | K | K |
| GB21 | 13/65 | 7 | O | O | M | L | L |
| Serostrains | | | | | | | |
| CCUG 10935 | 1 | - | P | P | N | D | A1 |
| CCUG 10936 | 2 | - | Q | Q | O | E | H2 |
| CCUG 10938 | 4 | - | R | R | O | M | M |
| CCUG 10950 | 19 | - | L1 | S | C | N | B |
| CCUG 10954 | 23 | - | S | T | P | D | N |
| CCUG 10965 | 35 | - | S1 | U | Q | O | O |
| CCUG 10966 | 36 | - | T | V | R | G | G |
| CCUG 24868A | 64 | - | U | W | S | P | P |
| Enteritis | | | | | | | |
| 98-623 | - | - | V | X | T | G | Q |
| 98-624 | - | - | W | Y | U | Q | R |
| 98-652 | - | - | X | Z | V | R | K |
| 98-682 | - | - | Y | AA | W | S | A |
| 98-706 | - | - | Z | A1 | X | I | I |
| 98-1033 | - | - | AA | BB | Y | T | S |
| 98-1039 | - | - | BB | CC | Z | U | T |
| 98-1040 | - | - | CC | DD | AA | V | U |
| 98-1087 | - | - | DD | EE | BB | T | V |

UT = untypable.

tical flaA patterns that were shared with other strains GB2 (O:UI) and GB16 (O:13/65), but not with the O:19 reference strain. Other GBS strains had flaA patterns that were highly related to flaA patterns of enteritis-related strains and reference serotype strains (Table 1, Fig. 1). The heterogeneity of flaA patterns of the GBS/MFS strains was comparable to that of the

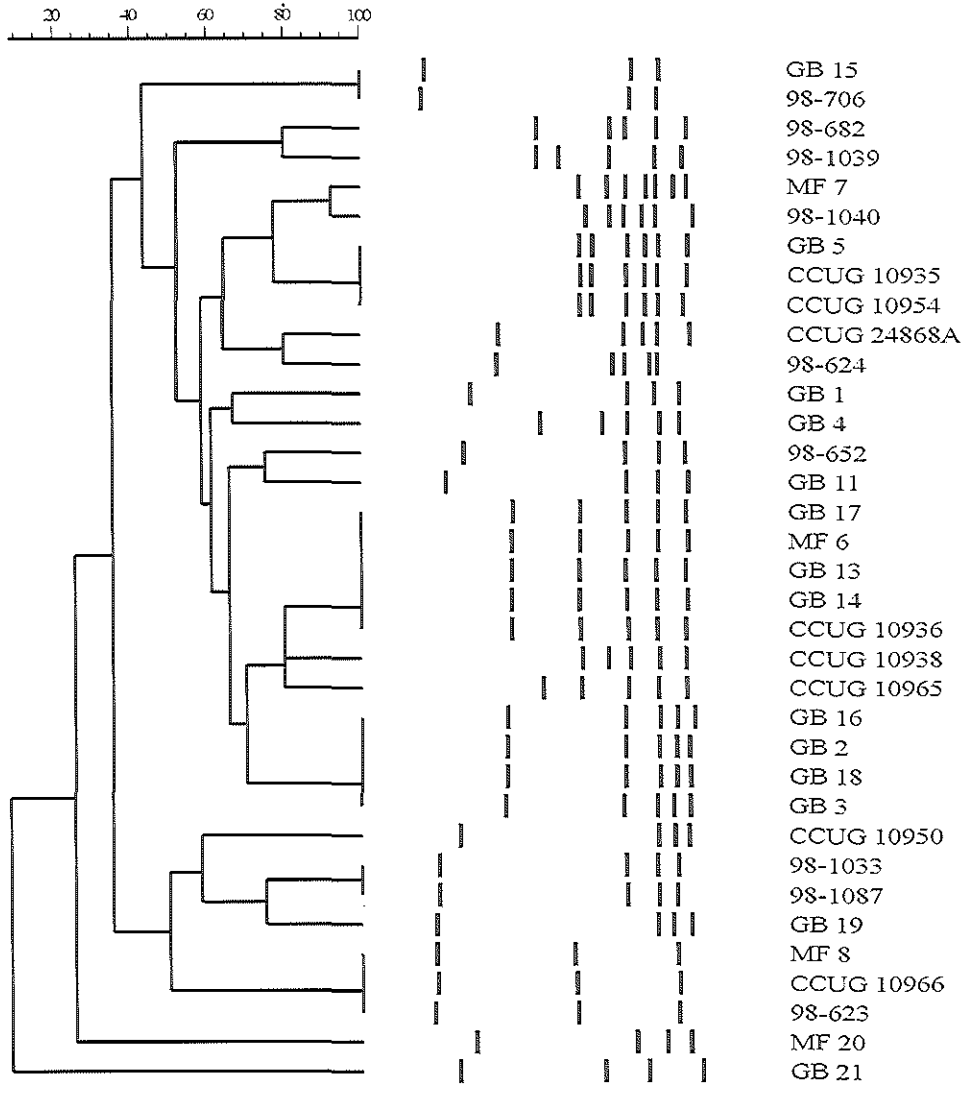


**Figure 1.** FlaA PCR-RFLP patterns of 18 GBS- or MFS-related *Campylobacter jejuni* strains, 9 enteritis control strains, and 8 Penner O serostrains. The dendrogram was constructed with band-based analysis and unweighted pair group method with averages clustering. The sizes of standard DNA fragments (in kilobase pairs) are indicated below.

enteritis and Penner reference strains. With cluster analysis, no specific flagellin type was present among the GBS/MFS-related *Campylobacter* strains tested (Fig 1).

**AFLP**

AFLP detected 12 distinct fingerprints within the 18 GBS/MFS *C. jejuni*. Two small clusters of strains were found. (Table 1). The O:19 GBS strains (GB3 and GB18) and O:19 serostrain showed highly related AFLP fingerprints. A high level of homology was also observed when comparing AFLP fingerprints of GB13, GB14, GB16, GB 19 and the O:2 serostrain. Although strain GB11 showed some band differences compared to the patterns of GB13 and GB14, a genetic relationship between these strains was evident. Two GBS strains, GB1 and GB15, and strain MF20 shared AFLP fingerprints with some of the enteritis strains (Table 1). Cluster analysis showed no separate clustering of GBS and MFS-related strains. Within each cluster of AFLP fingerprints, strains from GBS and MFS patients as well as reference serotype strains and strains from enteritis patients were present.

**RAPD and PFGE**

The PFGE analysis of 18 GBS/MF-related *C. jejuni* revealed the presence of 15 distinct types (Table 1). The two *C. jejuni* O:2, Lior 4, (GB13 and GB14) isolated from family members of a GBS patient, were indistinguishable from each other and were related to GB11. However, these strains were unrelated to MF20, a strain with the same O and HL serotype. *C. jejuni* GB5 isolated from a GBS patient appeared subclonally related to *C. jejuni* MF6, a strain obtained from a patient with MFS. On computer-aided analysis no clustering of GBS- or MFS-related strains was found. With the RAPD analysis of the 18 GBS/MF strains 15 (ERIC1) and 13 (ERIC2) distinct types were obtained (Table 1). No clustering of GBS/MFS strains was found on computer analysis.

## DISCUSSION

This study illustrates the substantial heterogeneity of *C. jejuni* strains associated with GBS/MFS in a restricted geographical area of the world. Of the GBS/MFS-related strains in our study 2/18 (11%) were of serotype O:19 and none were of serotype O:41. The most frequently observed serotype was O:2. Strains reacting with one or more of the antisera O:4, O:13, O:50, O:64 and O:65 are often related and classified as O:4-complex. *C. jejuni* O:4-complex was observed in five GBS patients and in one MFS patient. The O:2 serotype was found in two GBS/MFS-related strains and in two strains from family members of a GBS patient. *C. jejuni* O:2 is the prevailing serotype in collections of strains from patients with enteritis and according to Oosterom *et al.* account for approximately 25% of the enteritis strains in The Netherlands (Oosterom *et al.*, 1985). The Penner O serotyping scheme has been used in several previous studies to characterize *C. jejuni* strains isolated from patients with GBS or MFS, and those associated with these conditions include: O:1, O:2, O:4, O:4/50, O:5, O:10, O:16, O:19, O:23, O:37, O:41, O:44, and O:64 (Nachamkin *et al.*, 1998). We report two new *C. jejuni* O serotypes, *C. jejuni* O:35 and O:13/65, that have not been described previously in association with GBS. The great variety of O serotypes that are found in the litera-

ture and in this study, confirms our previous suggestion that GBS and MFS are not exclusively associated with a specific Penner O serotype (Jacobs *et al.*, 1995). However, *C. jejuni* serotype O:19 appears to be overrepresented among strains isolated from patients with GBS or MFS from the USA and Japan. In a Japanese study (Saida *et al.*, 1997), serotype O:19 comprised 12/16 (75%) of the GBS-related *C. jejuni* isolates while in a US based study (Mishu Allos *et al.*, 1998), 2/7 (29%) were serotype O:19. In both countries this serotype is encountered in less than 3% of the *C. jejuni* strains from patients with enteritis in the absence of neurological involvement. In South Africa, 9/9 (100%) of *C. jejuni* from GBS patients were of serotype O:41, whereas this serotype was found in less than 2% of enteritis control strains (Lastovica *et al.*, 1997). In addition, some authors suggested that Penner O:19 and O:41 strains, whether isolated from patients with GBS or from enteritis patients without neurological involvement, were clonally related, thereby indicating that these strains were particularly virulent (Fujimoto *et al.*, 1997; Lastovica *et al.*, 1997; Saida *et al.*, 1997). The data presented here demonstrate that the overrepresentation of specific O serotypes, as reported by others, is a phenomenon not seen in The Netherlands. Therefore, there would appear to be much variety in the distribution of O serotypes in different geographic locations.

In addition to the more traditional phenotypic analysis, a variety of molecular typing techniques were used to unravel the genomic differences or similarities of the *C. jejuni* strains. The conclusions drawn from the results of serotyping were corroborated by the compiled data of the different molecular typing methods. In general, the analysis of the GBS/MFS-related *C. jejuni* strains demonstrated substantial genetic heterogeneity. No clustering of GBS or MFS-related strains was found, irrespective of the method used. Although small clusters of related strains were found, strains from GBS and MFS as well as enteritis patients were present in these clusters. In some cases, a remarkable correlation was found between the results of serotyping and the different molecular techniques. GB11, GB13 and GB14, all were *C. jejuni* O:2, Lior 4 and showed great homology with PFGE, RAPD and RFLP. The two *C. jejuni* O:19, Lior 77 were highly related with flaA and AFLP but had different PFGE and RAPD patterns. Thus, although the discriminatory power of the different techniques varies significantly, the clustering of some strains was comparable.

Fujimoto *et al.* recently determined the extent of genetic variation among *C. jejuni* strains including 9 strains from GBS patients (Fujimoto *et al.*, 1997). Although the strains were isolated from patients residing in countries as diverse as the US, Japan and Germany, the authors found that by flaA-RFLP and RAPD analysis, at least 5/9 GBS strains were closely related. The five strains, however, were all of serotype O:19. In addition, the data indicated that all O:19 strains, whether GBS-related or not, were clonally related. RAPD and RFLP of other O-serotypes, in contrast, were reported to be different (Fujimoto *et al.*, 1997). In a recent Japanese study, 12/16 (75%) of the GBS-related *C. jejuni* that had been serotyped, were of O serotype 19 (Saida *et al.*, 1997). FlaA PCR/RFLP patterns of the 12 strains and of enteritis-related *C. jejuni* O:19 were identical and distinguishable from other O serotypes (Nishimura *et al.*, 1997). In the present study, clonal relationships among the three O:19 strains were only observed with AFLP. The two GBS strains had identical flaA types, while

the pattern was quite distinct from the O:19 serotype strain. Although this collection of Dutch GBS/MFS-related *C. jejuni* strains is characterized by a high degree of heterogeneity, there were too few isolates from each type present in the study. Therefore, the existence of clonality among certain O-serotypes cannot be excluded.

The heterogeneity among phenotypes and genotypes in the present study may reflect the heterogeneity of clinical symptoms and anti-ganglioside antibodies in GBS patients. *C. jejuni* infections are associated with a severe pure motor variant of GBS (Visser *et al.*, 1995), but have been reported in MFS, GBS with ophthalmoparesis, and patients with isolated abducens paresis as well (Roberts *et al.*, 1987; Van der Kruijk *et al.*, 1992). The clinical heterogeneity may be related to the variety of ganglioside-like structures in LPS of GBS/MFS-related *C. jejuni*, although this was not the subject of study.

No common molecular markers separating pathogenic GBS/MFS from non-GBS/MFS-related enteritis strains were identified. Based on the present results, it would be premature at this stage to dismiss the hypothesis of the "bad bug that causes GBS". The methods used in the study may simply lack the power to detect the particular determinants of *C. jejuni* related to GBS/MFS. Several authors have provided evidence that the molecular basis of the mimicry between *C. jejuni* and sialidated gangliosides resides in the LPS fraction of *Campylobacter* (Jacobs *et al.*, 1995; Moran, 1997; Nachamkin *et al.*, 1998). Analysis of genes involved in the synthesis of LPS and the incorporation of sialic acid may turn out to be of great importance to further clarify the particularity of the *C. jejuni* strains involved in the pathogenesis of GBS and MFS. Although ganglioside-like epitopes have recently been described in a significant percentage of *C. jejuni* isolates from patients with uncomplicated enteritis (Nachamkin *et al.*, 1999b), it seems likely that additional genotypic methods may detect markers of pathogenicity in the near future (Wassenaar and Newell, 2000).

Finally, the results of serotyping and genotyping of the two strains from a family outbreak of *C. jejuni* enteritis followed by one case of GBS, demonstrate a clonal relationship of the strains and, therefore, suggest the importance of host factors in the pathogenesis of GBS (Ang *et al.*, 2000d).

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# Chapter 10

## STRUCTURE OF *CAMPYLOBACTER JEJUNI* LIPOPOLYSACCHARIDES DETERMINES ANTI-GANGLIOSIDE SPECIFICITY AND CLINICAL FEATURES OF GUILLAIN-BARRÉ AND MILLER FISHER PATIENTS

**CW Ang[1,2], JD Laman[2], HJ Willison[3], ER Wagner[3], HP Endtz[4], MA de Klerk[1,2], AP Tio-Gillen[1,2], N van den Braak[4], BC Jacobs[1,2], PA van Doorn[1]**

Departments of Neurology[1], Immunology[2] and Microbiology & Infectious Diseases[4], Erasmus University Rotterdam, The Netherlands
University Department of Neurology[3], Southern General Hospital, Glasgow, G51 4TF, Scotland

*Submitted*

## ABSTRACT

We determined the presence of ganglioside mimicry in Guillain-Barré (GBS) and Miller Fisher syndrome (MFS)-related *C. jejuni* strains and compared it with strains from patients with an uncomplicated enteritis. Furthermore, we compared the antibody response to *C. jejuni* LPS and gangliosides between neuropathy patients and controls. LPS from GBS and MFS-associated strains more frequently contained ganglioside-like epitopes compared to control strains (p=0.03). Almost all neuropathy patients showed a strong antibody response against LPS and multiple gangliosides in contrast to enteritis patients (p=0.001). Strains from GBS patients more frequently had a GM1-like epitope than strains from MFS patients. GQ1-like epitopes in LPS occurred more frequently in MFS-associated strains and was associated with anti-GQ1b antibody reactivity and the presence of oculomotor symptoms. Our results demonstrate that expression of ganglioside mimics is a risk-factor for the development of post-*Campylobacter* neuropathy. This study provides strong evidence for the hypothesis that the structure of the LPS fraction determines the anti-ganglioside specificity and clinical features.


## INTRODUCTION

*Campylobacter jejuni* infection is the most frequent triggering event in post-infectious neuropathies such as the Guillain-Barré (GBS) and Miller Fisher syndrome (MFS) (Jacobs *et al.*, 1998; Kuroki *et al.*, 1991; Rees *et al.*, 1995b; Willison and O'Hanlon, 1999). The exact pathogenesis of post-*Campylobacter* neuropathy is unknown but molecular mimicry between bacterial glycoconjugates and peripheral nerve gangliosides has been implicated (Hughes *et al.*, 1999; Yuki, 1997). Cross-reactive antibodies between *C. jejuni* lipopolysaccharides (LPS) and ganglioside GM1 have been identified in GBS patients (Jacobs *et al.*, 1997b; Yuki *et al.*, 1995b), while cross-reactive anti-GQ1b/LPS antibodies are present in the serum of more than 90% of MFS patients (Chiba *et al.*, 1992; Jacobs *et al.*, 1995; Neisser *et al.*, 1997). The specificity of this cross-reactive anti-ganglioside/LPS response is associated with different disease profiles. Anti-GM1 antibody reactivity is associated with pure motor GBS (Jacobs *et al.*, 1996b; Kuwabara *et al.*, 1998b; Rees *et al.*, 1995a) and anti-GQ1b antibody reactivity has a strong association with the manifestation of oculomotor symptoms and ataxia (Carpo *et al.*, 1998; Kusunoki *et al.*, 1999a).

Ganglioside-mimics have been identified in *C. jejuni* LPS with biochemical and serological methods (Moran, 1997). Mass spectometry revealed the presence of GM1 and GD1a-like structures in LPS from GBS patients (Prendergast *et al.*, 1998; Yuki *et al.*, 1993a) and GD3-like structures in MFS-associated LPS (Salloway *et al.*, 1996). Serological studies using ganglioside-binding toxins and monoclonal anti-ganglioside antibodies confirmed and extended these findings (Sheikh *et al.*, 1998; Yuki *et al.*, 1995a; Yuki *et al.*, 1994b). All *C.*

*jejuni* strains from GBS and MFS patients that were investigated using biochemical and/or serological methods exhibit ganglioside-mimicry but this may have been caused by selection bias. Little information is available concerning the expression of ganglioside mimics in strains from uncomplicated *Campylobacter* enteritis cases when compared to neuropathy associated strains (Nishimura *et al.*, 1997; Sheikh *et al.*, 1998). Furthermore, detailed studies on antibody responses to glycolipid antigens such as LPS and gangliosides in *Campylobacter* enteritis patients have been limited.

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

## METHODS

### Patients and strains

We collected 18 GBS or MFS-related *C. jejuni* strains from patients in The Netherlands and Belgium between 1991 and 1998 (Endtz *et al.*, 2000). Two isolates were cultured from the diarrheal stools of two family members of a GBS patient. The GBS patient remained culture negative, but showed a serological response highly suggestive of a recent *Campylobacter* infection (Ang *et al.*, 2000d). Clinical information on oculomotor symptoms and ataxia was obtained from patient records. All GBS patients fulfilled the diagnostic criteria (Asbury and Cornblath, 1990) and all MFS patients had oculomotor symptoms and ataxia in the absence of limb weakness (Miller Fisher, 1956).

23 control *C. jejuni* strains were obtained from patients with an uncomplicated *Campylobacter* enteritis without neurological symptoms. From 15 of these control patients, serum samples taken in the first week of the disease were available. All control strains were from sporadic enteritis cases and the seasonal distribution of neuropathy associated and control strains was the same with a broad summer peak.

As additional controls for serotyping of *C. jejuni* strains, we used reference strains from which the core oligosaccharides have been shown to contain a GM1 mimic (Penner O:19, CCUG#10950)(Aspinall *et al.*, 1994a), a GD3 mimic (Penner O:10, CCUG#10943)(Nam Shin *et al.*, 1998) and no ganglioside mimic (Penner O:3, CCUG#10937)(Aspinall *et al.*, 1995).

### Serology

Acute phase serum samples were tested for IgM and IgG antibody reactivity against GA1, GM1, GM2, GD1a, GD1b, GD3 and GQ1b using ELISA and confirmed by thin-layer chromatography (TLC) as described previously (Jacobs *et al.*, 1995). Antibody reactivity

against a protein extract of *C. jejuni* was determined as described before (Herbrink *et al.*, 1988). All glycolipids were obtained from Sigma (St Louis, MO), except GQ1b (Biocarb, Lund, Sweden). LPS fractions from all strains were isolated with the hot-phenol water method (Westphal and Jann, 1965) and processed as described before (Jacobs *et al.*, 1997b). All LPS fractions showed a dense band migrating at 8-15 kD following electrophoresis on a polyacrylamide gel and silver staining (Novex, San Diego, CA), indicating the presence of LPS (Aspinall *et al.*, 1994a). For the detection of anti-LPS reactivity, serum samples were tested in ELISA at a dilution of 1:100 and 1:1000 as described previously (Jacobs *et al.*, 1997a). Samples were considered positive when the corrected optical density (OD) value was >3 standard deviations (SD) of the mean OD-value of a group of 12 healthy controls without serological evidence of a recent *C. jejuni* infection.

To assess serum samples for cross-reactive antibodies between LPS and gangliosides, serum was incubated with *C. jejuni* LPS conjugated to Octyl-Sepharose CL4B beads. 250 µg LPS was added to 1 mL of Octyl-Sepharose CL4B in methanol:water (1:1, v/v) containing 0.1M KCl and mixed for 1.5 hrs. After several washes with phosphate buffered saline, pH 7.8, serum samples were incubated with LPS-Sepharose conjugates for 5 hrs at 4°C. Following centrifugation, absorbed serum samples were tested for anti-LPS and anti-glycolipid reactivity as described above. Serum samples that were incubated with beads coupled to LPS from the Penner O:3 serostrain that does not contain a ganglioside-mimic (Aspinall *et al.*, 1995) or uncoupled beads served as controls.

**Serological typing of *C. jejuni* LPS**

*GBS/MFS serum panel*

Purified LPS was tested in an ELISA-system for the presence of ganglioside-like epitopes using a panel of sera divided into four groups. The first group (GM1) consisted of 10 serum samples from GBS patients with IgG anti-GM1 reactivity. 9/10 anti-GM1 positive patients also had reactivity against GA1 and 4/10 against GD1b. The second group (GQ1b) consisted of 10 serum samples from MFS and GBS patients with IgG anti-GQ1b reactivity. The third group (other) consisted of 4 serum samples from GBS patients with anti-ganglioside reactivity other than anti-GM1 or GQ1b. This group was included to detect ganglioside mimics that can not be defined with sera from the GM1 and GQ1b group. The fourth group (HC) was formed by 12 healthy controls without serological evidence for a recent *C. jejuni* infection. An LPS was considered to have a GM1-like epitope when at least 3/10 serum samples from the GM1-group had an OD>3 SD above the mean of the healthy controls, with a minimum OD of 0.300 (to avoid false positive samples due to the low levels of anti-LPS reactivity seen in healthy control samples). The criteria for LPS bearing a GQ1b-like epitope were the same, using the GQ1b-panel of sera. An LPS was considered to have another ganglioside-like epitope, which could not be designated GM1-like or GQ1b-like, when the LPS showed reactivity with only one or two serum samples in either the "GM1" or "GQ1b" group or reactivity with one or more serum samples from the "other" group.

*Toxins, lectin and monoclonal antibodies*

Specificity of binding of cholera toxin B subunit (CT), tetanus toxin fragment C (TT), peanut agglutinin (PNA) and monoclonal anti-ganglioside antibodies (mAbs) Sm1 (Willison *et al.*, 1994), Wo1 (Paterson *et al.*, 1995), Ha1rbc (Willison *et al.*, 1996), CGM3 (Goodyear *et al.*, 1999) and EG1 (Goodyear *et al.*, 1999) was validated with binding of these ligands to GA1, GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b and GQ1b. 96-wells plates (Nunc Immunosorb, Roskilde, Denmark) were coated with glycolipids in ethanol and non-specific binding was blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (PBS-BSA) as described previously. Uncoated wells served as controls. Peroxidase conjugated CT (List Biologicals, Campbell, CA and Sigma), biotin conjugated CT (List Biologicals), peroxidase conjugated TT (List Biologicals), peroxidase conjugated PNA (Sigma), or the mAbs diluted in PBS-BSA were added for 1 hour at room temperature (toxins) or overnight at 4°C (mAbs). After washing with PBS, plates containing peroxidase conjugated ligands were developed with o-phenyldiamine. Biotin-conjugated CT was incubated with peroxidase conugated avidin (ABC kit, Vector, Burlingame, CA) for 1 hour at room temperature, followed by washing and developing. Plates containing mAbs were incubated with peroxidase conjugated anti-human or anti-mouse antibodies and washed with PBS before developing. Substrate reactions were stopped by the addition of 2N HCl. Plates were read at 492 nm and the ODs were corrected by subtracting ODs from uncoated wells. Specificity of binding to purified glycolipids was confirmed with TLC overlay (Jacobs *et al.*, 1996b).

Binding of the same ligands to LPS was investigated with a similar method, using LPS coated 96-wells plates (1 μg/well). Dilutions of ligands were 4,2 μg/mL for peroxidase and biotin conjugated CT, 50 μg/mL for PNA, 7.5 μg/mL for TT and 10 μg/mL for the mAbs, except Wo1 which was used at a dilution of 1 μg/mL.

**Statistical analysis**

Differences in proportions were tested with the chi-square or Fisher exact test. Binding of toxins, lectin and mAbs was evaluated with the Mann Whitney U test. A p-value $<0.05$ was considered to be significant.

## RESULTS

**Patient characteristics**

Fourteen of the GBS and MFS patients had suffered a period of diarrhea in the weeks before the development of neurological symptoms, 4 patients did not report a gastro-intestinal illness. Two GBS patients had oculomotor symptoms in addition to their limb weakness and can be considered as overlap cases between GBS and MFS. All control enteritis patients had suffered from diarrhea. From most of the patients, there was no information concerning the duration of the diarrhea but based on a limited number of patients, there were no differences between the neuropathy and the control enteritis patients.

**Patient serology**

To confirm that all GBS and MFS patients indeed had experienced a recent infection with *Campylobacter*, we determined serum antibody reactivity against *Campylobacter* protein antigens. All but two neuropathy patients had IgA and/or IgM antibodies against a protein extract of *C. jejuni*, indicative of a recent infection (Herbrink *et al.*, 1988). One of the remaining two patients had high titer IgG anti-*Campylobacter* protein antibodies and a history of diarrhea. All but one of the GBS and all MFS patients had IgM and/or IgG antibody reactivity against purified *C. jejuni* LPS (Table 1). Most serum samples contained reactivity not only against LPS from their autologous strain but also from other *Campylobacter* strains.

13 out of 16 neuropathy patients showed antibody reactivity to a variety of peripheral nerve glycolipids (Table 2). All MFS patients had anti-GQ1b reactivity although the titers were low in some cases. Two GBS patients displayed no reactivity against the purified glycolipids, although they both had IgG anti-LPS reactivity. Depletion experiments with LPS conjugated to Sepharose beads demonstrated that the anti-glycolipid and anti-LPS antibodies were cross-reactive, not only in patients with anti-GM1 and anti-GQ1b antibodies (Jacobs *et al.*, 1997b), but also in patients with anti-GD1a and high titer anti-GA1 antibodies (data not shown).

In the enteritis group, only one patient had IgG anti-GM1 antibodies and this titer was very low at 100. Two other patients had anti-GA1 IgG antibodies. IgM antibody reactivity against GM1 was only present in one patient and against GM2 in another patient but also in these two patients the antibody titers were low. Anti-GA1 IgM antibodies were present in four patients. Antibody reactivity to gangliosides with two or more sialic acids could not be detected in any of the enteritis patients. Three enteritis patient had anti-LPS antibodies and one of them also had antibody reactivity against GA1. Antibody reactivity against any of the panel of purified neural glycolipids or *C. jejuni* LPS was more frequent in neuropathy patients (15/16) than in control enteritis patients (6/15; p=0.001).

**Serological typing of *Campylobacter* strains with GBS/MFS patient sera**

Penner serotyping of GBS and MFS associated *C. jejuni* strains identified a heterogeneous group of 10 different serotypes including O:19 as reported before (Endtz *et al.*, 2000). To detect ganglioside mimics, purified LPS fractions were typed using GBS and MFS patient sera containing anti-ganglioside reactivity. Examples of LPS reactivity with anti-ganglioside antibody containing serum samples are given in Figure 1. The O:19 serostrain reacted with serum samples from the GM1 group (Figure 1 D), the O:10 strains reacted predominantly with GQ1b-positive sera (Figure 1 E), whereas the O:3 serostrain, which does not contain a GM1 or GQ1b-like structure, did not react with any serum sample with anti-ganglioside reactivity (Figure 1 F). The anti-ganglioside serum panel reacted with all but one LPS from GBS and MFS patients (Table 2), indicating the presence of ganglioside mimics in almost all neuropathy associated strains. Remarkably, the LPS that did not react with the serum samples was derived from the only GBS patient (GB15) without anti-LPS reactivity. LPS from most

**Table 1. Anti-glycolipid titers and anti-LPS reactivity in Guillain-Barré and Miller Fisher syndrome patients.**

| | | IgG | | | | | | | | IgM | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Patient | GA1 | GM1 | GM2 | GD1a | GD1b | GD3 | GQ1b | LPS | GA1 | GM1 | GM2 | GD1a | GD1b | GD3 | GQ1b | LPS |
| Guillain-Barré | GB1 | 1600 | 800 | - | - | - | - | - | + | 1600 | 400 | - | - | 100 | - | - | + |
| | GB2 | nt[a] | nt | nt | nt | nt | nt | nt | nt | nt | nt | nt | nt | nt | nt | nt | nt |
| | GB3 | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | GB4 | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | GB5 | 400 | - | 100 | - | - | - | - | + | 400 | - | - | - | - | - | - | + |
| | GB11 | 200 | 800 | - | - | 3200 | - | - | + | - | - | - | - | - | - | - | + |
| | GB13/14 | 1600 | 400 | - | - | - | - | - | + | 800 | 200 | - | - | 200 | - | - | + |
| | GB15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | GB17 | 25600 | 100 | - | - | - | 100 | - | + | 200 | - | - | - | - | - | - | + |
| | GB18 | 3200 | 12800 | - | - | 1600 | - | - | + | - | 200 | - | - | - | - | - | + |
| | GB21 | 800 | 100 | - | 6400 | - | - | - | + | - | - | - | - | - | - | - | + |
| Guillain-Barré with oculomotor symptoms | GB16 | 200 | - | - | - | - | 3200 | >102,400 | + | 100 | - | - | - | - | - | - | + |
| | GB19 | - | - | - | - | - | 100 | >12,800 | + | 100 | - | - | - | - | - | - | + |
| Miller Fisher | MF6 | - | - | - | - | 100 | 200 | 1600 | + | - | - | - | - | - | - | - | - |
| | MF7 | - | - | - | - | - | - | 400 | + | - | - | - | - | - | - | 200 | + |
| | MF8 | - | - | - | - | 200 | 200 | 800 | + | - | - | - | - | 200 | - | - | + |
| | MF20 | 1600 | - | - | - | - | - | 100 | - | 400 | - | - | - | - | - | - | + |

a not tested.
+, titer >100.
-, titer <100.

**Table 2. Ganglioside-epitopes in *Campylobacter jejuni* strains from Guillain-Barré syndrome (GBS), Miller Fisher syndrome (MFS) and uncomplicated enteritis**

| Ganglioside-epitope | GBS (n=13) | MFS (n=4) | Enteritis (n=23) | p-value (chi-square) GBS/MFS vs Enteritis | p-value (Fischer exact) GBS vs MFS |
|---|---|---|---|---|---|
| GM1-like | 11 (85%) | 2 (50%) | 13 (57%) | n.s.[a] | n.s. |
| GQ1b-like | 2 (15%) | 4 (100%) | 2 (9%) | 0.04 | 0.006 |
| Other ganglioside-like | 8 (62%) | 4 (100%) | 12 (52%) | n.s. | n.s. |
| No ganglioside | 1 (8%) | 0 | 8 (35%) | 0.03 | n.s. |

a not significant.

strains reacted with serum samples from more than one group, suggesting the presence of multiple ganglioside mimics. The GM1-GQ1b typing did not correlate with the Penner serotyping method thereby confirming that the Penner serotype is not related to the expression of ganglioside-like epitopes on the LPS (Karlyshev *et al.*, 2000).

The presence of GM1-like epitopes did not differ significantly between neuropathy patients and enteritis controls or between GBS and MFS patients, although the frequency of

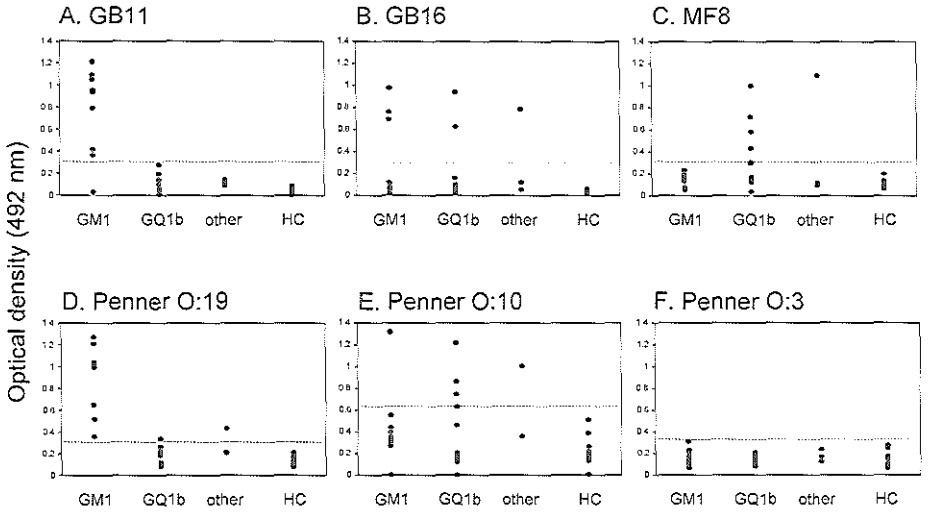


**Figure 1.** *Campylobacter jejuni* LPSs react differentially with anti-ganglioside antisera. Purified LPSs were tested for reactivity against a panel of serum samples containing anti-ganglioside reactivity against GM1/GA1 (GM1), GQ1b/GD3 (GQ1b)or other gangliosides/combination of GA1 and GQ1b (other). LPS from Guillain-Barré syndrome (GBS) patients GB11 and GB16 reacted with sera of the GM1-group (**A**,**B**). LPS from GBS patient GB16, who has an overlap between GBS and Miller Fisher syndrome (MFS), reacts with sera of the GM1, GQ1b and other group (**B**). LPS from MFS patient MFS reacted predominantly with GQ1b-sera (**C**). LPS from the O:19 serostrain reacted with sera of the GM1-group but not with the GQ1b-group (**D**) while LPS from the O:10 serostrain reacted with sera from the GQ1b group (**E**). LPS from the O:3 serostrain, which does not contain a GM1 or GQ1b-like structure, did not react with any of the sera (**F**). None of the LPS fractions reacts with sera from healthy controls (HC). Dashed lines indicate cut-off values.

GM1-like epitopes was higher in GBS-associated strains (Table 2). GQ1b-like epitopes occurred more frequently in MFS-associated strains than in GBS or control strains (p=0.006, Table 2). 35% of control enteritis strains did not react with any of the anti-ganglioside sera indicating the absence of ganglioside mimics. In contrast, only one GBS associated strain lacked ganglioside mimics (p=0.03, Table 2).

To investigate whether ganglioside-mimicry in *Campylobacter* LPS predicts the anti-ganglioside specificity and clinical features in GBS and MFS patients, we correlated the results from the GM1-GQ1b typing with these features (Table 3). The presence of a GQ1b-like epitope was strongly associated with anti-GD3 and anti-GQ1b antibody reactivity. In addition, we observed a relation between oculomotor symptoms and a GQ1b-like epitope (p=0.01). Four out of five patients with ataxia had a GQ1b-like LPS (Table 3). All GBS patients with anti-GM1 antibodies had a GM1-bearing LPS. In addition, two MFS-associated strains also contained a GM1-like LPS although no anti-GM1 antibodies could be detected in those patients. Thus, the structure of the ganglioside mimics is related to the anti-ganglioside specificity and clinical features. Furthermore, ganglioside mimics do not always elicit an immune response.

**Serological typing of *Campylobacter* strains with toxins, lectin and mAbs**

**Table 3. Association of LPS structure with anti-ganglioside reactivity and clinical features.**

| | LPS structure | |
|---|---|---|
| | GM1-like (n=13) | GQ1b-like (n=6) |
| Anti-ganglioside reactivity | | |
| Anti-GA1 | 9/13 (n.s.[a,b]) | 1/6 (n.s.[c]) |
| Anti-GM1 | 6/13 (n.s.[a]) | 3/6 (n.s.[c]) |
| Anti-GD3 | 4/13 (n.s.[a]) | 4/6 (p=0.04[c]) |
| Anti-GQ1b | 4/13 (n.s.[a]) | 5/6 (p=0.008[c]) |
| Clinical features | | |
| Oculomotor symptoms | 4/13 (n.s.[a]) | 5/6 (p=0.01[c]) |
| Ataxia | 3/13 (n.s.[a]) | 4/6 (n.s.[c]) |

a when compared to patients without a GM1-like *Campylobacter* LPS.
b not significant.
c when compared to patients without a GQ1b-like *Campylobacter* LPS.

Validation of CT binding, which in its unconjugated form binds highly selectively to GM1 (Angstrom *et al.*, 1994), showed differential binding specificity of CT, depending on the conjugated molecule (Figure 2 A, B). These patterns were independent from the concentration of glycolipids used and these results were confirmed using TLC (data not shown). Peroxidase conjugated PNA specifically bound to GA1 (Figure 2 C) and peroxidase conjugated TT bound to gangli-osides containing an internal disialosyl group such as GT1b, GD1b and GQ1b (Figure 2 D). The mAbs reacted with purified glycolipids as described before (Goodyear *et al.*, 1999; Paterson *et al.*, 1995; Willison *et al.*, 1996). Sm1 only reacts with GM1 while Wo1 binds to GA1, GM1 and GD1b. Ha1rbc binds to glycolipids with a disialosyl group while CGM3 and EG1 show a preference for GQ1b and GD3 (data not shown).

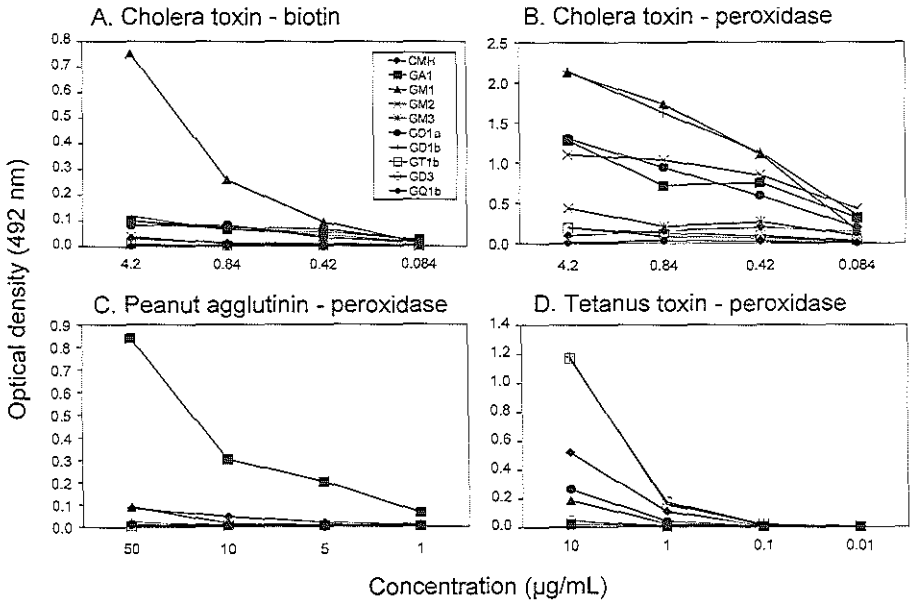


**Figure 2.** Specificity of toxins and lectin for purified glycolipids. A panel of glycolipids was coated onto 96-well plates and incubated with glycolipid binding ligands. Biotin labelled cholera toxin specifically binds to GM1 (**A**), while peroxidase labelled cholera toxin has a much broader binding pattern (**B**). Peroxidase labelled peanut agglutinin specifically binds to GA1 (**C**) and peroxidase labelled tetanus toxin binds to gangliosides with an internal disialosyl group (GD1b, GT1b, GQ1b) but not with a terminal disialosyl group (GD3) (**D**). Note different scales of abscissa.

As expected, all ligands reacted with purified LPS fractions. CT, PNA, Sm1 and Wo1 all reacted with LPS from the Penner O:19 reference strain, which has a GM1 mimic. Ha1rbc, CGM3 and EG1 reacted with LPS from the Penner O:10 serostrain, which has a GD3 mimic. None of the toxins, lectin or mAbs reacted with LPS from the Penner O:3 strain (data not shown). In general, the two serum panel and toxin/mAb typing systems correlated well. Strains that were designated "GM1-like" or "GQ1b-like" with the serum panel had a significantly higher level of reactivity with CT and the mAbs with corresponding specificity (data not shown).

Both CT conjugates clearly discriminated GBS-strains from MFS-strains ($p=0.03$, Figure 3 A), although not all GBS strains bound CT. In addition, the level of CT binding was higher in neuropathy-associated strains than in control strains ($p=0.02$). The level of PNA-binding was also higher in GBS-related LPS but this was not significant (Figure 3 B). TT-binding did not differ either between GBS and MFS-related LPS or between neuropathy and control strains (Figure 3 C). Both anti-GM1/GA1 mAbs reacted with a few GBS-strains and control enteritis strains but not with MFS-strains (Figure 3 D,E). However, the difference was not significant and many GBS strains were not recognized by the mAbs although they were recognized by CT. The anti-disialosyl mAbs clearly distinguished MFS-strains from GBS-

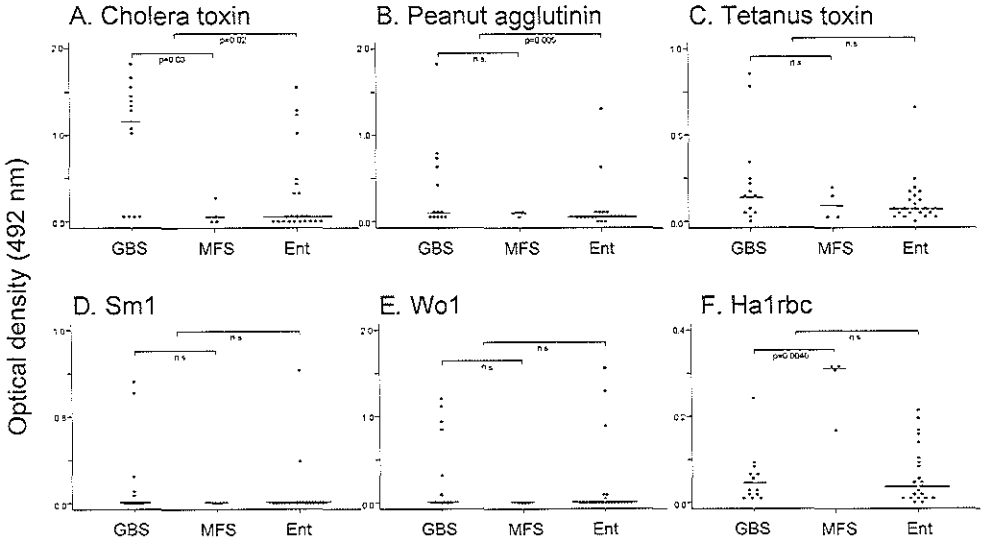


**Figure 3.** Differential binding of toxins, lectins and monoclonal anti-ganglioside antibodies to *Campylobacter jejuni* LPS from patients with Guillain-Barré syndrome (GBS), Miller Fisher syndrome (MFS) and uncomplicated enteritis (Ent). Purified *C. jejuni* LPS was coated onto 96-well plates and incubated with glycolipid binding ligands. **(A)** cholera toxin (biotin labelled) **(B)** peanut agglutinin **(C)** tetanus toxin **(D)** Sm1 **(E)** Wo1 and **(F)** Ha1rbc. Difference in binding was compared between GBS and MFS strains and between neuropathy (GBS & MFS) and uncomplicated enteritis strains.

strains (Figure 3 F).

CT binding was positively correlated with the presence of anti-GM1 antibodies (p=0.04) and there was an inverse relation between binding of CT and antibody reactivity against GQ1b and GD3 (p=0.007 and p=0.01). There was the same trend for Sm1 and Wo1 but with these mAbs the difference did not reach significance. Binding of the Ha1rbc to LPS was strongly correlated with anti-GQ1b antibody reactivity (p=0.009), the presence of oculomotor weakness (p=0.01) and ataxia (p=0.04) and the absence of motor weakness (p=0.006). Binding of CGM3 and EG1 to LPS gave comparable but less pronounced results.

## DISCUSSION

This study demonstrates that *Campylobacter* strains from GBS and MFS patients more frequently express of ganglioside mimics in their LPS than strains from uncomplicated enteritis patients. Cross-reactive anti-LPS/glycolipid responses can be detected in almost all culture proven cases of *Campylobacter* related Guillain-Barré and Miller Fisher syndrome but not in serum from patients with an uncomplicated enteritis. Differences in LPS-structure, detected with serological methods, determine the specificity of the antibody response and thereby the

clinical features of the patients.

In all but one neuropathy patient, we demonstrated IgA, IgM and/or high titer IgG antibodies against a protein extract from *C. jejuni*. This indicates that, despite the lack of gastrointestinal complaints, the patients from whom the *Campylobacter* was cultured had indeed experienced a recent infection.

The GBS patients were highly diverse in their anti-ganglioside specificity but almost all had antibody reactivity against the LPS from the strain with which they had been infected. In addition, anti-glycolipid and anti-LPS antibodies were cross-reactive. This serves as further evidence that the anti-ganglioside antibodies in GBS and MFS patients result from the previous infection with *C. jejuni*. In contrast to the findings in neuropathy patients, the response to bacterial and neural glycolipids was significantly lower in patients with an uncomplicated *Campylobacter* enteritis, despite the presence of ganglioside-mimics in the LPS of some enteritis strains. This finding underscores the influence of host-related factors, in addition to bacterium related factors, in the development of neurological symptoms following an infection with *C. jejuni*.

For the present study we developed a method using serum samples containing anti-ganglioside antibody reactivity to detect and define ganglioside-like epitopes on *C. jejuni* LPS. We validated the method with the O:19, O:10 and O:3 serostrains, from which the core oligosaccharide structures have been biochemically defined and found the method to be highly reproducible between individual tests and between different batches of purified LPS (data not shown). The serum samples showed a diverse binding pattern to various LPS fractions, possibly reflecting the fine specificity of the anti-LPS/ganglioside antibodies. Serum samples containing antibody reactivity to gangliosides other than GM1 or GQ1b reacted strongly with LPS from several strains which indicates the presence of other as yet undefined ganglioside-mimics. In addition, most LPS fractions reacted with serum samples from more than one serum-group, suggesting the presence of multiple ganglioside epitopes on one strain (Linton *et al.*, 2000; Yuki *et al.*, 1995a).

We used CT as a GM1-specific ligand but we observed a broadened reactivity to glycolipids when CT was conjugated to peroxidase as has been described before (Schwerer *et al.*, 1995). In contrast, CT conjugated to biotin was highly specific for purified GM1. The altered specificity may be explained by influence of the peroxidase molecule on the structure of CT, thereby altering the three-dimensional structure and specificity. However, CT-biotin and CT-peroxidase showed strong reactivity with the same LPS fractions. The serum panel typing system for GM1-like epitopes correlated well with binding to CT (Nachamkin *et al.*, 1999b; Prendergast *et al.*, 1999; Sheikh *et al.*, 1998; Wirguin *et al.*, 1994) and with the two anti-GA1/GM1 mAbs. CT binding showed a clear difference between GBS and MFS-related strains although not all GBS-related strains bound CT, and CT binding was not restricted to GBS patients with anti-GM1 antibodies. The lack of CT binding of all MFS-related strains and some GBS-related strains is important since CT-binding assays are currently being used to identify strains that have a higher potential to induce neurological symptoms (Nachamkin *et al.*, 1999b; Sack *et al.*, 1998). PNA binding was specific for purified GA1 (Molin *et al.*,

1986) and showed a preference for GBS-associated strains. There was no additional value of PNA typing over CT typing.

The anti-GA1/GM1 mAbs reacted selectively with a limited number of GBS-related strains. These results indicate that the use of very specific ligands is not sufficient to detect the wide variety of ganglioside-mimics in *C. jejuni* LPS. Consequently, GBS patients may have anti-ganglioside antibodies that are directed against GM1-like gangliosides (e.g. GM1b or GalNAc-GD1a)(Ang *et al.*, 1999; Yuki *et al.*, 1996). Together, these results indicate that CT and the GM1-panel recognize GM1-like immunoreactive epitopes on *C. jejuni* LPS as distinct from defining an exact structural mimic of the GM1 oligosaccharide.

Depending on the method used, we found that about half of the strains from uncomplicated enteritis patients bear a GM1-like epitope. This is slightly more than was reported in strains from the United States but this difference may be due to technical reasons (Nachamkin *et al.*, 1999b).

The serum panel typing method for GQ1b-like epitopes correlated well with binding of anti-GQ1b mAbs and showed a very strong correlation with the diagnosis MFS, the presence of anti-GQ1b antibodies, oculomotor symptoms and ataxia. TT bound to gangliosides containing a disialosyl group (Critchley *et al.*, 1986) but did not preferentially bind to MFS-associated strains although there was a slight preference for LPS from patients with antibodies against gangliosides with a disialosyl group. This might be due to the preference of TT for an internal disialosyl group because disialosyl groups in GBS and MFS related *C. jejuni* strains were demonstrated to be attached terminally (Aspinall *et al.*, 1994b; Salloway *et al.*, 1996). Based on these results, typing with an anti-GQ1b-serum panel or a mAb seems currently the only option for reliable detection of GQ1b-like epitopes.

In conclusion, our data provide strong support for the hypothesis that molecular mimicry between *C. jejuni* LPS and gangliosides plays a key role in the induction of anti-ganglioside antibodies and neurological symptomes in patients with GBS or MFS. Furthermore, we have demonstrated that heterogeneity in LPS structure determines the specificity of the anti-glycolipid response and thereby the clinical features in patients with a post-*Campylobacter* neuropathy.

**Acknowledgements**

The authors thank Dr P Herbrink, Diagnostic Centre SSDZ, Delft, The Netherlands for performing *Campylobacter* infection serology.

# Chapter 11

## *CAMPYLOBACTER JEJUNI* LIPOPOLYSACCHARIDES FROM GUILLAIN-BARRÉ SYNDROME PATIENTS INDUCE IGG ANTI-GM1 ANTIBODIES IN RABBITS

**CW Ang[1,2], HP Endtz[3], BC Jacobs[1,2], JD Laman[2], MA de Klerk[1,2], FGA van der Meché[1], PA van Doorn[1]**

Departments of Neurology[1], Immunology[2], Medical Microbiology & Infectious Diseases[3], Erasmus University Rotterdam, The Netherlands

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## ABSTRACT

Lipopolysaccharides (LPS) from *Campylobacter jejuni* strains isolated from patients with Guillain-Barré syndrome (GBS) display molecular mimicry with GM1. We immunized rabbits with *C. jejuni* LPS from GBS-associated strains containing a GM1-like epitope. All animals produced high titer anti-LPS antibodies that were cross-reactive with GM1. We conclude that *C. jejuni* strains from GBS patients are able to induce antibodies that cross-react with gangliosides and LPS. This study further confirms the role of molecular mimicry in the induction of anti-ganglioside antibodies in GBS patients.


## INTRODUCTION

Antibodies that cross-react with gangliosides and lipopolysaccharides (LPS) from *Campylobacter jejuni* can be found in serum from patients with Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) with a preceding infection with *C. jejuni* (Gregson *et al.*, 1997; Jacobs *et al.*, 1997b; Sheikh *et al.*, 1998; Yuki *et al.*, 1994b). At present, the exact role of anti-ganglioside antibodies in the pathogenesis of GBS and MFS is unknown but several groups have documented binding of anti-ganglioside antibodies to human neural tissue (Kusunoki *et al.*, 1993; O'Hanlon *et al.*, 1998). In addition, sera containing anti-ganglioside antibodies were found to interfere with neuromuscular transmission (Plomp *et al.*, 1999; Takigawa *et al.*, 1995).

Ganglioside-like structures (e.g. GM1, GD1a) were found in the LPS of all GBS associated *C. jejuni* strains studied so far (Aspinall *et al.*, 1994a; Yuki *et al.*, 1993a). It has been hypothesized that molecular mimicry between LPS and gangliosides accounts for the induction of anti-ganglioside antibodies in patients with GBS and MFS (Jacobs *et al.*, 1997b; Sheikh *et al.*, 1998), although the data from inhibition studies are more clearcut for MFS-associated strains than for GBS-associated strains.

Interestingly, Ritter et al (1996) demonstrated an anti-GM2 and anti-GM1 response in rabbits that were immunized with LPS from *C. jejuni* reference strains, derived from enteritis patients without neurological involvement. To confirm and extend these observations, we immunized rabbits with LPS fractions from two GBS associated *C. jejuni* strains and determined the antibody response to gangliosides and LPS. In addition, we investigated whether the antibodies were cross-reactive with LPS and gangliosides.

## MATERIALS AND METHODS

For immunization studies, two *C. jejuni* strains were used. GB3 was isolated from a GBS patient and has Penner serotype O:19. GB13 was isolated from the father of a GBS

patient who had diarrhoea and positive *C. jejuni* serology without neurological symptoms and has Penner serotype O:2 (Ang *et al.*, 1998; Chapter 7). Both strains were shown to express a GM1-like epitope demonstrated by binding to cholera toxin as described by Sheikh et al. (Sheikh *et al.*, 1998) and with reactivity against a panel of GM1-positive serum sample (Jacobs *et al.*, 1997a; Chapter 10). LPS was isolated with water-hot phenol method and purified as described before (Jacobs *et al.*, 1997b).

New Zealand White rabbits (2.0-2.5 kgs) were immunized with 400 μg LPS in complete Freund's adjuvant (DIFCO Laboratories, Detroit, MI) in a total volume of 1 mL with four subcutaneous injections on the back, according to the protocol of Ritter et al. (Ritter *et al.*, 1996). Two animals were immunized with GB3 LPS, two with GB13 LPS and two animals received injections with adjuvant only . Booster injections were given at days 14, 28 and 42 with the same amount of LPS in incomplete Freund's adjuvant (DIFCO). Blood was collected from the ear vein prior to each immunization. The animals were bled at day 56. The faeces of all animals was cultured for *C. jejuni* prior to immunization and at day 56. This experiment was approved by the local animal ethics committee.

Anti-ganglioside reactivity was detected with ELISA and confirmed with thin layer-chromatography (TLC) as described before (Jacobs *et al.*, 1996b) using anti-rabbit IgG and IgM peroxidase labelled secondary antibodies (Southern Biotechnology, Birmingham, AL). The titer was defined as the highest serum dilution with an Optical density (OD)>0.1, corrected for binding to uncoated wells. Anti-LPS reactivity was detected with ELISA as described before (Jacobs *et al.*, 1997a) with the same modification as stated above. Anti-LPS reactivity was further investigated with affinity-purified anti-GM1 antibodies and with Western blotting. Rabbit anti-ganglioside antibodies were affinity-purified on a GM1~Octyl-Sepharose CL4B-column (Hirabayashi *et al.*, 1983)(Pharmacia, Uppsala, Sweden). LPS was separated on a 15% polyacrylamide gel and blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, England). Membranes were blocked with 5% skimmed milk and incubated with diluted serum samples for 16 hrs at 4°C. Bound antibody was visualized with peroxidase conjugated antibodies and a chemiluminescence method (Pierce, Rockford, IL).

To assess cross-reactivity, serum from immunized rabbits was incubated with *C. jejuni* LPS conjugated to Octyl-Sepharose CL4B beads. 250 μg LPS was added to 1 mL of Octyl-Sepharose CL4B in methanol:water (1:1, v/v) containing 0.1M KCl and mixed for 1.5 hrs. After several washes with phosphate buffered saline, pH 7.8, serum samples were incubated with LPS-Sepharose conjugates for 5 hrs at 4°C. Absorbed serum samples were tested for anti-LPS and anti-GM1 reactivity as described above. Serum samples that were incubated with beads coupled to LPS from a strain that was isolated from an MFS patient (Jacobs *et al.*, 1997b), unconjugated beads or without beads served as controls.

# RESULTS

All animals tolerated the injections well and none of the animals developed overt signs of weakness. All cultures for *C. jejuni* were negative, indicating that a rise in anti-LPS or anti-ganglioside reactivity did not result from an intercurrent infection with *C. jejuni*.

All four rabbits immunized with LPS showed a strong humoral immune response to LPS as well as to gangliosides. No anti-LPS nor anti-ganglioside response was seen in the adjuvant controls (Table). In general, anti-LPS responses could be detected a few weeks before anti-ganglioside responses (Figure 1). Elevated titers of IgM antibodies to LPS could be detected 14 days after the first injection and continued to rise during the immunization period (Figure 1). IgG responses to LPS were seen after 28 days. The serum titers to LPS ranged from 12.800 up to 204.800 for IgG antibodies while titers did not rise higher than 800

**Table. Serum anti-ganglioside antibody titers in rabbits immunized with *Campylobacter jejuni* lipopolysaccharides.**

| Rabbit | Immunogen | Days since first immunization | IgM | | | | | IgG | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | GM1 | GM2 | GD1b | GQ1b | GA1 | GM1 | GM2 | GD1b | GQ1b | GA1 |
| 1 | GB3 LPS | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 14 | - | - | - | - | 100 | - | - | - | - | - |
| | | 28 | - | - | - | - | 100 | - | - | - | - | - |
| | | 42 | - | - | - | - | 100 | - | - | - | - | - |
| | | 56 | 100 | - | - | - | 200 | 1600 | - | - | - | 200 |
| 2 | GB3 LPS | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 14 | - | - | - | - | - | - | - | - | - | - |
| | | 28 | - | - | - | - | - | - | - | - | - | - |
| | | 42 | 200 | - | - | - | 200 | 800 | - | 200 | - | - |
| | | 56 | 400 | - | - | - | 400 | 12800 | - | 3200 | - | 1600 |
| 3 | GB13 LPS | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 14 | - | - | - | - | 100 | - | - | - | - | - |
| | | 28 | 200 | - | 100 | - | 400 | - | - | - | - | - |
| | | 42 | 400 | - | 200 | - | 800 | - | - | - | - | - |
| | | 56 | 400 | - | 200 | - | 800 | 1600 | - | - | - | - |
| 4 | GB13 LPS | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 14 | - | - | - | - | 100 | - | - | - | - | - |
| | | 28 | - | - | - | - | 200 | 400 | - | - | - | - |
| | | 42 | - | - | - | - | 400 | 1600 | - | - | - | 100 |
| | | 56 | - | - | - | - | 200 | 1600 | - | - | - | 400 |
| 5 | CFA | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 14 | - | - | - | - | - | - | - | - | - | - |
| | | 28 | - | - | - | - | - | - | - | - | - | - |
| | | 42 | - | - | - | - | - | - | - | - | - | - |
| | | 56 | - | - | - | - | - | - | - | - | - | - |
| 6 | CFA | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 14 | - | - | - | - | - | - | - | - | - | - |
| | | 28 | - | - | - | - | - | - | - | - | - | - |
| | | 42 | - | - | - | - | - | - | - | - | - | - |
| | | 56 | - | - | - | - | - | - | - | - | - | - |

- = titer<100

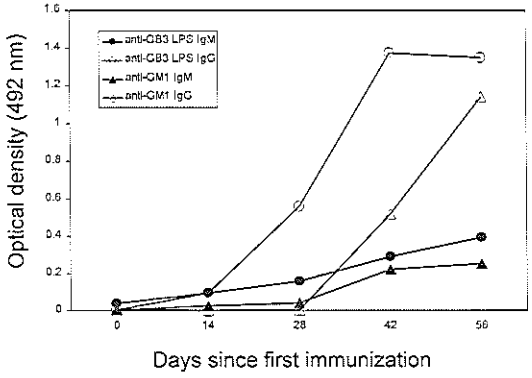


**Figure 1.** Kinetics of IgM and IgG anti-lipopolysaccharide (LPS) and anti-ganglioside responses. Sequential serum samples were tested for anti-LPS and anti-GM1 reactivity. The IgM response rises gradually, while IgG responses show a strong increase after 28 (anti-LPS) or 42 (anti-GM1) days.

for IgM antibodies.

Anti-GM1 IgM antibodies appeared only after 28 days and remained low throughout the experiment (Table). IgM antibodies against GA1 could already be detected after 14 days in three out of four animals, but the titers remained low throughout the experiment. We could detect IgG reactivity against GM1 from 28 days (rabbit 4). At day 56 anti-GM1 IgG titers ranged from 1600 to 12,800 (Table). All reactivity to glycolipids was confirmed with TLC (not shown).

In Western blots of *C. jejuni* LPS, rabbit immune serum reacted strongly with a band that co-migrated between 10 and 15 kD (Figure 2B). However, there was also a vague stain-

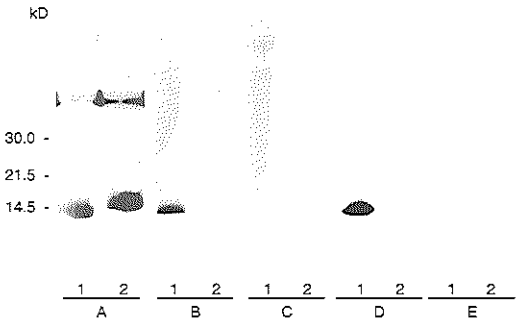


**Figure 2.** Affinity-purified rabbit anti-GM1 antibodies react with *Campylobacter jejuni* lipopolysaccharides in Western blot. Lipopolysaccharides from strains GB3 (lanes 1) and GB13 (lanes 2) were electrophoresed and silver stained (**A**) or blotted onto nitrocellulose and overlaid with serum from rabbit 2 (**B-E**). Serum from rabbit 2 (immunized with LPS GB3) taken on day 56 reacts with the low molecular weight fraction of GB3 LPS but not GB13 LPS (**B**). Serum depleted for anti-GM1 antibodies does not stain the lower band (**C**), whereas affinity-purified anti-GM1 antibodies only stain the lower band (**D**). Serum taken prior to immunization gives no staining (**E**).

ing of bands with higher molecular weight (Figure 2B). When we used serum that was depleted for anti-GM1 antibodies, the low molecular weight band disappeared whereas the high molecular weight staining remained (Figure 2C). Affinity purified anti-GM1 antibodies from animals that were immunized with GB3 consistently stained only the low-molecular weight band from GB3 LPS (Figure 2D). In contrast, no or only a faint staining of GB13 LPS could be seen (Figure 2D), indicating a different fine specificity of anti-GM1 antibodies.

To demonstrate that the anti-ganglioside antibodies indeed cross-react with LPS, we absorbed the sera with LPS conjugated to Sepharose CL4B beads. Incubation of serum from rabbit 1 with GB3 LPS coated beads resulted in a strong inhibition of anti-LPS reactivity and a concomitant decrease in anti-GM1 reactivity (Figure 3A,B). Uncoated beads and beads that were coated with LPS from a *C. jejuni* strain isolated from an MFS patient, not expressing a GM1-like epitope (Jacobs *et al.*, 1997b), affected neither anti-LPS nor anti-GM1 reactivity.

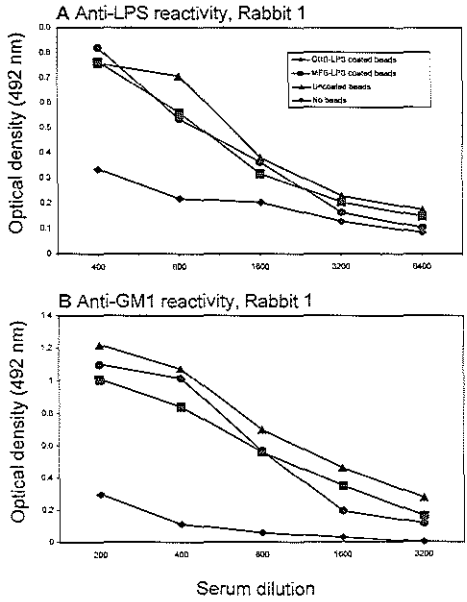


**Figure 3.** Inhibition of antibody reactivity to GM1 and *Campylobacter jejuni* lipopolysaccharides (LPS) in a rabbit immunized with LPS. Rabbit serum taken on day 56 was incubated with *C. jejuni* LPS from strain GB3, isolated from a GBS patient and a strain isolated from a Miller Fisher syndrome patient, conjugated to Octyl-Sepharose CL4B beads. Uncoated beads and serum that was incubated without beads were used as a control. Anti-GB3 LPS (**A**) and anti-GM1 (**B**) reactivity was decreased in rabbit serum following depletion with LPS from GB3 but not from a Miller Fisher syndrome patient, indicating the specificity of the anti-GM1 antibodies. Note differences in scales.

## DISCUSSION

We have demonstrated an IgG response to gangliosides and to LPS of *C. jejuni* following immunization of rabbits with LPS from GBS-related *C. jejuni* strains. Two groups have previously induced anti-ganglioside antibodies with *C. jejuni* LPS (Ritter *et al.*, 1996; Wirguin *et al.*, 1997). In these two studies the Penner O:19 serostrain was used for induction of anti-GM1 antibodies. However, in the present study we demonstrate that LPS from two GBS-associated *C. jejuni* strains, including Penner O:19 and O:2, are also able to induce an anti-GM1 response. LPS fractions from both strains were able to bind cholera toxin, a ligand for GM1 and reacted with a panel of polyclonal anti-GM1 antisera from GBS patients (Jacobs *et al.*, 1997a), indicating that LPSs from both strains have a GM1-like epitope. Although strain GB13 was isolated from a family member of a GBS patient who did have neurological symptoms or anti-GM1 antibodies (Ang *et al.*, 1998), immunization of rabbits with this strain resulted in an anti-GM1 response. This indicates that, in addition to a ganglioside-like mimic, host factors are important for the generation of a cross-reactive anti-LPS/ganglioside response.

Wirguin et al (1997) primed rats with keyhole-limpet hemocyanin and subsequently immunized with LPS from the Penner O:19 serostrain. This method only resulted in an IgM response to GM1. Using a similar protocol as in the present study, Ritter et al (1996) could detect IgG antibodies against GM1, GD1b and GM2 in rabbits following immunization with LPS from the Penner O:19 and Penner O:1 serostrain. The kinetics and isotype pattern of the anti-ganglioside response in our study was similar to that observed in the Ritter study (Ritter *et al.*, 1996).

The anti-GM1 antibodies cross-react with the LPS fraction with which the animals were immunized. Anti-GM1 reactivity could not be blocked by LPS from a *C. jejuni* strain that was isolated from a MFS patient, excluding the aspecific binding of anti-GM1 antibodies to *C. jejuni* LPS. The same pattern of cross-reactivity could be observed in GBS patients with a preceding *C. jejuni* infection and anti-LPS antibodies (data not shown and Jacobs *et al.*, 1997b). These very similar antibody reactivity patterns suggest that the anti-ganglioside response in GBS patients is induced by a similar mechanism as in this rabbit model. Remarkably, the anti-LPS response could be detected two weeks earlier than the anti-ganglioside response. There are two possible explanations for this observation. First, the ELISA system for detecting anti-LPS antibodies is more sensitive than the anti-ganglioside ELISA, thereby masking early anti-ganglioside responses. Second, the initial anti-LPS response may be directed against other epitopes than the ganglioside-like core structure.

The strong IgG response suggests that a T cell dependent mechanism is involved, in contrast to the predominant IgM response that is generally found against glycolipid antigens (Alfonso *et al.*, 1995; Freimer *et al.*, 1993; Kusunoki *et al.*, 1996b).

Although both LPS fractions were able to induce anti-GM1 antibodies, the fine specificity of these antibodies was different as demonstrated by Western blot. This may result from the fact that the GM1-like epitopes in strain GB3 en GB13 are slightly different. In addition,

host factors may influence the specificity and isotype distribution of anti-ganglioside antibodies as animals that were immunized with the same LPS showed a slightly different pattern of antibody reactivity.

None of the animals developed any overt neurological signs. This may be due to several factors. The antibodies may not bind to peripheral nerve and thus not lead to neurological symptoms. Another possibility is that these antibodies are not pathogenic, although they can bind to the nerve. Alternatively, the duration of the experiment may have been too short to observe any clinical signs. In previous studies on experimental neuritis, some rabbits remained symptom free for several months (Kusunoki *et al.*, 1996b; Saida *et al.*, 1979b). We sacrificed all animals at day 56 so we were not able to investigate this. Finally, the animals may have had subclinical nerve dysfunction which can only be detected with *in vivo* or *in vitro* electrophysiological studies (Thomas *et al.*, 1991).

In conclusion, these results confirm the hypothesis that anti-ganglioside antibodies can be induced by an antecedent infection with *C. jejuni* through molecular mimicry of *C. jejuni* LPS with gangliosides. Whether these antibodies are crucial in the development of neurological symptoms remains to be determined.

**Acknowledgements**

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# Chapter 12

## GUILLAIN-BARRÉ AND MILLER FISHER SYNDROME ASSOCIATED *CAMPYLOBACTER JEJUNI* LIPOPOLYSACCHARIDES INDUCE ANTI-GM1 AND ANTI-GQ1B ANTIBODIES IN RABBITS

**CW Ang[1,2], MA de Klerk[1,2], HP Endtz[3], BC Jacobs[1,2], JD Laman[2], FGA van der Meché[1], PA van Doorn[1]**

Departments of Neurology[1], Immunology[2], Medical Microbiology & Infectious Diseases[3], Erasmus University Rotterdam, The Netherlands

*Submitted*

## ABSTRACT

*Campylobacter jejuni* infections are thought to induce anti-ganglioside antibodies in patients with Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) by molecular mimicry between *C. jejuni* lipopolysaccharides (LPS) and gangliosides. We used purified LPS fractions from five *Campylobacter* strains to induce anti-ganglioside responses in rabbits. The animals that received injections with LPS from GBS-associated strains developed anti-GM1 and anti-GA1 antibodies. Animals injected with LPS from one MFS-related *C. jejuni* strain produced anti-GQ1b antibodies. Rabbits that were injected with Penner O:3 LPS had a strong anti-LPS response but no anti-ganglioside reactivity was observed. The anti-ganglioside specificity in the rabbits reflected the specificity in the patients from whom the strains were isolated. In conclusion, our results indicate that an immune response against GBS and MFS associated *C. jejuni* LPS results in anti-ganglioside antibodies. These results provide strong support for molecular mimicry as a mechanism in the induction of anti-ganglioside antibodies following infections.


## INTRODUCTION

The Guillain-Barré syndrome (GBS) and the Miller Fisher syndrome (MFS) are acute immune-mediated neuropathies and are frequently preceded by an infectious illness. The most frequently identified micro-organism is the enteric pathogen *Campylobacter jejuni* (Jacobs *et al.*, 1998; Kuroki *et al.*, 1993; Rees *et al.*, 1995b).

In the majority of *Campylobacter*-associated GBS and MFS patients, serum antibodies that react with gangliosides are present (Hao *et al.*, 1998; Jacobs *et al.*, 1997b; Sheikh *et al.*, 1998). The presence of these antibodies is associated with specific clinical symptoms. Anti-GM1 antibodies are associated with motor neuropathy (Gregson *et al.*, 1991; Jacobs *et al.*, 1996b; Kuwabara *et al.*, 1998b) and anti-GQ1b antibodies with oculomotor symptoms (Chiba *et al.*, 1993; Willison *et al.*, 1993b). Anti-ganglioside antibodies exert an effect on the neuromuscular junction, indicating that these antibodies may contribute to the neurological deficits (Goodyear *et al.*, 1999; Plomp *et al.*, 1999).

The core oligosaccharides of *C. jejuni* lipopolysaccharides (LPS) contain ganglioside-like structures and anti-ganglioside antibodies from GBS and MFS patients do indeed react with *C. jejuni* LPS (Ho *et al.*, 1997; Jacobs *et al.*, 1997b; Moran, 1997; Neisser *et al.*, 1997) These results suggest that anti-ganglioside antibodies in neuropathy patients with an antecedent *C. jejuni* infection are induced through molecular mimicry. Several authors have addressed this issue in animal studies (Goodyear *et al.*, 1999; Ritter *et al.*, 1996; Wirguin *et al.*, 1997). Wirguin et al (Wirguin *et al.*, 1997) induced IgM anti-GM1 antibodies in rats following immunization with LPS from the Penner O:19 reference strain and a pre-immunization with keyhole limpet hemocyanin. Ritter et al (Ritter *et al.*, 1996) immunized rabbits with

LPS from several *C. jejuni* strains, resulting in IgG anti-GM2, anti-GD1b and anti-GM1 antibodies. These two groups used *C. jejuni* reference strains that were isolated from enteritis patients without neurological involvement. Goodyear et al (Goodyear *et al.*, 1999) and Ang et al. (Ang *et al.*, 2000b) used GBS-associated *C. jejuni* strains to induce an anti-ganglioside response in mice and rabbits respectively. However, the anti-ganglioside reactivity in the serum of the patients from which these neuropathy associated strains were cultured was not known and therefore the anti-LPS/ganglioside response in the animals could not be compared to the response in neuropathy patients.

The aim of the present study was to investigate whether immunization of rabbits with LPS from GBS and MFS-associated *C. jejuni* strains induces cross-reactive anti-ganglioside/anti-LPS antibodies. Furthermore, we wanted to compare the anti-ganglioside specificity in the rabbits with the specificity in the patients from whom the *C. jejuni* strains were isolated.

## METHODS

### Patients and *Campylobacter* strains

Strains GB17 and GB18 were isolated from two GBS patients, strains MF6 and MF8 were isolated from two MFS patients and have been described before as strain A en strain C respectively (Table 1)(Jacobs *et al.*, 1997b). For extraction of LPSs, bacteria were grown on blood agar plates with 5% sheep blood, at 37°C for 48 h under microaerophilic conditions. Cells were scraped from fresh grown plates and washed in phosphate-buffered saline (pH 7.8). LPSs were extracted with the hot phenol-water method of Westphal and Jann (Westphal and Jann, 1965). Combined water phases containing the extracted LPSs were dialyzed against water. After ultracentrifugation at 100,000 *g* for 2 h, the pellets were freeze-dried and weighed. After resuspension in water, all LPS fractions showed a single dense band migrating at 8-15 kD following electrophoresis on a polyacrylamide gel and silver staining (Novex,

**Table 1. Clinical characteristics of patients and Penner serotype of isolated Campylobacter jejuni strains.**

| Patient | GB17 | GB18 | MF6 | MF8 |
|---|---|---|---|---|
| Diagnosis | Guillain-Barré | Guillain-Barré | Miller Fisher | Miller Fisher |
| Age | 54 | 7 | 41 | 22 |
| Gender | male | male | male | male |
| Prodromal illness | diarrhea | vomiting/URTI[a] | diarrhea | diarrhea |
| Maximum clinical deficit | unable to walk unaided | bedbound | no limb weakness | no limb weakness |
| Sensory involvement paresthesias | paresthesias | | no | no |
| Ataxia | no | no | yes | yes |
| Oculomotor weakness | no | no | yes | yes |
| Electrophysiological studies | axonal neuropathy | not available | not available | normal |
| Penner serotype of strain | O:4,13,64 | O:19 | O:4,64 | O:23,36 |

a Upper respiratory tract infection.

San Diego, CA), indicating the presence of LPS (Aspinall *et al.*, 1994a). The anti-ganglioside and anti-LPS reactivity in the patients from whom the *C. jejuni* strains were isolated was determined with ELISA and confirmed with thin-layer chromatography (TLC) as described previously (Table 2)(Jacobs *et al.*, 1997a). Results from Penner serotyping of the strains are listed in Table 1. The Penner O:3 serostrain, which is known not to contain a GM1 or GQ1b-like structure was used as a control (Aspinall *et al.*, 1995; Moran *et al.*, 1991). To confirm the presence of ganglioside-like epitopes, LPSs from all five strains were tested in ELISA with a panel of polyclonal GM1/GA1 and GQ1b/GD3-reactive sera, cholera toxin (CT), peanut agglutinin (PNA) and a monoclonal anti-disialosyl antibody (Willison *et al.*, 1996), provided by HJ Willison, Glasgow (Chapter 10).

**Immunization protocol**

New Zealand White rabbits (2.0-2.5 kgs) were immunized as described before (Ang *et al.*, 2000b). Two animals were immunized with each LPS. As a control, two animals were injected with adjuvant only, without LPS. Booster injections with the same amount of LPS in incomplete Freund's adjuvant (DIFCO) were given at days 14, 28 and 42. Blood was collected from the ear vein prior to each immunization. The animals were bled at day 56. The faeces of all animals was cultured for *C. jejuni* prior to immunization and at day 56. This experiment was approved by the local animal ethics committee.

**Serology**

Anti-ganglioside reactivity was detected with ELISA and confirmed with TLC as described before (Ang *et al.*, 2000b). The titer was defined as the highest serum dilution with an optical density (OD)>0.1, corrected for binding to uncoated wells. Anti-LPS reactivity was detected with ELISA and Western blot as described before (Ang *et al.*, 2000b).

To assess cross-reactivity of anti-LPS antibodies with gangliosides, serum samples from immunized rabbits and from the patients from whom the *C. jejuni* strains were isolated were incubated with *C. jejuni* LPS conjugated to Octyl-Sepharose CL4B beads (Ang *et al.*, 2000b). In short, 250 μg LPS was added to 1 mL of Octyl-Sepharose CL4B in methanol:water (1:1, v/v) containing 0.1M KCl and mixed for 1.5 hrs. After several washes with PBS, serum samples were incubated with LPS-Sepharose conjugates for 5 hrs at 4°C. Absorbed serum samples were tested for anti-LPS and anti-ganglioside reactivity as described above. As controls, serum samples that were processed in an identical way but incubated with unconjugated beads and without beads were included in each experiment.

## RESULTS

Clinical data of the GBS and MFS patients are presented in Table 1. Serum from all patients reacted with several glycolipid antigens, although the fine-specificity and isotype distribution of the antibodies differed between patients (Table 2). In addition, all patients had

**Table 2. Serum anti-glycolipid and anti-LPS antibody titers in Guillain-Barré and Miller Fisher syndrome patients.**

| Patient | IgM | | | | | | | | IgG | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | GA1 | GM1 | GM2 | GD1a | GD1b | GD3 | GQ1b | Homologous LPS | GA1 | GM1 | GM2 | GD1a | GD1b | GD3 | GQ1b | Homologous LPS |
| GB17 | 200 | -[a] | - | - | - | - | - | 400 | 25,600 | 100 | - | - | - | 100 | - | 25,600 |
| GB18 | - | 200 | - | - | - | - | - | 800 | 3,200 | 12,800 | - | - | 1,600 | - | - | 51,200 |
| MF6 | - | - | - | - | - | - | - | - | - | - | - | - | 100 | 200 | 1,600 | 200 |
| MF8 | - | - | - | - | 200 | - | - | 200 | - | - | - | - | 200 | 200 | 800 | 800 |

a - = <100

antibodies to *C. jejuni* LPS (Table 2) and protein antigens (data not shown). Detailed electrophysiological data were only available for patients GB17 and MF8. Patient GB17 had an axonal neuropathy, with predominant distal weakness. Motor nerve conduction studies in patient MF8 revealed no abnormalities.

The presence of GM1 and GQ1b-like epitopes on the GBS and MFS associated strains was studied with ganglioside-binding ligands. GB18 LPS reacted strongly with cholera toxin, indicating a GM1-like epitope, while MF6 and MF8 LPS showed reactivity with the anti-disialosyl antibody, demonstrating a GQ1b-like epitope (data not shown). In addition, both GB17 LPS and MF6 LPS reacted with sera containing anti-GM1 and anti-GA1 reactivity and GB17 LPS reacted strongly with peanut agglutinin, a ligand specific for GA1, indicating the presence of GA1-like epitopes in these strains. In contrast, Penner O:3 LPS did not show reactivity any of the ganglioside binding ligands (data not shown).

To exclude the possibility that the observed antibody responses resulted from an intercurrent infection of the animals with *C. jejuni* and not from the immunization procedure, stool cultures for *C. jejuni* were performed at the start of the immunization procedure and when the animals were bled. Stool cultures remained negative during the immunization period. Furthermore, anti-ganglioside and anti-LPS reactivity could not be detected in pre-immune serum samples. Immunization of rabbits with *C. jejuni* LPS from neuropathy patients resulted in high titer anti-LPS and anti-glycolipid antibodies, while serum from animals that were immunized with LPS from the O:3 serostrain only contained anti-LPS but no antibodies against any of the purified glycolipids (Table 3). The adjuvant controls did not show any reactivity to LPS and only a slight elevation of anti-GA1 reactivity (Table 3). All anti-glycolipid responses were confirmed with TLC (data not shown).

The specificity of anti-ganglioside antibodies in the rabbits resembled the observed specificity in the patients from whom the strains were cultured. Serum from patient GB17 contained mainly antibody reactivity to GA1, as did the rabbits that were immunized with LPS from strain GB17. The other GBS patient, GB18, had a higher anti-GM1 than anti-GA1 titer. Serum from the two animals that were immunized with LPS from GB18 displayed the same pattern of anti-glycolipid reactivity as patient GB18. Both rabbits that were immunized with LPS from MF6 mounted an immune response against GQ1b. One animal had only IgM anti-GQ1b antibodies, the other had only IgG anti-GQ1b (Table 3). In addition, both animals had high titers of anti-GA1 antibodies, which was not found in the patient from whom MF6 was isolated but was in accordance with the presence of a GA1-like structure in MF6 LPS. Immunization with MF8 LPS did not result in an anti-GQ1b response although one of the animals had an IgG anti-GM2 response. Anti-LPS reactivity in these two rabbits was also much weaker compared to animals immunized with LPS from other *C. jejuni* strains.

The kinetics of anti-glycolipid and anti-LPS antibody response differed between animals, depending on the source of the LPS. In both animals that were immunized with LPS from GB17, a strong IgG response against GA1 and GB17 LPS could already be observed two weeks after the first immunization (Figure 1, A,B). In contrast, animals that were immunized with GB18 and MF6 LPS showed a gradual increase of the IgG anti-ganglioside and

**Table 3 - Serum anti-glycolipid and anti-LPS antibody titers in rabbits immunized with Campylobacter jejuni lipopolysaccharides.**

| Rabbit | C. jejuni strain | IgM | | | | | | | | IgG | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GA1 | GM1 | GM2 | GD1a | GD1b | GD3 | GQ1b | Homologous LPS | GA1 | GM1 | GM2 | GD1a | GD1b | GD3 | GQ1b | Homologous LPS |
| 1 | GB17 | a800 | 200 | b- | - | 100 | - | - | 400 | 409,600 | - | - | - | - | - | - | 819,200 |
| 2 | GB17 | 800 | - | - | - | - | - | - | 400 | 819,200 | - | - | - | 800 | 200 | - | 3,276,800 |
| 3 | GB18 | 100 | 100 | - | - | - | - | - | 100 | 400 | 51,200 | 200 | - | - | - | - | 12,800 |
| 4 | GB18 | 200 | 200 | - | - | 100 | - | - | 100 | 1,600 | 1,600 | - | - | 100 | - | - | 102,400 |
| 5 | MF6 | 800 | 100 | - | - | - | - | - | 200 | 102,400 | - | - | - | - | - | 400 | 102,400 |
| 6 | MF6 | 1,600 | - | - | - | - | - | 200 | 800 | 25,600 | - | - | - | - | - | - | 3,200 |
| 7 | MF8 | - | 100 | 100 | 100 | - | - | - | 100 | - | - | 800 | - | - | - | - | 200 |
| 8 | MF8 | - | - | 100 | 100 | - | - | - | 100 | - | - | - | - | - | - | - | 800 |
| 9 | Pen O:3 | - | - | - | - | - | - | - | 400 | - | - | - | - | - | - | - | 3,276,800 |
| 10 | Pen O:3 | - | - | - | - | - | - | - | 400 | - | - | - | - | - | - | - | 3,276,800 |
| 11 | CFA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 12 | CFA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

a Serum samples taken at day 56.

b - = <100..

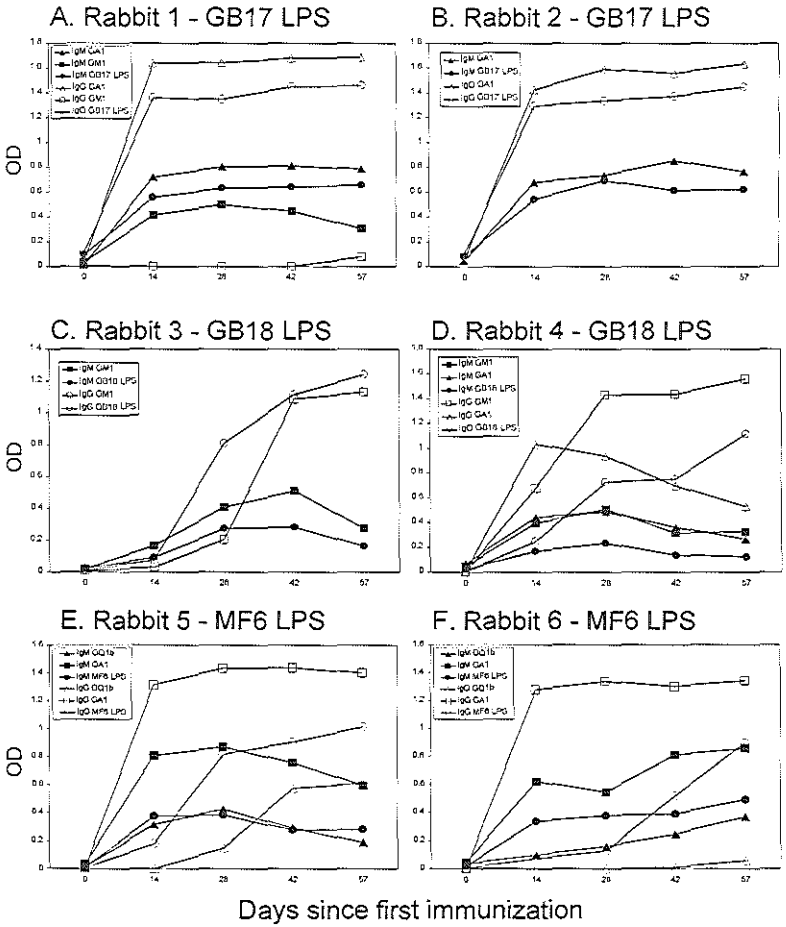


**Figure 1.** Kinetics of anti-lipopolysaccharide and anti-glycolipid reactivity in rabbits immunized with *Campylobacter jejuni* lipopolysaccharides. Sequential serum samples were tested for IgM and IgG reactivity against *C. jejuni* lipopolysaccharides (LPS) and glycolipids. **(A/B)** Rabbit 1 and 2, anti-GM1, anti-GA1 and anti-GB17 LPS reactivity IgM and IgG reactivity rose in parallel and were at high levels after 14 days; **(C/D)** Rabbit 3 and 4, IgM anti-GM1 and anti-GB18 LPS antibody reactivity decreased after an initial rise, while IgG reactivity continued to rise. Anti-GA1 and anti-GM1 reactivity in rabbit 4 showed different kinetics; **(E/F)** Rabbit 5 and 6, similar kinetics of IgM anti-GQ1b and anti-MF6 LPS. Different kinetics of IgG anti-GQ1b and IgG anti-GA1 reactivity compared to IgG anti-MF6 LPS reactivity. OD, optical density.

anti-LPS antibody reactivity during the immunization period (Figure 1, C-F). Remarkably, in one animal that was immunized with GB18, the IgG anti-GM1 and anti-GA1 response showed different kinetics. After an initial increase, the anti-GA1 reactivity decreased while the anti-GM1 reactivity still increased until the end of the immunization period (Figure 1, D).

On Western blot, the sera from all LPS-immunized rabbits reacted with the LPS they had been injected with. Silver staining revealed a band that co-migrated between 11-15 kD

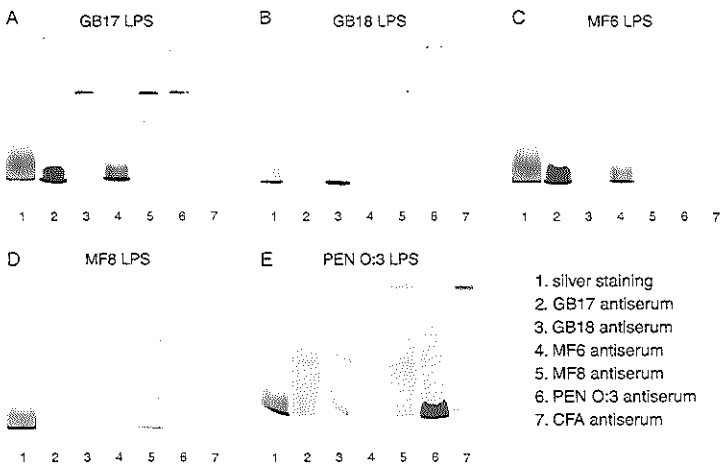


**Figure 2.** Strain specific anti-lipopolysaccharide responses detected by Western blotting. *C. jejuni* lipopolysaccharides (LPS) were subjected to electrophoreses and silver stained (lanes 1) or transferred to nitrocellulose and overlaid with serum from immunized rabbits (lanes 2-6). (**A**) GB17 LPS reacted with serum from a GB17 LPS-immunized animal but also with serum from a MF6 LPS-immunized animal; (**B**) GB18 LPS only reacted with serum from a GB18 LPS immunized animal; (**C**) MF6 LPS reacts with serum from a MF6 LPS-immunized animal but also with serum from a GB17 LPS immunized animal; (**D**) MF8 LPS reacted weakly with serum from a MF8 immunized animal; (**E**) Penner O:3 LPS reacted strongly with serum from a Penner O:3 LPS immunized animal.

(Figure 2), consistent with earlier observations on *C. jejuni* LPS (Preston and Penner, 1989) Serum samples predominantly reacted with this low molecular weight fraction of the LPS. We did not observe ladder-like patterns as described for *C. jejuni* (Preston and Penner, 1989) although in some instances single bands with higher molecular weight were present. Serum from some rabbits not only reacted with the LPS with which they were immunized, but also with LPS from other *C. jejuni* strains. Serum from animals immunized with MF6 also reacted strongly with LPS from GB17 and vice versa (Figure 2 A lanes 2 and 4, 2 C lanes 2 and 4). Both strains have Penner serotype O:4. Only serum from animals that were immunized with Penner O:3 had a strong response to Penner O:3 LPS (Figure 2 E).

To prove that the anti-LPS antibodies and the anti-ganglioside antibodies are cross-reactive, we conducted a series of depletion experiments with *C. jejuni* LPS conjugated to Sepharose CL4B beads. The results from experiments on cross-reactive anti-GQ1b/anti-MF6 LPS antibodies are summarized in Figure 3. Anti-GQ1b/anti-MF6 LPS reactivity in a MF6-immunized rabbit could only be inhibited by incubation of serum with Sepharose beads conjugated to MF6 LPS and not with LPS from GB18 and Penner O:3 (Figure 3 A, C). The observed specific pattern of cross-reactivity was identical to that found in the patient from which the MF6 strain was isolated (Figure 3 B,D). In a similar fashion, cross-reactivity of anti-GA1/GM1 antibodies in patients GB17 and GB18 and rabbits immunized with corresponding LPS was demonstrated. In the GB17 immunized rabbits, anti-GA1 reactivity could only be decreased by incubation with GB17 LPS and not GB18 LPS, indicating that the anti-

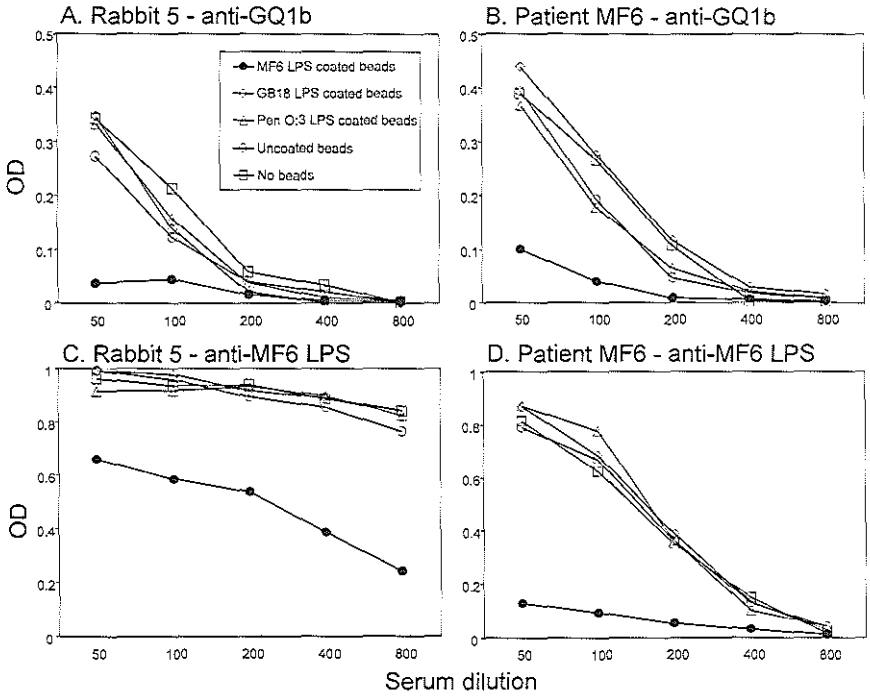


**Figure 3.** Identical pattern of cross-reactivity of anti-ganglioside/lipopolysaccharide antibodies in patients and immunized rabbits. Serum from patient MF6 and from rabbit 5, immunized with MF6 lipopolysaccharide (LPS), was incubated with Sepharose CL4B beads that were coated with *C. jejuni* LPS and diluted serum samples were tested for residual reactivity against GQ1b and MF6 LPS. Specificity of both human and rabbit serum is identical. Anti-GQ1b reactivity in the rabbit and the patient was only reduced by incubation with MF6 LPS coated beads (**A, B, filled circles**). The same pattern could be observed when tested for anti-MF6 LPS reactivity (**C, D**). OD, optical density.

GA1 antibodies are specifically induced by the GA1-like GB17 LPS.

Almost all rabbits tolerated the injections well and did not show any overt signs of weakness or ataxia and did not lose weight. However, one animal that was immunized with GB18 LPS started losing weight four weeks after the first injection. In addition, this animal had less spontaneous movements although there were no signs of muscle weakness. After a few weeks, the animal recovered and gained weight again. Histological examination of the sciatic nerve of this animal, taken 56 days after the first injection did not show any signs of neuropathy or inflammation.

## DISCUSSION

This study demonstrates that immunization of rabbits with LPS from GBS and MFS-

associated *C. jejuni* strains that contain ganglioside-like structures can induce anti-ganglioside antibodies while immunization with the Penner O:3 serostrain did not lead to anti-ganglioside responses despite a strong anti-LPS response. The specificity of the anti-ganglioside response in the rabbits reflected the specificity in the patients. This suggests that the immune response in the patients was directed against the same structures as the immune response in the rabbits, thereby giving support for the hypothesis that anti-ganglioside antibodies can arise through molecular mimicry with ganglioside-like structures of an infectious agent.

The anti-ganglioside antibody response in the animals was the result of the immunization procedure and not from a intercurrent infection of the animals with *C. jejuni* as all animals had negative stool cultures at the onset and end of the experiment. Furthermore, pre-immunization titers of anti-LPS and anti-ganglioside antibodies were <100. Rabbits that were immunized with only CFA, which contains mycobacterial antigens, did not show a rise in anti-LPS nor anti-ganglioside antibodies. In addition, immunization with O:3 LPS which does not contain a GM1-like or GQ1b-like structure (Aspinall *et al.*, 1995), resulted in a strong anti-LPS response without any anti-ganglioside reactivity. These results confirm that immunization solely with adjuvants, such as CFA and LPS, does not lead to anti-ganglioside antibody formation and indicates that these antibodies do not result from polyclonal B cell stimulation (Ang *et al.*, 2000b).

Depletion experiments using LPS-coated Sepharose beads showed that the rabbit anti-LPS and anti-ganglioside antibodies are cross-reactive. This pattern of cross-reactivity was identical to the pattern that was observed using serum from the patient from which the *C. jejuni* strains were derived. This serves as further evidence that anti-ganglioside antibodies in GBS and MFS patients with an antecedent *Campylobacter* infection are directed against ganglioside-like structures of bacterial antigens.

Two animals that were injected with LPS from MF8 did not produce anti-GQ1b antibodies, although the patient's serum contains anti-GQ1b reactivity and the LPS has a GQ1b-like structure. This may be due to the lack of immunogenicity of the LPS from the MF8 strain in rabbits. On Western blot, these animals showed a weaker anti-LPS response than rabbits that were immunized with LPS from other strains. It is improbable that the GQ1b-like epitopes on MF8 LPS were destroyed during extraction and purification because we confirmed the presence a GQ1b-like epitope on the LPS with a monoclonal anti-disialosyl antibody. Furthermore, in mice we were able to induce an anti-GQ1b response with the MF8 strain (CW Ang et al, unpublished observations). Ritter et al were unable to induce an anti-ganglioside response in four animals that were immunized with LPS from the O:23 and O:36 serostrains, both having a GM2-like LPS (Aspinall *et al.*, 1993b; Ritter *et al.*, 1996) The MF6 strain also has the O:23 serotype and remarkably, one of the MF6 immunized animals had a moderate anti-GM2 response.

There were considerable differences in the kinetics of the anti-ganglioside response when comparing strains and isotypes. An explanation for the differences between strains might be that rabbits more easily mount an immune response to GM1-like structures than to GQ1b-like structures. The titers of anti-GM1 and anti-GA1 antibodies were much higher than

the titers of anti-GQ1b antibodies. In animals that were immunized with GB18 and MF8 LPS, IgM and IgG responses against LPS and gangliosides deviated during the immunization period. IgG responses gradually increased and reached a high titer, while IgM responses remained at the same level or even decreased. This stepwise increase in IgG reactivity suggests a booster effect of the repeated immunizations and T cell involvement in the response to these non-protein antigens. In GBS and MFS patients, the IgG response against gangliosides and LPS is of the IgG1 and IgG3 subclass (Jacobs *et al.*, 1997a; Ogino *et al.*, 1995; Willison and Veitch, 1994; Yuki *et al.*, 1995b) Unfortunately, lack of information on IgG subclasses in rabbits and lack of specific reagents prohibited further investigation of this issue.

Contrary to our expectations, we observed a difference in the kinetics of anti-LPS and anti-ganglioside reactivity in some animals. One would expect a simultaneous rise in anti-LPS and anti-ganglioside reactivity because these antibodies are cross-reactive. There are several explanations for this phenomenon. First, the ELISA systems to detect anti-ganglioside and anti-LPS reactivity may have a differential sensitivity, thereby masking an early anti-ganglioside response that is still below the detection limit. Second, in the early phase of the immunization period, the anti-LPS antibodies may be directed against epitopes on the LPS molecule other than the ganglioside-like oligosaccharide while the specificity against the core oligosaccharide develops in the later phase of the immunization period.

During the 56 days of the experiment, none of the animals developed clear clinical signs of neuropathy. One animal that was immunized with GB18 LPS showed remarkable weight loss but had no clinical or histological signs of neuropathy. This lack of clinical symptoms despite the apparent good antibody response against LPS may depend on several factors. First, the animals may have had subclinical nerve damage detectable by electrophysiological examination only (Thomas *et al.*, 1991) Second, the duration of the experiment may have been too short for the animals to develop clinical symptoms. Some galactocerebroside and GD1b-immunized animals that developed neuropathy had the first clinical symptoms only after several months (Kusunoki *et al.*, 1996b; Saida *et al.*, 1979b), the period in which the animals in our experiment had already been sacrificed. Third, an intact blood nerve barrier might exclude the anti-ganglioside antibodies from the nerve and fourth, species differences in glycolipid composition might render the rabbit nerves relatively insensitive to the action of the anti-ganglioside antibodies.

In conclusion, we have demonstrated an anti-ganglioside response following immunization of rabbits with LPS from GBS and MFS-associated *C. jejuni* strains. The specificity of the anti-ganglioside antibodies in the rabbits is similar to that observed in the patients, thereby confirming the role of molecular mimicry in the development of anti-ganglioside antibodies in post-infectious neuropathy patients. The pathogenic potential of this anti-LPS/ganglioside response remains to be determined.

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# Chapter 13

# GANGLIOSIDE MIMICRY OF *CAMPYLOBACTER JEJUNI* LIPOPOLYSACCHARIDES DETERMINES THE ANTI-GANGLIOSIDE SPECIFICITY IN RABBITS

**CW Ang[1,2], PG Noordzij[1,2], MA de Klerk[1,2], HP Endtz[3], PA van Doorn[1], JD Laman[2]**

Departments of Neurology[1], Immunology[2], Medical Microbiology & Infectious Diseases[3], Erasmus University Rotterdam, The Netherlands

*Submitted*

## ABSTRACT

The core oligosaccharides of *Campylobacter jejuni* lipopolysaccharides (LPS) display molecular mimicry with gangliosides. Cross-reactive anti-LPS/anti-ganglioside antibodies have been implicated to show a crucial role in the pathogenesis of the Guillain-Barré and Miller Fisher syndrome. The specificity of the anti-ganglioside response response is thought to depend on the structure of the ganglioside mimic. To test this hypothesis and to investigate the potential of LPS from *Campylobacter* strains from enteritis patients to induce an anti-ganglioside response, we immunized 16 rabbits with purified LPS from 8 *Campylobacter jejuni* reference strains (Penner O:1, O:2, O:3, O:4, O:10, O:19, O:23 and O:36) with biochemically well-defined distinct ganglioside mimics and determined the presence of anti-ganglioside antibodies. All rabbits produced IgM and IgG anti-LPS antibodies and the specificity of the cross-reactive anti-ganglioside response indeed corresponded with the biochemically defined mimic. Exceptions were Penner O:23 LPS which did not induce an anti-ganglioside response despite a GM2-mimic and Penner O:10 LPS which induced a strong anti-GA1 response in contrast to its reported GD3-mimic. Most rabbits also had antibody reactivity against additional gangliosides and there were slight differences in the fine-specificity of the antibody response between rabbits that had been immunized with LPS from the same strain. High anti-LPS and anti-ganglioside titers persisited over a 10-month period. In conclusion, the structure of the LPS is the major determinant of the anti-ganglioside specificity. Other strain-specific as well as host-related factors determine the induction and fine-specificity of the cross-reactive anti-LPS/anti-ganglioside response.


## INTRODUCTION

The core oligosaccharide fraction of *Campylobacter jejuni* lipopolysaccharides (LPS) displays mimicry with mammalian gangliosides (Moran, 1997). An antibody response against these ganglioside-like structures leads to cross-reactive anti-LPS/ganglioside antibodies. The Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) are frequently preceded by an infection with *C. jejuni* and GBS and MFS patients have cross-reactive antibodies against LPS/gangliosides (Jacobs *et al.*, 1997b; Yuki, 1997). These antibodies have been implicated to play a role in the pathogenesis of both neurological diseases (Van der Meché and Van Doorn, 1995; Willison *et al.*, 1997).

The specificity of the anti-ganglioside antibodies differs between GBS and MFS patients. *Campylobacter*-related GBS patients have anti-ganglioside antibodies that react with GM1, GM1b, GalNAc-GD1a, GD1b and GD1a (Ang *et al.*, 1999; Ho *et al.*, 1999; Yuki *et al.*, 1996). In MFS patients, serum antibodies against GD3 and GQ1b occur in up to 90% of the cases (Willison and O'Hanlon, 1999). The differences in anti-ganglioside specificity are probably caused by differences in the LPS structure of the *Campylobacter* strain that triggered the

neurological disease. Inhibition studies demonstrated that anti-GM1 reactivity could be decreased following incubation with LPS from a GBS-related *Campylobacter* strain but not from a MFS-related strain. Conversely, anti-GQ1b reactivity could only be inhibited by incubation of serum with LPS from a MFS-related strain (Jacobs *et al.*, 1997b; Neisser *et al.*, 1997). In previous studies we have investigated whether the anti-ganglioside antibody response following immunization of rabbits with purified LPS from GBS and MFS-related strains (Ang *et al.*, 2000a; Ang *et al.*, 2000b). The exact biochemical structure of the LPS of these strains is not known but the rabbits had an anti-ganglioside response comparable to the anti-ganglioside response in the patients from which the strains were cultured (Chapter 12).

Using biochemical methods, LPS from a GBS-related *C. jejuni* strain was shown to contain a GM1-mimic, whereas LPS from a MFS-related strain had a GD3-mimic (Salloway *et al.*, 1996; Yuki *et al.*, 1993a). Mimics of GM1, GM2, GM3, GD1a and GD3 were also demonstrated in several *Campylobacter* reference strains of the Penner serotyping system, derived from patients with an uncomplicated enteritis (Moran *et al.*, 1996). For *C. jejuni* strains bearing a GM1 and GM2 mimic, Ritter et al demonstrated the ability to induce an anti-ganglioside response against gangliosides GM1 and GM2 respectively (Ritter *et al.*, 1996). Patients with an uncomplicated *C. jejuni* enteritis do have a low titer antibody response against LPS, but they do not have a cross-reactive anti-glycolipid response, in contrast to patients with GBS and MFS (Blaser and Perez-Perez, 1992; Jacobs *et al.*, 1997a).

In the present study, we investigated whether the specificity of the anti-ganglioside response in rabbits immunized with *Campylobacter* LPS corresponds with the biochemical structure of the ganglioside-mimic in the LPS. We used LPS from the O:1, O:2, O:3, O:4, O:10, O:19, O:23 and O:36 reference strains, all with a biochemically defined core-oligosaccharide structure.

## MATERIALS AND METHODS

### *Campylobacter* strains

Penner reference strains for serotypes O:1 (CCUG #10935, GM2 mimic) (Aspinall *et al.*, 1993b), O:2 (CCUG #10936, GM3-mimic) (Aspinall *et al.*, 1993b), O:3 (CCUG #10937, no ganglioside mimic) (Aspinall *et al.*, 1995), O:4 (CCUG #10938, GM1 and GD1a-mimic) (Aspinall *et al.*, 1994a; Aspinall *et al.*, 1993b), O:10 (CCUG #10943, GD3-mimic) (Aspinall *et al.*, 1993a; Nam Shin *et al.*, 1998), O:19 (CCUG #10950, GM1 and GD1a mimic) (Aspinall *et al.*, 1994b), O:23 (CCUG #10954, GM2-mimic) (Aspinall *et al.*, 1993b) and O:36 (CCUG #10966, GM2-mimic) (Aspinall *et al.*, 1993b) were used. A summary of the LPS structure and corresponding ganglioside-mimics is given in Figure 1.

For extraction of LPSs, bacteria were grown on blood agar plates with 5% sheep blood, at 37°C for 48 h under microaerophilic conditions. Cells were scraped from fresh grown plates and washed in phosphate-buffered saline (pH 7.8). LPSs were extracted with the hot phenol-water method of Westphal and Jann (Westphal and Jann, 1965). Combined water

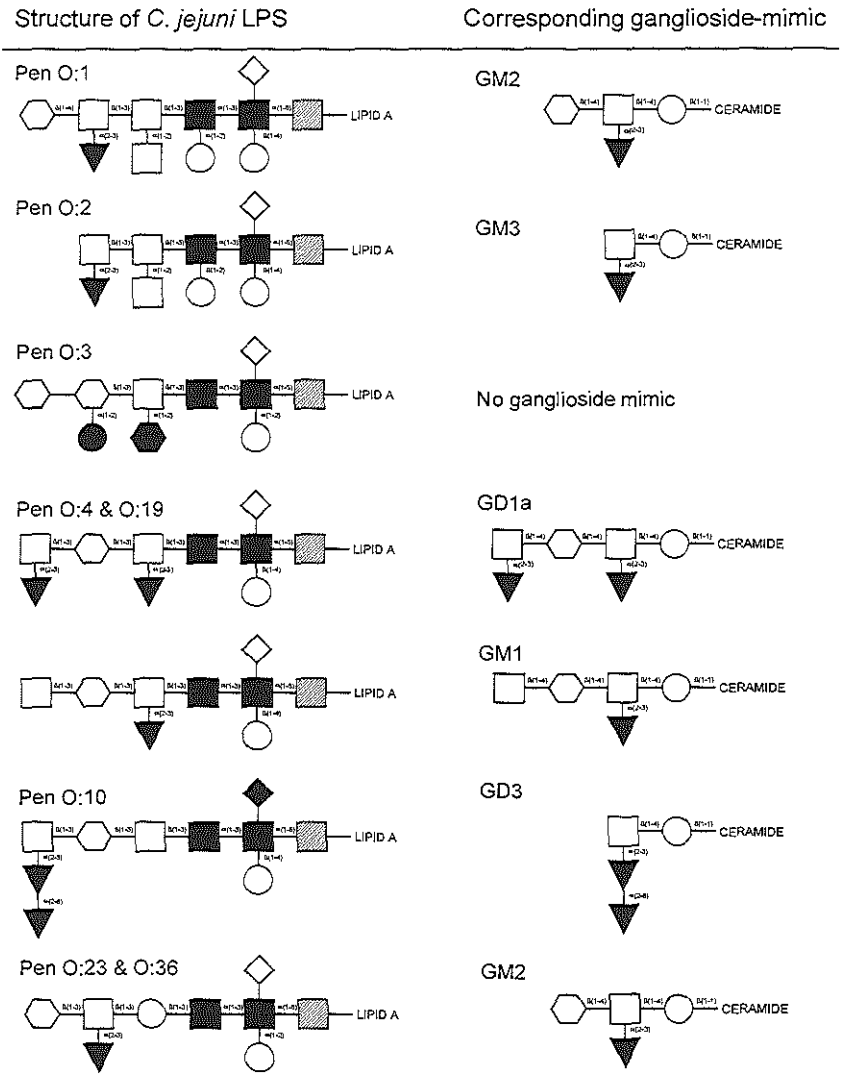


**Figure 1.** Structure of *Campylobacter jejuni* LPS used for immunization of rabbits and corresponding ganglioside mimics. ⬡ N-acetyl-galactosamine, □ galactose, ▼ sialic acid, ○ glucose, ◇ (phosphoryl)ethanolamine, ⬢ N-acetyl-glucosamine, ■ heptose, ▩ 3-deoxy-D-manno-octusolonic acid (KDO), ● dideoxyglucose, ◆ 2-aminoethylphosphate.

phases containing the extracted LPSs were dialyzed against water. After ultracentrifugation at 100,000 g for 2 h, the pellets were freeze-dried and weighed. After resuspension in water, all LPS fractions showed a single dense band migrating at 8-15 kD following electrophoresis on a polyacrylamide gel and silver staining (Novex, San Diego, CA), indicating the presence of LPS (Aspinall *et al.*, 1994a). To confirm the presence of ganglioside-like epitopes, LPSs from

all strains were tested in ELISA with a panel of polyclonal GM1/GA1 and GQ1b/GD3-reactive sera, cholera toxin (CT), peanut agglutinin (PNA) and a monoclonal anti-disialosyl antibody (Willison *et al.*, 1996).

**Immunization protocol**

New Zealand White rabbits (2.0-2.5 kgs) were immunized with 400 μg LPS in complete Freund's adjuvant (DIFCO Laboratories, Detroit, MI) as described before (Ang *et al.*, 2000b). Two animals were immunized with each LPS. Booster injections with the same amount of LPS in incomplete Freund's adjuvant (DIFCO) were given at days 14, 28 and 42 and 56. Blood was collected from the ear vein prior to each immunization. Follow up serum samples were taken every 2 months until 10 months after the first immunization. The faeces of all animals was cultured for *C. jejuni* prior to immunization and at day 56. This experiment was approved by the local animal ethics committee.

**Serology**

Antibody reactivity against GA1, GM1, GM2, GM3, GD1a, GD1b, GD3 and GQ1b was detected with ELISA and confirmed with thin-layer chromatography (TLC) as described before (Ang *et al.*, 2000b). The titer was defined as the highest serum dilution with an optical density (OD)>0.1, corrected for binding to uncoated wells. Anti-LPS reactivity was detected with ELISA and Western blot using diluted serum samples and affinity-purified anti-glycolipid antibodies as described before (Ang *et al.*, 2000b).

To assess cross-reactivity of anti-LPS antibodies with glycolipids, serum samples from immunized rabbits and from the patients from whom the *C. jejuni* strains were isolated were incubated with *C. jejuni* LPS conjugated to Octyl-Sepharose CL4B beads as described previously (Ang *et al.*, 2000b).

## RESULTS

All rabbits responded to the immunization with the production of IgM and IgG anti-LPS antibodies. One rabbit that was immunized with O:4 LPS had an adverse reaction following the second booster injection and was sacrificed at day 42. The stool cultures for *C. jejuni* were all negative, indicating that the antibodies had been induced by the immunization and not by a concurrent infection with *C. jejuni*. Data on anti-glycolipid antibody responses determined in serum taken at day 56 are summarized in Table 1. Immunization with LPS from the O:1, O:2, O:4, O:19 and O:36 serostrains resulted in an anti-glycolipid response with a specificity that could be inferred from the structure of the ganglioside-mimic in the LPS (Table 1). Rabbits that were immunized with O:1 and O:36 had an strong anti-GM2 antibody response. LPS from the O:19 serostrain induced IgM and IgG anti-GM1 antibodies. Rabbits that were immunized with O:2 LPS had an anti-GM3 response and one of the O:4 immunized animals had an IgM and IgG response against GD1a although the titers were low (Table 1).

**Table 1. Serum anti-glycolipid titers in rabbits immunized with *Campylobacter jejuni* LPS.**

| *C. jejuni* | IgM | | | | | | | IgG | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| strain | GA1 | GM1 | GM2 | GM3 | GD1a | GD3 | GQ1b | GA1 | GM1 | GM2 | GM3 | GD1a | GD3 | GQ1b |
| Pen 0:1 | [a]200 | 200 | 100 | [b]- | - | - | - | 100 | 800 | 25,600 | - | - | - | - |
| Pen 0:1 | 200 | 200 | 100 | - | - | - | - | - | 400 | 200 | - | - | - | - |
| Pen 0:2 | 400 | 100 | - | 100 | - | - | - | 400 | - | - | 100 | - | - | - |
| Pen 0:2 | 200 | 100 | - | - | - | - | - | 100 | - | - | 1,600 | 1,600 | - | - |
| Pen 0:3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Pen 0:3 | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| Pen 0:4 | 100 | - | - | - | 200 | - | - | 100 | - | - | - | 100 | - | - |
| Pen 0:4 | 200 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Pen 0:10 | 400 | - | - | - | - | - | - | >51,200 | - | - | - | - | - | - |
| Pen 0:10 | 200 | 100 | - | - | - | - | - | 51,200 | - | - | - | - | - | - |
| Pen 0:19 | 800 | 800 | - | - | - | - | - | >51,200 | 25,600 | 100 | 100 | - | - | - |
| Pen 0:19 | 400 | 800 | - | - | - | - | - | - | 3,200 | 100 | - | 1,600 | - | - |
| Pen 0:23 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Pen 0:23 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Pen 0:36 | 100 | - | - | - | - | - | - | - | - | 800 | - | - | - | - |
| Pen 0:36 | - | 200 | 200 | - | 800 | - | - | - | - | 800 | - | 400 | 400 | - |

Anti-ganglioside reactivity as can be expected from the corresponding ganglioside-mimic in the LPS is within boxes.
a. Results are expressed as titers.
b. titer <100.

Immunization with LPS from the O:3 serostrain, which does not bear any ganglioside-mimics, only resulted in a strong anti-LPS response, with no reactivity against any of the purified glycolipids.

In addition to this expected pattern of anti-ganglioside response, the specificity of the antibodies was broader than could be expected from the biochemical characterization of the LPS. The O:1 immunized animals both had additional anti-GM1 reacivity, and one of the rabbits that was immunized with O:3 LPS had an additional strong response against GD1a. The two animals that had received injections with O:19 LPS differed in the broadening of their anti-glycolipid specificity. One of the rabbits had, in addition to its anti-GM1 reactivity, a very strong response against GA1. The other rabbit had IgG antibodies against GD1a. Most patterns of broadening can be explained by sharing of similar di- or trisaccharides by the different gangliosides. Anti-GM2 antibodies may bind to the N-acetyl galactosamine ~ galactose ~ sialic acid trisaccharide which is also present in GM1 (Table 1).

There are two exceptions to the general pattern. Animals that were immunized with O:10 LPS did not mount a strong antibody response against GQ1b or GD3 except for a low titer IgM response during the first six weeks of the immunization procedure. In contrast, these animals produced high titer IgG GA1 antibodies already 14 days after the first immunization. The other exception is the lack of anti-ganglioside reactivity following immunization with O:23 LPS that contains a GM2-like structure (Tables 1, 2). Despite an IgM and IgG anti-LPS response, we could not detect antibody reactivity against any of the purified glycolipids.

Despite the presence of a GM2-mimic in O:1, O:23 and O:36, antisera had the highest titers against their homologous LPS (data not shown). Furthermore, on Western blot, purified anti-GM2 antibodies from a O:36 immunized animal, only reacted with O:36 LPS and not with O:1 or O:23 (data not shown).

To determine whether the induced anti-glycolipid antibodies are indeed cross-reactive with LPS, we performed inhibiton experiments using LPS-coated Sepharose beads. Incubation of antisera with beads coated with homologous LPS resulted in an inhibition of anti-glycolipid reactivity. The anti-glycolipid antibodies induced by O:1, O:2, O:4, O:19 and O:36 were all shown to be cross-reactive with their homologous LPS. The anti-GA1 reactivity in O:10 antiserum could not be decreased following incubation with O:10 LPS coated beads.

Antibody titers were followed until 10 months after the first immunization. Animals with IgG titers >800 have persisting anti-glycolipid reactivity. Lower titers of IgG antibodies and most of the IgM reactivity had decreased below detection level. During the whole immunization period, none of the animals developed any neurological signs such as limb weakness or ataxia.

## DISCUSSION

In accordance with the molecular mimicry hypothesis, immunization of rabbits with

*Campylobacter* LPSs mimicking different glycolipids leads to an anti-glycolipid response with a specificity that corresponds with the structure of the LPS. Our studies confirm and extend earlier reports using LPSs from *Campylobacter* strains isolated from GBS, MFS and uncomlicated enteritis patients (Ang *et al.*, 2000a; Goodyear *et al.*, 1999; Ritter *et al.*, 1996; Wirguin *et al.*, 1997).

In most rabbits, the anti-glycolipid response was not restricted to the biochemically defined mimic. There are several explanations for this broadened specificity. First, the purified LPS fraction we have used to immunized the rabbits may have contained LPS molecules displaying microheterogeneity in the ganglioside-mimicking core oligosaccharide. This may be due to incomplete biosynthesis of the LPS, loss of carbohydrate residues during the extraction and purification procedures or phase variation during culture (Aspinall *et al.*, 1994b; Linton *et al.*, 2000). All these mechanisms would lead to the expression of multiple LPS types with multiple ganglioside-mimics. For the O:1 serostrain, which bears a GM2-mimic, it has been decribed that due to phase variation in LPS bioysnthesis genes, isolated colonies revert to a cholera toxin (CT) binding phenotype (Linton *et al.*, 2000). CT does not bind to GM2 but has a high affinity for GM1, indicating that bulk quantities of LPS from the O:1 serostrain do not only contain GM2-mimics but also GM1-mimics. Serological studies using monoclonal anti-ganglioside antibodies to detect ganglioside-mimics in *Campylobacter* LPS have indicated that other serostrains such as O:4 also express multiple ganglioside mimics (Yuki *et al.*, 1994a).

Alternatively, the broadened specificity of the anti-glycolipid response may depend on the immune response of the rabbit. Animals that were immunized with highly purified glycolipids also demonstrate a slightly broadened antibody specificity (Prendergast *et al.*, 1998).

LPS from the O:10 serostrain did not induce high titer anti-GQ1b or anti-GD3 antibodies despite the reported GD3-mimic (Nam Shin *et al.*, 1998). Instead, the O:10-immunized animals had a strong anti-GA1 response. Purified O:10 LPS reacted with a monoclonal anti-GQ1b/GD3 antibody (data not shown) confirming the presence of a GD3-mimic in the preparation we used for the immunizations. In addition, the LPS also reacted with sera fro GBS patients containing anti-GM1 and anti-GA1 reactivity, indicating that the O:10 LPS also contains GA1-mimics (data not shown). However, depletion studies using O:10 LPS coated beads did not show inhibition of anti-GA1 reactivity by O:10 LPS. Therefore, we can not exclude that the anti-GA1 antibodies in O:10-immunized rabbits have been induced by an mechanism that is different from molecular mimicry. Polyclonal B cell activation by *Campylobacter* LPS seems unlikely because all immunized rabbits would have had high titer anti-GA1 antibodies.

We confirmed the results from previous studies that immunization with O:23 LPS did not induced an anti-GM2 antibody response, despite the biochemically defined presence of a GM2-mimic (Ritter *et al.*, 1996). It was unable to demonstrate binding of monoclonal anti-GM2 antibodies with O:23 LPS in Western blot or thin-layer chromatography, indicating the absence of a GM2-mimic (Ritter *et al.*, 1996; Yuki *et al.*, 1994a). Another explanation may be that differences in density of GM2-mimics and the adjuvant properties of the lipid A por-

tion and/or O-chain influence the induction of anti-glycolipid antibodies and the presentation of the GM2-mimics in the Western blot between the O:1, O:23 and O:36 serostrains (Ritter *et al.*, 1996; Willison *et al.*, 1997).

None of the rabbits showed any neurological signs during the 10-month follow-up period. Although this argues against a pathogenic role for anti-ganglioside antibodies there are several factors that might explain the lack of clinical symptoms. Several reports have indicated that a putative pathogenic role of anti-glycolipid antibodies is complement-mediated (Plomp *et al.*, 1999; Weber *et al.*, 2000). Ritter et al showed that the IgG anti-GM2 antibodies induced with the current immunization protocol had only a low level of complement-dependent cytotoxicity (Ritter *et al.*, 1996). Another factor might be the presence of an intact blood-nerve barrier, preventing access of anti-glycolipid antibodies to their presumed site of action. In addition, in contrast to a mucosal infection with the whole *Campylobacter* organism, systemic immunization with purified LPS might not generate additional T cell responses that may be needed to induce an immune mediated attack on the nerve (Pollard *et al.*, 1995).

We did not find a difference in the antibody response of rabbits to LPSs derived from *Campylobacter* strains from GBS patients compared to enteritis patients without neurological symptoms (Ang *et al.*, 2000a). This indicates that there are no intrinsic differences between LPSs from both groups of *Campylobacter* strains. The differential response to glycolipids in GBS and MFS patients compared to uncomplicated enteritis patients therefore probably depends on other factors. In the gut of uncomplicated enteritis patients, the density of ganglioside-mimics may be lower or absent, thereby not invoking a response. Alternatively, host-dependent factors such as polymorphisms in immune-response genes, may determine the induction of a cross-reactive anti-LPS/glycolipid response and concurrent neurological symptoms (Jeremias *et al.*, 1999; Pandey and Blaser, 1986; Wassenaar *et al.*, 1997).

In conclusion, this study demonstrates that the LPS structure is a major determinant of the specificity of a cross-reactive anti-LPS/glycolipid response. However, the equivalent capacity of LPS from enteritis strains and GBS or MFS strains to induce cross-reactive antibodies indicates that the sole presence of molecular mimicry between LPS and peripheral nerve glycolipids is not sufficient to induce an anti-glycolipid response in humans with GBS or MFS.

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# Chapter 14

## GENERAL DISCUSSION

- The relations between antecedent infections, anti-glycolipid reactivity and the pattern of clinical symptoms
- Ganglioside mimicry and the induction of anti-ganglioside antibodies by *Campylobacter jejuni*
- Are anti-glycolipid antibodies pathogenic?
- Directions for future research
- Conclusions

GBS and MFS are frequently preceded by infections. The studies described in this thesis have addressed the hypothesis that following infections, due to molecular mimicry between infectious agents and nervous tissue, cross-reactive anti-glycolipid antibodies are induced that contribute to nerve damage. This final chapter consists of four parts. In the first part, the evidence for heterogeneity of GBS based on different cross-reactive anti-glycolipid antibodies in subgroups of GBS patients defined by antecedent infection and clinical features will be reviewed. In the second part, molecular mimicry between peripheral nerve glycolipids and *C. jejuni* and the induction of anti-glycolipid antibodies in animals immunized with *Campylobacter* LPS will be discussed. In the third part, collective evidence for a role of anti-glycolipid antibodies in the pathogenesis of GBS and MFS will be critically evaluated. Finally, directions for future research will be indicated.

## THE RELATIONS BETWEEN ANTECEDENT INFECTIONS, ANTI-GLYCOLIPID REACTIVITY AND THE PATTERN OF CLINICAL SYMPTOMS

*Campylobacter jejuni*

In GBS patients, the specificity of anti-glycolipid antibodies is related with distinct antecedent infections and patterns of clinical symptoms. This is most prominent in the group of GBS patients infected with *C. jejuni*. In a large group of GBS patients, antecedent *Campylobacter* infections and the presence of diarrhea were significantly associated with antibody reactivity against GM1, GalNAc-GD1a and GM1b (Chapters 2-5, 7; Hao *et al.*, 1998; Jacobs *et al.*, 1996b; Rees *et al.*, 1995a). Furthermore, Dutch GBS patients with anti-GM1/GalNAc-GD1a/GM1b antibodies had a motor neuropathy with predominant distal weakness and showed a favorable response to intravenous immunoglobulins (IVIg) similar to Japanese patients (Hao *et al.*, 1999; Kaida *et al.*, 2000; Kuwabara *et al.*, 1998a). However, we could not confirm the correlation between anti-GM1/GalNAc-GD1a/GM1b reactivity and features of axonal GBS in the Dutch population as was described in several cohorts of Japanese patients (Kaida *et al.*, 2000; Kusunoki *et al.*, 1994; Yuki *et al.*, 1996).

The three gangliosides GM1, GalNAc-GD1a and GM1b share a similar tri-saccharide (Figure 3, Introduction) and groups of patients with either anti-GM1, anti-GalNAc-GD1a or anti-GM1b antibodies have similar clinical features, indicating that these differences in fine specificity of the antibodies do not influence the pattern of clinical features. An explanation for this might be that *C. jejuni* LPS does not bear exact GM1-mimics but GM1-like mimics, including GalNAc-GD1a and GM1b, thus leading to the induction of the corresponding antibodies (Chapter 10; Yuki *et al.*, 1996). Alternatively, host-related factors may influence the fine specificity of the anti-ganglioside response, leading to subtle differences in the anti-ganglioside specificity.

From the studies described in Chapters 2-5, it is not clear whether the association of

antibodies against GM1/GalNAc-GD1a/GM1b with motor neuropathy is mediated by the preceding *Campylobacter* infection or by the specificity of the anti-ganglioside response. In our study population very few patients without *Campylobacter* infections have antibodies with anti-GM1/GalNAc-GD1a/GM1b specificity but studies in Japanese patients indicate that the anti-ganglioside specificity and not the antecedent *Campylobacter* infection determines the pattern of clinical features (Ogawara *et al.*, 2000). We also found that in most GBS patients the anti-ganglioside reactivity was directed against GM1b and not GM1 (Chapter 5). Furthermore, many patients had antibody reactivity against more than one ganglioside. The abundance of reports describing the detection of anti-GM1 reactivity in serum from GBS patients does not necessarily mean that GM1 is the target-antigen in the nerve but merely reflects the commercial availability of purified GM1.

Strong evidence in favor of anti-ganglioside specificity and not just the presence of *Campylobacter* as a key factor in determining the pattern of clinical features is the observation that *Campylobacter*-infected MFS patients do not have anti-GM1 but anti-GQ1b antibodies (Chapter 10; Chiba *et al.*, 1993; Jacobs *et al.*, 1997b; Willison *et al.*, 1993b; Yuki *et al.*, 1997b). The presence of anti-GM1 antibodies in GBS patients and anti-GQ1b antibodies in MFS patients is correlated with the ganglioside mimic of the LPS of the strains with which the patients were infected. This indicates that strain differences can influence the pattern of clinical features, presumably mediated by anti-ganglioside antibodies with different specificity (Chapter 10).

*Cytomegalovirus*

Cytomegalovirus (CMV) infections are related to anti-GM2 antibodies and a subpopulation of GBS patients that is characterized by younger age, a higher proportion of females and a severe motor/sensory form of GBS (Irie *et al.*, 1996; Jacobs *et al.*, 1998; Khalili-Shirazi *et al.*, 1999; Visser *et al.*, 1996; Yuki and Tagawa, 1998). The anti-GM2 antibodies cross-react with CMV-infected fibroblasts (Chapter 8; Irie *et al.*, 1996). This strongly suggests that the anti-GM2 antibodies are indeed induced by molecular mimicry. The nature of the GM2-mimic expressed by CMV or CMV-infected tissue has not yet been identified. Viral glycoproteins may carry a GM2-like oligosaccharide. Alternatively, GM2 or GM2-bearing glycoproteins in the hosT cell membrane of infected cells may become immunogenic after virus infection due to the expression of GM2-mimicking viral glycoproteins, an upregulation of GM2 expression, or an altered ganglioside composition of the cells (Bai *et al.*, 1992; Evans and Webb, 1986; Lehmann *et al.*, 1992). Due to the limited number of patients with anti-GM2 antibodies, it was not possible to determine specific clinical features related to the presence of anti-GM2 antibodies. An increase in size of the studied GBS population will make such inferences feasible.

In *Campylobacter*, the expression of ganglioside mimics is strain dependent (Chapter 10). Although we found differences in the capacity of several CMV strains to decrease anti-GM2 reactivity in an inhibition assay, the small number of GBS-associated CMV strains has not been investigated for specific features. The capacity of laboratory CMV strain AD169 to

inhibit GM2-reactivity indicates that GM2-mimics are not exclusively expressed on GBS-associated CMV strains (Chapter 8).

*Mycoplasma pneumoniae*

Antibodies against galactocerebroside (GalC) are associated with antecedent infections with *M. pneumoniae* (Chapter 6; Kusunoki *et al.*, 1995, 1996c; Nishimura *et al.*, 1996b). The anti-GalC antibodies cross-react with *Mycoplasma* antigens, implying molecular mimicry between GalC and *Mycoplasma* as a possible mechanism for the induction of anti-GalC antibodies (Chapter 6). Anti-GalC reactivity could not be related to a specific subgroup of patients but most anti-GalC positive patients had an EMG with demyelinating features (Chapter 6). This observation is in concordance with the demyelinating effects of GalC-immunization, intraneural injection and ex vivo/in vitro application of anti-GalC antisera (Hahn *et al.*, 1993; Saida *et al.*, 1979b; Sergott *et al.*, 1984; Stoll *et al.*, 1986; Sumner *et al.*, 1982).

In conclusion, anti-glycolipid antibodies in GBS patients are related to specific antecedent infections, specific patterns of clinical symptoms and response to therapy (Figure 1). In these different subgroups, the anti-glycolipid antibodies cross-react with components of the corresponding infectious agents. This serves as strong evidence for the hypothesis that all glycolipid antibodies have been induced by molecular mimicry and that the specificity of the anti-glycolipid response is determined by the structure of the mimic on the corresponding pathogen.

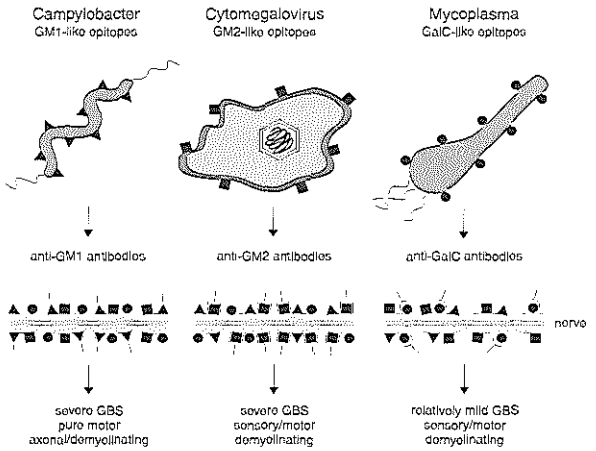


**Figure 1.** Proposed scheme for the role of differences between microbial agents regarding molecular mimics of peripheral nerve glycolipids, the presence of specific anti-glycolipid antibodies and heterogeneity in the Guillain-Barré syndrome.

# GANGLIOSIDE MIMICRY AND THE INDUCTION OF ANTI-GANGLIOSIDE ANTIBODIES BY *CAMPYLOBACTER JEJUNI*

A strong antibody response against ganglioside-mimics in *C. jejuni* LPS is associated with the development of neurological symptoms (Chapters 7, 10). For such a response to occur, the following conditions must be met. First, the *Campylobacter* strain must have a ganglioside-mimic (Chapters 12, 13); second, the mimic has to be immunogenic and third, the patient must mount an antibody response against the ganglioside-mimic (Chapters 7, 10). These factors will be discussed below.

*Campylobacter-related factors*

We investigated the presence of ganglioside-mimics in *Campylobacter* strains from GBS and MFS patients and compared this with strains from uncomplicated *Campylobacter* enteritis patients. Almost all GBS/MFS-related strains display ganglioside mimicry. 85% of GBS-related strains have a GM1-like epitope compared to 57% of *Campylobacter* strains from patients with an uncomplicated enteritis. For GQ1b-like epitopes and MFS this difference is even more pronounced (100% vs 9%; Chapter 10). This suggests that ganglioside mimicry by *C. jejuni* LPS is a risk factor for the development of neurological symptoms following *Campylobacter* infections. Furthermore, this observation indicates an important role for anti-ganglioside antibodies in the pathogenesis of *Campylobacter*-associated neuropathy.

Previous studies have investigated the presence of GM1-like epitopes on strains from neuropathy patients and from uncomplicated enteritis patients (Nishimura *et al.*, 1997; Sheikh *et al.*, 1998). In those studies no difference in expression of ganglioside-mimics was found between neuropathy associated strains and control strains but this may have been due to the methods of sampling of the control strains and the small numbers of neuropathy strains (Nishimura *et al.*, 1997; Sheikh *et al.*, 1998).

In Chapter 10 we confirmed previous reports that ganglioside-mimics can also be found on strains from uncomplicated enteritis patients (Moran, 1997; Nachamkin *et al.*, 1999b; Wirguin *et al.*, 1994; Yuki *et al.*, 1994a). In addition, LPSs from neuropathy patients and uncomplicated enteritis patients were equally able to induce a cross-reactive anti-LPS/glycolipid response in rabbits (Chapters 11-13; Ritter *et al.*, 1996). This leads to the conclusion that other factors, either *Campylobacter* or host-related, determine the induction or suppression of an anti-glycolipid response in patients with a *Campylobacter* enteritis. These other *Campylobacter*-related factors presumably concern the immunogenicity of the ganglioside-mimic.

Which factors may contribute to an increased immunogenicity of ganglioside-mimics on *Campylobacter*? It has been argued that a higher expression or slightly different chemical composition of other surface structures such as the O-chain, may contribute to an increase in immunogenicity of ganglioside mimics (Moran and O'Malley, 1995; Moran and Prendergast, 1998; Yuki, 1997). For example the presence of β-N-acetylglucosamine in *C. jejuni* has been

suggested to differ between neuropathy associated strains and strains from control enteritis patients with the O:19 serotype (Kuroki *et al.*, 1993).

Another mechanism that has been put forward as a possible enhancement of immunogenicity of LPS is the production of a ganglioside-binding toxin by *Campylobacter* (Daikoku *et al.*, 1990; Wassenaar, 1997; Willison and Kennedy, 1993). An immune response against the toxin would provide the $CD4^+$ T cell help that is needed to induce the isotype switch to an IgG1 and IgG3 anti-ganglioside response (Stavnezer, 1996; Willison and Kennedy, 1993; Zinkernagel, 2000).

Despite the focus on the cross-reactivity of anti-glycolipid antibodies with *Campylobacter* LPS, these anti-glycolipid antibodies are not necessarily induced by LPS. Glycoproteins of *Campylobacter*, including flagellin, also have sialic acid moieties (Doig *et al.*, 1996; Guerry, 1997; Guerry *et al.*, 1996). Furthermore, the enzymes that are responsible for glycosylation of lipids and proteins may be the same (Szymanski *et al.*, 1999). These results indicate that LPS may not be the only *Campylobacter* surface structure bearing ganglioside-mimics. The protein component of a glycoprotein with a ganglioside mimic may, like an LPS-binding toxin, provide T cell help for the IgG isotype switch.

A last factor that may influence the immunogenicity of ganglioside mimics is the number and distribution of mimics on the surface of a bacterium. Highly repetitive and organized surface structures are superior in inducing an antibody response to unorganized structures (Bachmann and Zinkernagel, 1996; Fehr *et al.*, 1998). There is currently no information about the quantitative expression of ganglioside-mimics in neuropathy-associated *Campylobacter* strains compared to strains from uncomplicated enteritis cases.

To test the hypothesis of the presence of a putative "GBS/MFS-factor" in neuropathy-associated *Campylobacter* strains, we investigated whether there were any special characteristics of GBS and MFS-associated *Campylobacter* strains. For this purpose we used additional serological and genetic typing methods (Penner and Hennessy, 1980). Penner serotyping of strains from Dutch GBS and MFS patients demonstrated a heterogeneous Penner serotype distribution, roughly the same as found in uncomplicated enteritis patients in the Netherlands (Chapter 9). The results from the Penner-serotyping are in contrast with the predominance of O:19 strains in Japan and O:41 strains in South Africa because only two *Campylobacter* strains from Dutch GBS patients had the O:19 serotype (Lastovica *et al.*, 1997; Nishimura *et al.*, 1996a, 1997). The Penner serotyping results indicate that in addition to O:19 and O:41 strains, other serotypes also have the potential to induce anti-ganglioside antibodies and neurological symptoms.

The genetic analysis was in concordance with the results from the Penner serotyping and also demonstrated a heterogeneous population of *Campylobacter* strains. There was no evidence for clustering of the GBS and MFS associated strains using several genotyping methods (flaA PCR-RFLP, AFLP, RAPD and PFGE) when comparing with strains from uncomplicated enteritis patients and chickens (Chapter 9; Duim *et al.*, 2000). Thus, the results from the genetic analyses did not support the hypothesis that GBS and MFS are caused by a subgroup of genetically related *Campylobacter* strains.

*Host-pathogen interactions*

Which other factors could be involved in the development of GBS following exposure of an individual to a potentially ganglioside-mimicking strain?

It is possible that ganglioside-mimicking LPS is normally not or less abundantly expressed by bacteria in the gut although the *Campylobacter* strains express the ganglioside-mimic when grown in vitro. The interaction between bacterium and host in the gut might lead to a tightly regulated expression of ganglioside mimics (Chart *et al.*, 1996; Preston *et al.*, 1996). It can be argued that molecular mimicry by micro-organisms occurs to escape immune detection (Damian, 1964; Moran *et al.*, 1996). An example of this phenomenon is that Lewis antigen expression in *Helicobacter pylori* depends on the Lewis phenotype of the carrier (Wirth *et al.*, 1997). The same might be true for the expression of ganglioside-mimics by *Campylobacter*. In the case of post-*Campylobacter* neuropathy, this mechanism of host-induced ganglioside-mimicry may have failed, leading to the improper expression of ganglioside mimics ("failure of molecular mimicry"), invoking an anti-LPS/ganglioside response rather than misleading the immune system by sharing of molecular structures.

Another mechanism that may be involved in the regulation of expression of ganglioside mimics is phase variation in LPS-biosynthesis genes (Linton *et al.*, 2000). It has been shown that the difference between a GM1-like LPS and a GM2-like LPS or a GD3-like and a GT1a-like LPS results from frame-shift mutations in genes coding for galactosyltransferases (Gilbert *et al.*, 2000; Linton *et al.*, 2000). In the family outbreak study described in Chapter 7, such a phenomenon may have occurred. The two family members with uncomplicated enteritis had their episode of *C. jejuni* enteritis just before the GBS patient. It is possible that both strains expressed a GM2-like epitope in the gut of the uncomplicated enteritis patients and a GM1-like epitope in the gut of the GBS patient. Surprisingly, the brother of the GBS patient had a low titer of anti-GM2 antibodies while the GBS patient himself had high titer anti-GA1 and anti-GM1 antibodies. The strains isolated from the father and the brother were shown to have a GM1-like epitope when the strains were grown in vitro but phase variation may have taken place during culture. It would be interesting to investigate the strains isolated from the father and the brother for the presence of phase-variation and the stools from all three members of the family for the presence of *C. jejuni* mimicking GM1 and GM2.

*Host-related factors*

In a study of Japanese *Campylobacter* strains from GBS patients, it was shown that infection with genotypically identical *Campylobacter* O:19 strains lead to an axonal GBS as well as a "classic" (demyelinating) GBS (Nishimura et al., 1997). This indicates that other factors, presumably host-related, determine the pattern of clinical features.

An example of a genetically determined response is the difference in quality and quantity of the antibody response between different inbred mouse strains following immunization or infection (Ljungqvist *et al.*, 1988; Markham and Pier, 1983; Reiner and Locksley, 1995; Velge-Roussel *et al.*, 1997). Knowledge about genetic factors that determine the antibody

response may help to identify genetic risk factors for susceptibility to develop GBS following infections and genetic factors that influence the severity of the disease, response to therapy and residual symptoms. Polymorphisms in genes that encode molecules which are essential for the response to ganglioside-mimics may determine the eventual outcome of an infection of a ganglioside-bearing *Campylobacter*: induction of cross-reactive antibodies or not.

Genetic factors of an individual person or a certain strain of mice may influence the ability to respond to infections and may also be involved in determining the severity and outcome of the disease and the response to therapy (Brinkman *et al.*, 1997; Jeremias *et al.*, 1999; Sawcer *et al.*, 1997). Polymorphisms in the IL-1ß and TNF-ß genes have been shown to lead to a different phenotype with regard to cytokine production and have been linked to progression of autoimmune diseases (Bouma *et al.*, 1996; Pociot *et al.*, 1991; 1992; Schrijver *et al.*, 1999). A pilot-study showed that homozygosity for the Fc-γ-IIaH131 polymorphism was related to a severe form of GBS (Van der Pol *et al.*, 2000). We have recently started a large-scale study on immune response gene polymorphisms in GBS patients and their relation to antecedent infections, anti-glycolipid responses, the pattern of clinical features and residual disability.

In addition to the more static genetic determinants, other host-related factors concerning the immune system may determine the development of post-infectious immune-mediated disease. The functional status of the immune system fluctuates in time as a function of the relative activity of many cells and molecules. This is clearly demonstrated by the observation that environmental factors such as stress or additional infections greatly influence the capacity of the immune system to deal with microbiological invaders (Cohen *et al.*, 1991; Glaser and Glaser, 1994; Riley, 1999). Priming or activation of the immune system by other infections may increase the susceptibility for autoimmune phenomena and predispose to a deviant immune response as seen in GBS patients (Rook and Stanford, 1998; Whitton and Fujinami, 1999).

*Anti-glycolipid responses in an LPS immunization model*

Immunization of rabbits with *Campylobacter jejuni* LPS leads to a cross-reactive anti-LPS/glycolipid response (Chapters 11-13). The anti-glycolipid antibodies specifically cross-react with the LPS used for immunizations and the specificity of the anti-ganglioside response is determined by the structure of the LPS. Most important, the specificity of the anti-glycolipid response in rabbits closely resembled the anti-glycolipid specificity in the patients from whom the *Campylobacter* strains were cultured (Chapter 12). Most rabbits that were immunized responded to LPS with the induction of cross-reactive anti-glycolipid antibodies. However, LPS from Penner serostrain O:23 did not induce an anti-glycolipid response despite the presence of a GM2-mimic in the LPS from this strain (Chapter 13; Ritter *et al.*, 1996). This indicates that other LPS-related factors co-determine the immunogenicity of a ganglioside mimic.

The anti-ganglioside response in this systemic immunization model using a high dose of LPS in a strong adjuvant consists of high titer IgG and low titer IgM antibodies, in con-

trast to what would be expected for ganglioside-like antigens (Alfonso *et al.*, 1995; Weng *et al.*, 1994). In rabbits, no IgG subclasses have been identified so we could not investigate whether the subclass distribution of the IgG anti-LPS/ganglioside antibodies in rabbits resembles the predominance of IgG1 and IgG3 anti-glycolipid antibodies in GBS and MFS patients (Garcia Guijo *et al.*, 1992; Jacobs *et al.*, 1997a; Ogino *et al.*, 1995; Willison and Veitch, 1994; Yuki *et al.*, 1995b). The presence of these high titer IgG antibodies in immunized rabbits and in neuropathy patients suggests the involvement of T cells in the induction of the anti-ganglioside response (Stavnezer, 1996). The role of $CD4^+$ T-helper cells and co-stimulation in the induction of cross-reactive anti-LPS/ganglioside antibodies can be better investigated in the mouse. This species is immunologically better characterized than the rabbit and the availability of genetically modified mouse strains allows us to investigate several aspects of the immune-response in detail.

The present immunization experiments also provided evidence for host-related factors in the anti-ganglioside antibody response. When pairs of rabbits were immunized with LPS from the same *Campylobacter* strain, a slightly different fine-specificity of the anti-ganglioside response could be observed between rabbits (Chapters 11-13). Furthermore, we found that LPS from strains GB17, from a GBS patient and strain MF6, from an MFS patient, closely resembled each other both genetically and regarding the presence of ganglioside mimics (Chapters 9, 10). Both strains express a GA1 and a GD3/GQ1b mimic. However, in patient GB17 the strain elicited an anti-GA1 response and in patient MF6 an anti-GQ1b response. In rabbits, both strains induced a strong anti-GA1 response and a weaker anti-GD3 or anti-GQ1b response (Chapter 12). This can be explained by any of the *Campylobacter* or host-related factors described above. In addition, species differences might exist. In our studies, rabbits generally responded much stronger to asialo- and monosialogangliosides such as GA1, GM1 and GM2 than to complex gangliosides with disialosyl groups such as GD3 and GQ1b (Chapters 11-13). Additional evidence for species differences in the specificity of an anti-glycolipid response is provided by the observation that mice respond preferentially to these disialosyl LPSs compared to GM1-mimicking LPSs (Ang and De Vos, unpublished observations).

In rabbits that were immunized with LPS from uncomplicated enteritis patients bearing GM2 and GM3 mimics, a corresponding anti-ganglioside response was induced. However, in *Campylobacter*-induced GBS and MFS cases, anti-GM2 and anti-GM3 responses were absent or low (Chapter 10). This indicates that GM2 and GM3-mimics on *Campylobacter* may not be immunogenic in humans, in contrast to GM2-mimics on CMV (Chapter 8; Jacobs *et al.*, 1997d; Yuki and Tagawa, 1998).

In conclusion, ganglioside-mimicry in *Campylobacter*-strains can be considered as a risk factor for the development of neurological symptoms. Ganglioside-mimicry is necessary but not sufficient to induce a cross-reactive anti-LPS/ganglioside response. The difference in LPS structure between *C. jejuni* strains from GBS and MFS patients and the correspondingly distinct specificity of the anti-ganglioside response suggests a causal link between gan-

glioside-mimicry, the specificity of the anti-ganglioside response and the pattern of clinical symptoms. The ability of strains from uncomplicated enteritis cases to express ganglioside-mimics and to induce an anti-ganglioside response in rabbits indicates that other factors, *Campylobacter*-related, host-related or both, determine the induction and the fine-specificity of a cross-reactive anti-LPS/ganglioside antibody response.

Additional serological and genetic typing did not identify a *Campylobacter*-related factor that could explain the differential response to ganglioside-mimics in GBS/MFS patients compared to uncomplicated enteritis patients. Analysis of specific genes will become feasible now that the complete sequence of the *Campylobacter* genome is available (Parkhill *et al.*, 2000). The resolution of the genotyping methods we have used in the current studies may have been insufficient to detect any special genetic features of GBS and MFS-associated strains, if present.

## ARE ANTI-GLYCOLIPID ANTIBODIES PATHOGENIC?

In this thesis it is argued that anti-glycolipid antibodies can be induced by an antecedent infection with a micro-organism expressing glycolipid-mimicking structures. Furthermore, the specificity of the anti-glycolipid response is associated with clinical subtypes. An unresolved issue is the relation between the presence of circulating anti-glycolipid antibodies and neurological symptoms in GBS and MFS patients. In other words: do the anti-glycolipid antibodies cause disease? In the following section, the arguments in favor and the arguments against a pathogenic role of anti-glycolipid antibodies will be critically discussed.

**Evidence in favor**

***Anti-ganglioside antibodies are consistently found in serum from patients with immune-mediated neuropathies.*** Many studies have demonstrated the presence of anti-ganglioside reactivity in GBS and MFS patients from countries in different parts of the world (Ho *et al.*, 1998; Hughes *et al.*, 1999; Quarles and Weiss, 1999).

***Differences in anti-glycolipid specificity are related with specific pattern of clinical features.*** Several studies have described a relation between the specificity of anti-ganglioside antibodies and the pattern of clinical features. Anti-GM1/GM1b/GalNAc-GD1a antibody reactivity is associated with motor GBS, anti-GD1a with axonal involvement in Chinese GBS patients and anti-GQ1b with MFS and oculomotor weakness in GBS patients (Chapters 2, 3; Carpo *et al.*, 1998; Chiba *et al.*, 1993; Ho *et al.*, 1999; Jacobs *et al.*, 1996b; Kaida *et al.*, 2000; Kuwabara *et al.*, 1998b; Visser *et al.*, 1995; Willison and O'Hanlon, 1999).

***Anti-ganglioside antibodies bind to nerve and motor neurons.*** Studies with polyclonal and monoclonal antibodies show that anti-ganglioside antibodies with different specificity are able to bind to nodes of Ranvier, myelin, dorsal root ganglion cells and motor neurons (Chiba *et al.*, 1993; Corbo *et al.*, 1993; Kusunoki *et al.*, 1993; O'Hanlon *et al.*, 1996,

1998; Willison *et al.*, 1996).

***Anti-ganglioside antibodies can fix complement.*** The isotype and IgG1 and IgG3 subclass distribution of the anti-glycolipid antibodies indicates that they are capable of binding complement (Jacobs *et al.*, 1997a; Papadea and Check, 1989; Willison and Veitch, 1994). Both in vivo and in vitro studies have demonstrated the binding of activated complement by anti-GM1 and anti-GQ1b antibodies (Paparounas *et al.*, 1999; Uetz-Von Allmen *et al.*, 1998). Histological studies of biopsies from GBS patients with axonal features revealed the presence of immunoglobulin deposits and complement on the axonal surface, although the exact specificity of the immunoglobulins is not known (Hafer-Macko *et al.*, 1996a). In addition, some of the effects of anti-ganglioside antibodies on neurophysiological parameters have been shown to be complement dependent (Plomp *et al.*, 1999; Takigawa *et al.*, 1995; Weber *et al.*, 2000).

***Anti-ganglioside antibodies can interfere with neurophysiological processes.*** In vitro and ex vivo studies indicate that anti-GM1 and anti-GQ1b antibodies can block ion-channel function and disturb neuromuscular transmission (Arasaki *et al.*, 1998; Goodyear *et al.*, 1999; Plomp *et al.*, 1999; Takigawa *et al.*, 1995; Weber *et al.*, 2000; Willison *et al.*, 1997).

***Anti-glycolipid antibodies can impair blood-nerve barrier function.*** Endoneurial blood vessels contain gangliosides that can be the target of circulating anti-glycolipid antibodies (Kanda *et al.*, 1997, 2000b). Anti-GM1 antibodies can impair the function of a blood-nerve barrier model and may also have an effect on disruption of the blood-nerve barrier in GBS (Kanda *et al.*, 2000a). Finally, anti-glycolipid antibodies and activated T cells may synergistically alter the blood-nerve barrier function (Pollard *et al.*, 1995; Spies *et al.*, 1995).

**Evidence against**

***Not all GBS patients have anti-glycolipid antibodies.*** A substantial proportion of GBS patients does not have anti-glycolipid antibodies (Hao *et al.*, 1998; Jacobs *et al.*, 1998). The cumulative percentage of patients with antibody reactivity against any of 13 different glycolipids tested in our laboratory is about 50% (Chapters 2, 3, 5, 6; Jacobs *et al.*, 1998). There is a possibility that the other 50% of the GBS patients do not have anti-glycolipid reactivity. On the other hand, we have tested only a limited quantity of the more than 30 species of glycoconjugates that are present in neurons, suggesting that the cumulative percentage may increase when testing more glycolipid species (Ledeen, 1985). At least in *Campylobacter*-related GBS this seems to be the situation. Almost all *Campylobacter*-related GBS patients had anti-LPS reactivity against the LPS from the strain they had been infected with, despite the lack of antibody reactivity in some patients against purified glycolipids (Chapter 10). These patients may have antibodies directed against yet unknown glycolipid antigens. Alternatively, other anti-neural antibodies, directed against protein antigens and not glycolipids may be present in these other patients (Gabriel *et al.*, 2000; Hughes *et al.*, 1984, 1999; Khalili-Shirazi *et al.*, 1993). Thus, the absence of anti-glycolipid reactivity in some patient groups does therefore not undermine the potential pathogenicity of anti-glycolipid antibodies.

***Anti-ganglioside antibodies are not disease specific.*** Anti-glycolipid antibodies occur in HIV-infected patients (Petratos *et al.*, 1998; Wu *et al.*, 2000). GM2-vaccines induce IgG

and IgM anti-GM2 antibodies in melanoma patients without inducing neurological symptoms (Chapman *et al.*, 2000). Furthermore, there are many other diseases in which an elevated titer of anti-GM1 antibodies has been demonstrated (Bansal *et al.*, 1994; Endo *et al.*, 1984; Sadiq *et al.*, 1990).

***Anti-glycolipid antibodies occur in healthy individuals.*** Depending on the detection method used, it is possible to demonstrate anti-glycolipid reactivity in healthy individuals (Garcia Guijo *et al.*, 1995; Marcus, 1990; Willison *et al.*, 1993a). However, in most cases the antibody reactivity is directed against asialo-glycolipids (Garcia Guijo *et al.*, 1995; Ang and Jacobs, unpublished observations). This reactivity may represent natural antibodies which may be very important in the first line of defence against infections (Ochsenbein *et al.*, 1999). Alternatively, anti-glycolipid reactivity in healthy persons may be linked to antecedent infections without neurological symptoms.

***Anti-glycolipid reactivity does not always correlate with clinical severity.*** Although in most studies a relation was found between reduction of anti-ganglioside antibody titers and clinical improvement, some GBS patients have persistent high titers of anti-ganglioside antibodies despite obvious clinical improvement (Jacobs *et al.*, 1997a, 1998; Nobile-Orazio *et al.*, 1992; Ang, unpublished observations).

***Anti-glycolipid specificity does not always correlate with the pattern of clinical features.*** Despite the correlation of anti-GM1/GM1b/GalNAc-GD1a reactivity with pure motor GBS, not all patients with antibody reactivity against these glycolipids have this pattern of clinical features and vice versa (Chapters 2, 3). The same situation applies to anti-GQ1b reactivity and the presence of oculomotor symptoms (Carpo *et al.*, 1998). These inconsistencies may be explained by the observed differences in binding of anti-glycolipid antibodies in different conditions. Binding of serum antibodies at 4°C in an ELISA system may only partially reflect the binding properties of the antibodies in vivo (Willison *et al.*, 1997).

***Effects of anti-glycolipid antibodies in model systems do not reflect the pattern of clinical symptoms in patients.*** In the mouse phrenic nerve-diaphragm model, application of anti-GQ1b antibodies ultimately leads to paralysis of the diaphragm. Patients with anti-GQ1b antibody reactivity have oculomotor weakness instead of weakness of the diaphragm (Carpo *et al.*, 1998; Chiba *et al.*, 1993). The difference in symptoms between patients and the experimental situation leaves the possibility that the effect of anti-GQ1b antibodies might not be related to the clinical symptoms in MFS and GBS patients. However, species differences in expression of GQ1b-like epitopes may account for the observed inconsistencies.

***Animals with high titer anti-ganglioside antibodies do not develop a neuropathy.*** Immunization-studies with *Campylobacter* LPS indicate that animals with high titer circulating anti-ganglioside antibodies do not develop weakness (Chapters 11-13; Goodyear *et al.*, 1999; Ritter *et al.*, 1996; Wirguin *et al.*, 1997). Furthermore, our histological analysis does not show nerve damage or an inflammatory infiltrate (Chapters 11, 12). However, several factors may prevent a pathological action of anti-ganglioside antibodies. First, the antibodies may not reach the targets in the nerve due to an intact blood-nerve barrier (Hughes *et al.*, 1999). Second, the subclass distribution and consequent physiological properties of the anti-

ganglioside antibodies may differ between humans and rabbits. Third, a mucosal immune response and concomitant response against other antigens or a more generalized activation of the immune system may be necessary for impairment of the blood-nerve barrier and development of clinical symptoms (Hartung and Kieseier, 2000; Pollard *et al.*, 1995; Westland *et al.*, 1999).

Thorough examination of the current knowledge on anti-glycolipid antibodies and neurological dysfunction leads to the conclusion that although there is strong evidence for a pathogenic role of molecular mimicry-induced anti-glycolipid antibodies, a causal role remains unproven. Experimental data indicate that anti-glycolipid antibodies can bind to peripheral nerves, activate complement and affect neurophysiological function. The relationship between immunoglobulin deposits in nerves from GBS patients and the presence of neurological symptoms remains unclear.

Some issues remain unexplained but it must be kept in mind that as yet, for none of the human immune-mediated diseases mentioned in Table 1 of the Introduction (page 14), a definite role for molecular-mimicry induced antibodies and/or T cells has been demonstrated (Rose and Bona, 1993; Rose and Mackay, 2000; Whitton and Fujinami, 1999). It is therefore a challenge to further explore the mechanisms of the induction of anti-glycolipid responses and pathogenic action of anti-glycolipid antibodies.

**Table 1. Evidence in favor and against a pathogenic role of anti-glycolipid antibodies.**

| In favor | Against |
|---|---|
| Consistent presence in serum of GBS patients | No uniform marker |
| Relation between specificity and clinical features | No disease specificity |
| Binding to nerve | Present in healthy persons |
| Interference with physiological functions | No absolute correlation with clinical severity |
| Impairment of blood nerve barrier | No absolute correlation with clinical features |
| | Effect on neurophysiology not directly related to symptoms in patients |
| | Induction of anti-glycolipid antibodies in animals does not lead to neurological symptoms |

## DIRECTIONS FOR FUTURE RESEARCH

Which experiments should be performed to further analyze the role of molecular mimicry-induced anti-glycolipid antibodies in the pathogenesis of GBS and MFS?

### Determining the role of anti-glycolipid antibodies in demyelination and axonal degeneration

It is currently not known whether the immunoglobulin deposits in the nerve that can be found in biopsies and autopsy material from GBS patients contain anti-ganglioside antibodies. Therefore, new and available techniques using labelled glycolipids could be used to

identify the specificity of these immunoglobulin deposits. Furthermore, although anti-glycolipid antibodies bind to several structures in the nerve and cause electrophysiological dysfunction, their exact target is unknown. Immunoprecipitation and/or expression systems for neuronal molecules can be used to address these issues (Kwa *et al.*, 2000; Lugaresi *et al.*, 1991). Finally, the in vitro and ex vivo assays that to study the action of anti-ganglioside antibodies on neurophysiological parameters should be further developed to investigate the relationship between the observed effects in the models and clinical observations in patients (Paparounas *et al.*, 1999; Weber *et al.*, 2000).

**Developing an animal model for GBS/MFS based on infection or immunization with bacterial/viral antigens**

In the current animal model for GBS, experimental allergic neuritis, the relationship with antecedent infections is not taken into account (Vriesendorp, 1997). Therefore, to study the post-infectious divergence of the immune response towards nerve antigens, it is necessary to develop a disease model by infection or immunization of animals with an infectious agent. *C. jejuni* would be the first choice infectious agent for this purpose because weakness in animals following experimental *Campylobacter jejuni* infection has been described and because of the obvious link with anti-glycolipid antibodies (Li *et al.*, 1996; Yuki, 1997). The conclusive experiment to demonstrate the crucial role of ganglioside-mimicry in the pathogenesis of GBS could be performed in such a model. Animals should be infected with a ganglioside-bearing *Campylobacter* strain resulting in an immune-mediated neuropathy. If ganglioside-mimicry is crucial for the induction of neurological symptoms, infection with the adequate control, a genetically modified mutant version of this GBS-causing strain lacking the ganglioside mimic, will not lead to disease. In addition, using transgenic and knock-out mice, the routing and handling of glycoconjugates can be investigated. Special emphasis should be given to studies on the role of T cells in the anti-glycoconjugate response.

**Developing experimental systems to investigate the human immune-response to carbohydrate antigens at the B and T cell level**

To understand why only a small proportion of people develop a neuropathy after infection with a ganglioside-bearing infectious agent, it is important to study the normal immune response against glycoconjugates (glycolipids and glycoproteins). To investigate host-specific responses to glycoconjugates, in vitro systems should be developed, in which it is possible to compare the response of former GBS-patients with anti-glycolipid antibodies with patients who experienced an uncomplicated enteritis with the same infectious agent (Heidenreich *et al.*, 1994; Wassenaar *et al.*, 1997).

Furthermore, it is unknown whether GBS patients have any circulating or infiltrating T cells directed against glycolipids. Tetramer technology and the use of glycolipid-pulsed antigen presenting cells will allow the detection of circulating and nerve-infiltrating T cells that are specific for GM1 and other glycolipids (Benlagha *et al.*, 2000; Shamshiev *et al.*, 1999; Shamshiev *et al.*, 2000).

## CONCLUSIONS

The working hypothesis for the current studies was that acute immune-mediated neuropathies are caused by anti-glycolipid antibodies that are induced by molecular mimicry between microbial and peripheral nerve antigens. The nature of the antecedent infections determines the anti-glycolipid specificity and consequently the pattern of clinical symptoms. The studies described in this thesis lead to the following conclusions relating to the research questions formulated in the last part of the Introduction.

1. There is a relation between anti-glycolipid reactivity, specific antecedent infections and specific clinical features in patients with GBS (Chapters 2, 3, 4, 5, 6). Antibodies against GM1, GM1b and GalNAc-GD1a are associated with antecedent infection with *C. jejuni*, and a predominant distal motor neuropathy. Anti-GM2 antibodies are associated with CMV infections and anti-GalC antibodies with antecedent infection with *M. pneumoniae*.
2. The frequency of antecedent *C. jejuni* infections and anti-glycolipid reactivity between GBS patients from Japan and The Netherlands does not differ. However, there are slight differences in isotype distribution and fine specificity of the anti-ganglioside antibodies between patients from both countries (Chapters 4, 5).
3. Anti-GM2 and anti-GalC antibodies in CMV and *Mycoplasma*-infected patients cross-react with antigens from the corresponding infectious agents (Chapters 6, 8). These observations suggest that all anti-glycolipid antibodies have been induced by infections with different microbiological agents.
4. GBS and MFS-associated *C. jejuni* strains more frequently display ganglioside mimicry than strains from uncomplicated enteritis patients. However, we could not identify specific serological or genetic determinants that discriminate neuropathy-associated *Campylobacter* strains from other strains (Chapters 9, 10).
5. The observations mentioned under 4. indicate that ganglioside mimicry in necessary but not sufficient to induce a cross-reactive anti-LPS/ganglioside response. Host-pathogen interactions and/or host-specific responses to ganglioside-mimics after *C. jejuni*-infection eventually direct an aberrant response to ganglioside-mimics (Chapters 7, 10).
6. Immunization of rabbits with *C. jejuni* LPS induces a cross-reactive anti-glycolipid/LPS response. The specificity of the anti-glycolipid response is determined by the structure of the LPS. Despite the induction of high titer anti-glycolipid antibodies the animals did not develop clinical symptoms indicating that the mere presence of anti-glycolipid antibodies does not induce neurological symptoms. Additional activity of other components of the immune-system is probably needed to induce neurological disease (Chapters 11, 12, 13).

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# ABBREVIATIONS

| | |
|---|---|
| AFLP | amplified fragment length polymorphism |
| AIDP | acute inflammatory demyelinating polyneuropathy |
| AMAN | acute motor axonal neuropathy |
| AMSAN | acute motor sensory axonal neuropathy |
| BSA | bovine serum albumin |
| CER | ceramide |
| CFA | complete Freund's adjuvant |
| CIDP | chronic inflammatory demyelinating polyneuropathy |
| CJ | *Campylobacter jejuni* |
| CJC | *Campylobacter jejuni* control |
| CMV | cytomegalovirus |
| CT | cholera toxin |
| EAN | experimental allergic neuritis |
| EBV | Epstein-Barr virus |
| ELISA | enzyme-linked immunosorbent assay |
| EMG | electromyogram |
| Ent | enteritis |
| GA1 | asialo-GM1 |
| GalC | galactocerebroside |
| GBS | Guillain-Barré syndrome |
| HC | healthy control |
| HLA | human leucocyte antigen |
| HSV | herpes simplex virus |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| IgM | immunoglobulin M |
| IFN | interferon |
| IL | interleukin |
| IVIg | intravenous immunoglobulins |
| KDO | 3-deoxy-D-manno-octusolonic acid |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| MAG | myelin-associated glycoprotein |
| MFS | Miller Fisher syndrome |
| MGUS-PNP | neuropathy associated with monoclonal gammopathy of undetermined significance |
| MHC | major histocompatibility complex |
| MMN | multifocal motor neuropathy |
| MP | *Mycoplasma pneumoniae* |
| MPC | *Mycoplasma pneumoniae* control |
| MRC | Medical Research Council |

| | |
|---|---|
| NK | natural killer |
| ns | not significant |
| nt | not tested |
| OD | optical density |
| OND | other neurological disease |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PE | plasma exchange |
| PFGE | pulsed-field gel electrophoresis |
| PNA | peanut agglutinin |
| RAPD | random amplified polymorphic DNA |
| RFLP | restriction fragment length polymorphism |
| TGF | transforming growth factor |
| TLC | thin-layer chromatography |
| TNF | tumor necrosis factor |
| TT | tetanus toxin |
| URTI | upper respiratory tract infection |
| UT | untypeable |
| VZV | varicella-zoster virus |

# SUMMARY

The Guillain-Barré syndrome (GBS) is an acute immune-mediated neuropathy with a heterogeneous clinical presentation. Approximately two-thirds of the GBS patients report symptoms of an infectious illness in the weeks before the development of neurological disease. *Campylobacter jejuni*, cytomegalovirus (CMV) and *Mycoplasma pneumoniae* are the most frequent identified infectious agents.

Antibodies against different peripheral nerve glycolipids such as gangliosides GM1, GM2 and GQ1b are present in serum of patients with GBS and its cranial nerve variants such as the Miller Fisher syndrome (MFS). These anti-glycolipid antibodies bind to nerve and have an effect on nerve conduction and neuromuscular transmission. Therefore, anti-glycolipid antibodies are thought to play a crucial role in the pathogenesis of GBS. In *C. jejuni* infected GBS and MFS patients, anti-glycolipid antibodies cross-react with *C. jejuni* LPS. In addition, *C. jejuni* LPS contains ganglioside-like structures. This suggests that the anti-glycolipid antibodies are induced by molecular mimicry between *C. jejuni* LPS and peripheral nerve glycolipids.

Molecular mimicry refers to a structural resemblance between host and pathogen. Due to this resemblance, an antibody and/or T cell response that is originally directed against the invading pathogen also leads to an immune-mediated attack on host tissue. **The hypothesis addressed in this thesis is that the structure of the ganglioside mimic of an infectious agent determines the specificity of the anti-glycolipid antibody response, and consequently the pattern of clinical features.**

The studies described in this thesis can be divided into three parts. In the first part (**Chapters 2-8**), it was investigated whether anti-glycolipid reactivity in a large group of clinically well defined GBS patients could be related to specific antecedent infections and patterns of clinical features. It was found that antibodies against GM1, GM1b and GalNAc-GD1a were linked to antecedent infections with *C. jejuni*. Furthermore, patients with antibodies against any of these three glycolipids had a motor neuropathy with predominant distal weakness and no cranial nerve deficits. With regard to the frequency of *C. jejuni* infections, anti-GM1, GM1b or GalNAc-GD1a reactivity, there was no difference between Japanese and Dutch GBS patients (**Chapters 4, 5**).

**Chapter 6** describes the relation between *M. pneumoniae* infections and antibody reactivity against galactocerebroside (GalC). These anti-GalC antibodies were shown to cross-react with *M. pneumoniae* antigens. In addition, CMV-infected patients frequently have antibodies against GM2. In **Chapter 8**, it was shown that these anti-GM2 antibodies cross-react with CMV-infected fibroblasts, providing strong evidence that anti-GM2 antibodies are induced by molecular mimicry.

Together, these studies indicate that all anti-glycolipid antibodies in GBS patients may have been induced by molecular mimicry with different pathogens. Furthermore, these studies provide evidence for the hypothesis that clinical heterogeneity of GBS is mediated by anti-

glycolipid antibodies with different specificity and binding patterns.

The second part of the thesis investigates *C. jejuni* strains that were isolated from GBS and MFS patients from The Netherlands (**Chapters 9, 10**). Only a small proportion of individuals that are infected with *C. jejuni* develop anti-glycolipid antibodies and neurological symptoms (**Chapter 7**). Therefore, it was investigated whether the GBS/MFS-related strains have specific properties or constitute a subgroup of *C. jejuni* strains. We developed new serotyping methods to compare the expression of ganglioside-mimics in LPS between *C. jejuni* strains from GBS/MFS patients and cases with an uncomplicated *C. jejuni* enteritis. Although ganglioside-mimicry was also detected on strains from uncomplicated enteritis patients, GBS/MFS-related strains displayed ganglioside-mimicry more frequently. Furthermore, LPS from GBS and MFS patients have distinct ganglioside-mimicking structures. GBS patients had a GM1-like LPS whereas MFS patients had a GQ1b-like LPS. The difference in LPS-structure corresponds with the anti-glycolipid antibody specificity and pattern of clinical features of GBS and MFS patients. Additional genotyping methods did not provide evidence for clustering of GBS/MFS-related *C. jejuni* strains (**Chapter 9**). Thus, ganglioside-mimicry is a risk factor for the development of neurological symptoms following infection with *C. jejuni*. However, the presence of ganglioside-mimics in strains from uncomplicated enteritis patients indicates that ganglioside-mimicry is necessary but not sufficient to induce GBS or MFS. Furthermore, for the Dutch situation there is no indication that there is a neuropathic subgroup of *C. jejuni* strains.

In the third part of the thesis (**Chapters 11-13**), it was demonstrated that immunization of rabbits with *C. jejuni* LPS leads to a cross-reactive anti-LPS/glycolipid antibody response. In accordance with the molecular mimicry hypothesis, the structure of the LPS is the major determinant of the specificity of the anti-glycolipid response. Moreover, the anti-glycolipid specificity in rabbits closely resembled the anti-glycolipid specificity in the patients from whom the strains were isolated. None of the immunized animals showed any neurological symptoms and LPS from *C. jejuni* strains isolated from uncomplicated enteritis patients had the same capacity of anti-glycolipid antibody induction. This indicates that the presence of circulating anti-glycolipid antibodies alone can not explain the neurological deficits of GBS patients. Additional factors such as activated T cells and/or a dysfunction of the blood-nerve barrier are also needed for the anti-glycolipid antibodies to cause nerve dysfunction.

Collectively, the studies described in this thesis provides ample evidence for a causal role of molecular mimicry-induced anti-glycolipid antibodies in the pathogenesis of GBS and its clinical variants. Different antecedent infections are related to specific anti-glycolipid antibodies and patterns of clinical features. The absence of clinical symptoms in animals with circulating anti-glycolipid antibodies indicates that additional factors are required to induce nerve dysfunction following infections. Expanded knowledge of cross-reactive anti-glycolipid antibody responses following infections and the action of anti-glycolipid antibodies in GBS patients will elucidate the role of molecular mimicry between microbial and peripheral nerve glycoconjugates in the pathogenesis of GBS.

# SAMENVATTING

Het Guillain-Barré syndroom (GBS) is een immuun-gemedieerde aandoening van het perifere zenuwstelsel, die zich uit in een verlamming van de ledematen en een variabele aantasting van de sensibele functies. De klinische presentatie van GBS is heterogeen. De ernst van de verlamming verschilt sterk tussen patiënten en een exclusieve aantasting van de motore zenuwen (puur motore GBS) of van hersenzenuwen is ook mogelijk. De meest voorkomende hersenzenuwvariant is het Miller Fisher syndroom (MFS), gekenmerkt door oogbolmotoriek stoornissen. Ongeveer tweederde van de GBS patiënten heeft een infectie doorgemaakt in de weken voorafgaand aan de neurologische stoornissen. *Campylobacter jejuni*, cytomegalovirus (CMV) en *Mycoplasma pneumoniae* zijn de meest frequent voorkomende microbiologische verwekkers. *C. jejuni* komt voornamelijk voor op kipproducten en in vervuild drinkwater en infectie met *C. jejuni* leidt tot een gastro-enteritis. CMV infecties leiden tot een griepachtig ziektebeeld en *M. pneumoniae* veroorzaakt een atypische pneumonie.

In serum van patiënten met GBS zijn antistoffen tegen glycolipiden aanwezig. Deze glycolipiden zoals GM1, GM2 en GQ1b vormen een belangrijk bestanddeel van zowel axon als myeline. Vanwege het vermogen van anti-glycolipide antistoffen om te kunnen binden aan de zenuw en het effect van anti-glycolipide antistoffen in diverse neurofysiologische modellen wordt gedacht dat deze antistoffen een cruciale rol spelen in de pathogenese van GBS. Enkele jaren geleden werd aangetoond dat de lipopolysaccharide (LPS) fractie van *C. jejuni* zeer sterke structurele overeenkomst vertoont met glycolipiden in de zenuw.

Met de term moleculaire mimicry wordt een overeenkomst in structuur tussen microbe en gastheer-weefsel aangeduid. Antistoffen en/of T cellen die oorspronkelijk gericht zijn tegen microbiële structuren kruisreageren met lichaamseigen moleculen, leidend tot een immuun-gemedieerde aanval op lichaamseigen weefsel, in dit geval de zenuw. **In dit proefschrift wordt onderzocht of anti-glycolipide antistoffen bij GBS patiënten zijn geïnduceerd door de voorafgaande infecties door middel van "moleculaire mimicry".** De moleculaire mimicry hypothese vormt ook een verklaring voor de klinische heterogeniteit van GBS. Verschillende infectieuze agentia met verschillende glycolipide-mimics induceren verschillende anti-glycolipide antistoffen. Door verschillende bindingskarakteristieken zal een verschil in anti-glycolipide specificiteit mogelijk leiden tot een verschil in klinische verschijnselen.

In het eerste deel van het proefschrift wordt aangetoond dat verschillende voorafgaande infecties inderdaad gerelateerd zijn aan specifieke anti-glycolipide antistoffen en patronen van klinische verschijnselen. Anti-GM1, GM1b en GalNAc-GD1a antistof reactiviteit is gerelateerd aan voorafgaande infecties met *C. jejuni* en de puur motore vorm van GBS, zonder hersenzenuwuitval. CMV-infecties zijn geassocieerd met anti-GM2 antistoffen en een ernstige vorm van GBS met zowel motore als sensibele uitval. Antistof reactiviteit tegen galactocerebroside is gerelateerd aan voorafgaande *M. pneumoniae* infecties. Voor al

deze verschillende soorten anti-glycolipide antistoffen werd aangetoond dat ze kruisreageren met de corresponderende bacterie of virus. Dit is een sterke aanwijzing dat anti-glycolipide antistoffen bij GBS patiënten ontstaan door moleculaire mimicry met microbiële structuren.

Omdat niet iedereen met een *C. jejuni* infectie GBS krijgt werd in het tweede deel van het proefschrift onderzocht of *C. jejuni* bacteriën van GBS en MFS patiënten specifieke eigenschappen hebben. De aanwezigheid van GM1 en GQ1b-achtige structuren in de LPS fractie van *C. jejuni* bacteriën van GBS repectievelijk MFS patiënten correleert zeer sterk met de aanwezigheid van anti-GM1 en anti-GQ1b antistoffen bij GBS en MFS patiënten. GM1 en GQ1b-achtige structuren kwamen vaker voor bij *C. jejuni* bacteriën van GBS/MFS patiënten dan bij patiënten met een ongecompliceerde *C. jejuni* infectie, hoewel het voorkomen van GM1 en/of GQ1b-achtige structuren niet exclusief was voorbehouden aan GBS/MFS gerelateerde *C. jejuni* stammen. Dit impliceert dat mimicry tussen *C. jejuni* LPS en zenuwweefsel noodzakelijk maar niet voldoende is om te leiden tot neurologische verschijnselen. Genetische typering van de *C. jejuni* stammen leverde geen aanwijzingen op voor het bestaan van een genetisch gerelateerde subgroep *C. jejuni* stammen die in staat zou zijn om neurologische symptomen uit te lokken.

In het derde deel van het proefschrift werd aangetoond dat konijnen die werden geïmmuniseerd met gezuiverd *C. jejuni* LPS inderdaad worden aangezet tot de productie van anti-glycolipide antistoffen. Ondanks de aanwezigheid van circulerende anti-glycolipide antistoffen vertoonden de konijnen geen neurologische symptomen.

Het werk beschreven in dit proefschrift toont aan dat er zeer veel aanwijzingen zijn voor een causale rol van anti-glycolipide antistoffen, geinduceerd door moleculaire mimicry, bij het ontstaan van GBS. Verschillende infecties zijn gerelateerd aan specifieke anti-glycolipide antistoffen en patronen van klinische verschijnselen. Het ontbreken van klinische verschijnselen bij proefdieren met circulerende anti-glycolipide antistoffen wijst erop dat andere factoren zoals geactiveerde T cellen en/of een dysfunctie van de bloed-zenuw barrière ook benodigd zijn voor het ontstaan van immuun-gemedieerde zenuwschade na infecties. Verder onderzoek naar de inductie van anti-glycolipide antistoffen na infecties en de pathogene mechanismen van anti-glycolipide antistoffen is noodzakelijk om de rol van moleculaire mimicry in de pathogenese van GBS verder te verhelderen.

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## CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 26 juli 1969 te Hoogeveen. Hij haalde in 1987 zijn VWO diploma aan het Menso Alting College te Hoogeveen en begon hetzelfde jaar aan de studie Geneeskunde aan de Vrije Universiteit te Amsterdam. In 1989 begon hij met de studie Medische Biologie, tevens aan de Vrije Universiteit. Hij deed wetenschappelijke stages op de afdeling Neuroanatomie van de Vrije Universiteit (o.l.v. Prof. dr. MP Witter) en op het Nederlands Instituut voor Hersenonderzoek (o.l.v. Dr FW van Leeuwen). In 1993 werd het doctoraalexamen Geneeskunde behaald. Het daaropvolgende jaar studeerde hij af als Medisch Bioloog. Na het afleggen van het arts-examen in 1996 (Vrije Universiteit, Amsterdam) begon hij op de afdelingen Neurologie (Prof dr van der Meché) en Immunologie (Prof dr R Benner) van de Erasmus Universiteit te Rotterdam aan het promotie-onderzoek dat wordt beschreven in dit proefschrift. Begin 2001 zal hij starten met de opleiding tot arts-microbioloog in het Dijkzigt Ziekenhuis te Rotterdam (Prof dr Verbrugh).

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