Immunogenetic polymorphisms in Guillain-Barré syndrome

Immunogenetische polymorfismen in het Guillain-Barré syndroom

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Voor mijn ouders

Immunogenetic polymorphisms in Guillain-Barré syndrome

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Chapter 1

General introduction

Guillain-Barré syndrome

Guillain-Barré syndrome (GBS) is a heterogeneous post-infectious immune-mediated polyneuropathy, characterized by an acute flaccid paralysis and loss of deep tendon reflexes. The clinical entity was described for the first time by Jean-Baptiste Octave Landry in 1859.[1] In 1916, Georges Guillain, Jean-Alexandre Barré and André Strohl reported patients with an acute ascending paresis with an increased protein level with normal cell count in the cerebrospinal fluid (CSF).[2] Since that year, the entity is referred to as GBS. The last few decades research groups focused on the important role of antecedent infections and the immune system in the pathogenesis of GBS. Pathogen-derived factors as well as host factors are likely to be involved in determining whether a person develops GBS after a common infection. This thesis focuses on the contribution of genetic host factors in the pathogenesis of GBS.

Diagnosis, course and treatment

The diagnosis of GBS is mainly based on its clinical characteristics: a progressive symmetric muscle weakness in more than one limb in combination with a loss or decrease of tendon reflexes. A variety of other clinical signs and symptoms, such as sensory and/or cranial deficits and autonomic dysfunction can occur. Most patients have an increased protein level in CSF by the second week of disease, but this is not required to confirm the diagnosis (see Appendix 1).[3]

The maximum level of weakness in GBS patients by definition should be reached within 4 weeks. The maximum weakness is also called the nadir. Two to four weeks after the patients reach their nadir, spontaneous recovery usually starts. Recovery, however, can take months and many patients have residual deficits.

Monitoring the respiratory capacity and autonomic functions of GBS patients is of great importance to prevent severe complications or even death by respiratory or autonomic dysfunction. The mortality rate of GBS is about 2-5%. Besides general supportive care, immune-modulating therapy helps to improve patients who are severely affected (unable to walk unaided). Several randomized controlled trials reported a beneficial effect on outcome by plasma exchange and intravenous immunoglobulins (IVIg).[4-7] A recent trial demonstrated that the combination of IVIg and methylprednisolone might be superior in those patients who are in the first two weeks of disease and unable to walk unaided and who have no contra-indication for the use of high dose steroids.[8]

Incidence/Epidemiology

GBS is the most common cause of a flaccid paralysis in the Western world. In The Netherlands, about 200 new cases are diagnosed per year (incidence rate 1.18/100.000/year).[9] GBS may affect persons of all ages, but the incidence slightly increases with age and GBS is slightly more common in males than females. Although some studies have indicated that GBS tends to occur more often during the colder months,

seasonal variation in general is not reported. Two exceptions are: a higher incidence of GBS in summer occurs in Northern-China,[10] and during the wet season in Curaçao.[11]

Heterogeneity of Guillain-Barré syndrome

Clinical features

GBS is a very heterogeneous disorder with respect to the severity and extent of clinical features and the prognosis. Some patients have a mild weakness of distal extremities, which recovers spontaneously without residual deficits. Other patients develop within a day a rapidly progressive weakness leading to tetraparalysis, ophthalmoplegia, diplegia facialis, bulbar weakness or respiratory deficiency for which they need ventilation and long term treatment in intensive care units and later in rehabilitation centers. The patients also vary with respect to the peripheral nerves involved, i.e. the extent of sensory deficits and cranial nerve involvement. This is also illustrated by the presence of some variants of GBS. Some patients have only distal weakness, while others have facial or bulbar weakness. Other patients have a clinical triad of ophthalmoplegia, areflexia and ataxia referred to as Miller-Fisher syndrome (MFS).[12] Some patients may have minor symptoms and other patients may need respiratory support (20-30%).[9, 13] Although GBS is considered to be a self-limiting disorder, most patients have residual deficits. Only about 35% of the patients fully recover, 35% have a mild handicap and about 30% remain with a severe handicap during years of follow-up.[14] Moreover, about 80% of the patients who have neurologically well recovered complain about severe fatigue.[15] This clinical heterogeneity makes it difficult to predict to what extent the patient will be disabled, both during the acute phase of disease and during long-term follow-up.

Electrophysiology

Electrophysiological characteristics are also diverse. Some patients have a reduced conduction velocity and partial conduction block indicative of a demyelinating form of GBS, while others have a reduction in distally evoked compound muscle action potentials with relatively spared conduction velocity in combination with other features of an axonal form of GBS.[16] Moreover, results of a single-fiber electrodiagnostic examination (EMG) study surprisingly indicated that the neuromuscular junction may also be involved in the pathophysiology of GBS.[17]

Patients fulfilling the electrophysiological criteria of demyelination are referred to as having acute inflammatory demyelinating polyneuropathy (AIDP), which is the most common form of GBS in the Western-world (56-87%).[16] Patients fulfilling the electrophysiological criteria of an axonal degeneration without demyelination are referred as having an acute motor axonal neuropathy (AMAN) or an acute motor-sensory axonal neuropathy (AMSAN) when sensory deficits are also present. These forms of GBS are most frequently found in Chinese and Japanese GBS patients, but also in patients from Central-America and India.[18, 19] Axonal degeneration can also occur secondary to severe demyelination.

Pathology

Multifocal and segmental infiltration with predominantly macrophages in spinal roots and peripheral nerves has been shown in cases with primary demyelination (AIDP). The macrophages invade and strip off the myelin sheaths.[20] Complement deposits can be found on the outer membrane of the Schwann cell.[21] In severe cases, the axon degenerates as well. In the case of primary axonal damage (AMAN), the macrophages are present under an intact myelin layer and complement depositions are present on the axolemma of the motor fibres with minimal mononuclear infiltration.[20, 22]

Pathogenesis of Guillain-Barré syndrome

Antecedent infections

GBS is a typical post-infectious disorder. About 2/3 of the patients report an upper respiratory tract infection (URTI), diarrhea or influenza-like symptoms one to three weeks prior to the onset of weakness. The monophasic course of GBS also illustrates the role of infections. The most commonly found pathogens are *Campylobacter jejuni*, cytomegalovirus (CMV), Epstein-Barr virus (EBV), *Mycoplasma pneumoniae* and *Haemophilus influenzae*.[23, 24] Although several case-reports have suggested that GBS in some patients is provoked by the administration of a ganglioside mixture, certain vaccinations, pregnancy, surgery or cancer, these relations are anecdotal.[25] The delay between infection and onset of symptoms suggests that not the infection itself but the immune response to the infectious agent plays a role in the pathogenesis of GBS. In addition there is no evidence for direct infection of the peripheral nerves.

Anti-ganglioside antibodies

Antibodies directed to gangliosides can be detected in serum from subgroups of GBS patients. Gangliosides are glycosphingolipids consisting of a ceramide moiety, that is anchored in the plasma membrane and a sialic acid containing oligosaccharide, which is located extracellularly.[26] Gangliosides are widely distributed and abundantly expressed in neuronal plasma membranes and play a role in cell growth and differentiation.[27]

Most antibodies directed to these gangliosides are not only present in serum from GBS patients, but can also be found in low titers in serum from healthy controls and in a variety of patients with other neurological or autoimmune disorders.[28] In serum from healthy controls these antibodies generally are of the IgM isotype suggesting that they belong to the naturally occurring antibody repertoire.[29] These antibodies may play a role in first line defense against invading pathogens. With increasing age low titers of IgG anti-ganglioside antibodies, usually directed to asialo-glycolipids, may also be present in healthy controls.[30]

In GBS patients the anti-ganglioside antibodies are usually of the IgG1, IgG3 or IgA isotype, indicating a mucosal origin.[31] Isotype switching is usually the result of a cognate interaction between T-helper cells and B-cells. However, this might not be the case for polysaccharide antigens, as these are generally regarded as T-cell independent antigens. Interestingly, a recent paper showed that cross-linking of pattern recognition

receptors (PPR) and B-cell receptors can induce isotype switching without T-cell help.[32]

Whether anti-ganglioside antibodies are pathogenic in GBS is still a matter of debate, but there are several arguments favoring a pivotal role of these antibodies:

(i) Anti-ganglioside antibodies are found consistently in serum from a subgroup of GBS patients.[33] In MFS patients, IgG anti-GQ1b antibodies are highly specific and can be found in up to 95% of these patients.[19] Other anti-ganglioside antibodies are also related with specific clinical entities (Table 1). For example, anti-GM1/GM1b/GD1a/GalNac-GD1a antibodies are associated with a pure motor variant of GBS.[31, 34, 35] Moreover, different subclasses of anti-ganglioside antibodies are associated with different clinical outcomes. In the case of anti-GM1 antibodies, IgG1 is associated with diarrhea and a preceding *C. jejuni* infection and a slow recovery, while IgG3 is associated with an upper respiratory tract infection and rapid recovery.[36] The IgG3 anti-GM1 antibodies are likely to be evoked by a preceding *H. influenzae* infection.[37]

(ii) The titer of anti-ganglioside antibodies generally decreases with clinical recovery, but sometimes remains high despite clinical recovery.[38, 39]

(iii) Anti-ganglioside antibodies are able to bind to nodes of Ranvier, myelin, dorsal root ganglion cells, motor neurons and perisynaptic Schwann cells.[40-44]

(iv) Anti-ganglioside antibodies are able to fix complement. Moreover, the IgG1 and IgG3 subclasses are capable of activating the complement system.[28, 45]

(v) Anti-ganglioside antibodies are capable of inducing leucocyte degranulation and phagocytosis via Fcγ receptor mediated mechanisms.[46]

(vi) Anti-ganglioside antibodies can block ion-channel function and disturb neuromuscular transmission.[47, 48]

(vii) Anti-ganglioside antibodies can impair the function of the blood-nerve barrier in an *in vitro* model.[49]

(viii) Passive transfer of human and rabbit anti-GM1 by intra-neural injection results in axonal degeneration.[50]

(ix) Active immunization with gangliosides GM1, GD1a, GD1b induces an immune-mediated neuropathy with neurological deficits in rabbits.[51]

Besides these anti-ganglioside antibodies, other components of the immune response are also involved (Figure 1). The interaction between antigen presenting cells (APC), T-cells and B-cells will eventually lead to the production of these antibodies. Macrophages which are present in the peripheral nerve infiltrates seem to play an important role in (i) promotion of inflammation by releasing pro-inflammatory cytokines, (ii) destruction of nerve tissue and also in (iii) remyelination and axonal regeneration.[52] The pro-inflammatory cytokines and other mediators are likely to be involved the immune response, blood-nerve barrier breaching, demyelination and remyelination.[53]

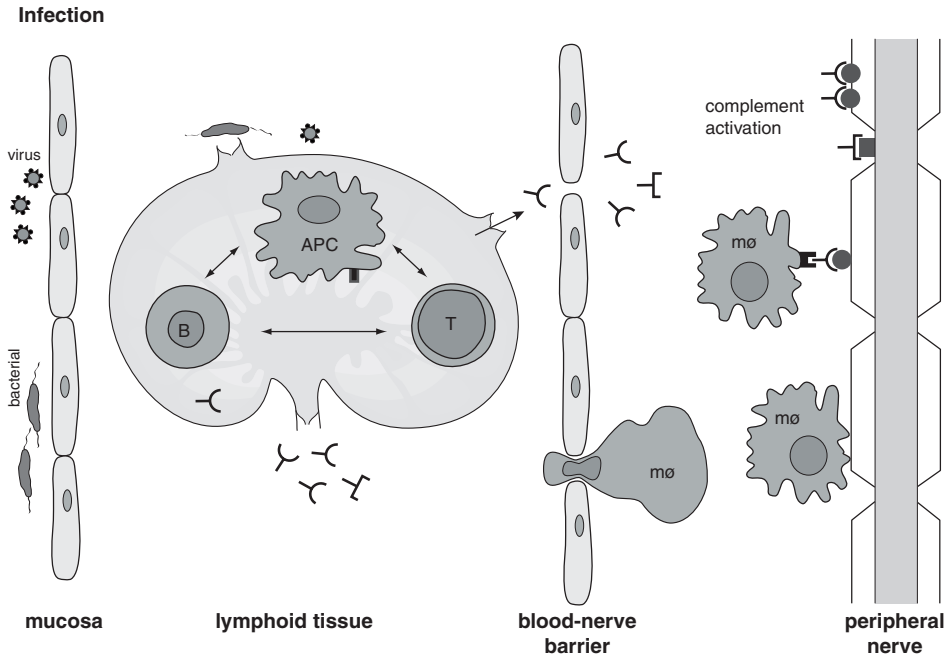

Figure 1. Pathogenesis of Guillain-Barré syndrome.
Upon infection the immune system is activated to eradicate the invading pathogen, in which antigen-presenting cells (APC), T- and B-cell interactions are of major importance. In GBS patients, however, the immune response is not only directed to the pathogen, but also to structures present on the peripheral nerves, the so-called gangliosides, which mimic those on infectious agents (molecular mimicry). Within the peripheral nervous system macrophages and complement factors play an important role in tissue destruction causing a polyneuropathy.

Table 1. Relation between antecedent infection, anti-ganglioside antibodies and clinical features in GBS.

Antecedent infection	Antibodies to	Clinical features
Campylobacter jejuni	GM1, GM1b, GD1a GalNac-GD1a	rapidly progressive, pure motor variant [31, 34, 35, 110]
	GQ1b, GT1a	Miller Fisher syndrome oculomotor weakness in GBS patients [111]
Cytomegalovirus	GM2	severe motor and sensory deficits high frequency of respiratory insufficiency [112]
Epstein-Barr virus	not known	autonomic dysfunction in one case-report [113]
Mycoplasma pneumoniae	Galactocerebroside	mild form [9]
Haemophilus influenzae	GM1	pure motor variant, favorable outcome [23]

Molecular mimicry

In the last decade an intriguing concept of molecular mimicry in GBS has been postulated, indicating a clear relation between antecedent infection and production of anti-ganglioside antibodies. Molecular mimicry is the mechanism by which infections trigger cross-reactive antibodies or T-cells that cause the symptoms of autoimmune diseases. One way to prove a role for molecular mimicry is the identification of antibodies or T-cells that recognize both pathogen and host.[54] In GBS several lines of evidence support the concept of molecular mimicry:

(i) The strong epidemiological association between distinct acute infectious diseases and GBS.[19]

(ii) The strong association between specific types of antecedent infections and specific types of anti-ganglioside antibodies (Table 1).

(iii) Lipo-oligosaccharide (LOS) fraction of the outer cell wall of *C. jejuni* contains structures that mimic gangliosides. This mimic is consistent with the antibodies to gangliosides in patients in whom the *C. jejuni* was isolated.[55]

(iv) The anti-ganglioside antibodies cross-react with *C. jejuni*. The molecular mimicry of the infectious agent therefore determines to a large extent cross-reactive antibodies and the clinical features of the patient.[33]

(v) Immunization of animals with *C. jejuni* or purified LOS results in antibodies to LOS that cross-react with gangliosides. The antibody specificity is in accordance with the anti-ganglioside antibodies in serum from the patient from who the *C. jejuni* was isolated.[48, 56]

(vi) In case of immunization of Japanese white rabbits not only the cross-reactive antibody response is present, but also clinical, electrophysiological and histopathological features resembling GBS.[50]

Hence, it was postulated that GBS is a true case of molecular mimicry.[57]

The above-mentioned arguments focus on molecular mimicry in the case of preceding *C. jejuni* infection. However, molecular mimicry seems also to be present in the case of a preceding CMV or *M. pneumoniae* infection. The anti-ganglioside antibodies in CMV-infected patients seem to be evoked by antigens that are induced by a CMV infection and cross-react with GM2 gangliosides.[58] The anti-galactocerebroside antibodies present in *M. pneumoniae* infected patients seem to be induced by *M. pneumoniae* antigens.[59] In the case of a preceding *H. influenzae* infection, the anti-GM1 antibodies present in sera from GBS patients or the anti-GQ1b or anti-GT1a antibodies present in MFS-patients seem to be evoked against epitopes present on *H. influenzae*.[60, 61]

Several research groups tried to identify differences between GBS or MFS-related *C. jejuni* strains and *C. jejuni* from patients with an uncomplicated gastro-enteritis to assess whether pathogen-derived factors are necessary for the presence of ganglioside-mimicking epitopes. Differences between GBS or MFS related *C. jejuni* strains and *C. jejuni* from patients with an uncomplicated gastro-enteritis are:

(i) The Penner serotyopes 19 and 41 were more frequently found in GBS-related strains than in control isolates from patients with uncomplicated gastro-enteritis in North-China[62] and Japan[63] respectively South Africa.[64] This predominance of some Penner serotypes was not found in *C. jejuni* isolated from Dutch/ Belgium patients indicating geographical differences.[65]

(ii) Ganglioside-like structures are more frequent in *C. jejuni* isolates from GBS patients compared to controls.[45, 66]

(iii) Genes involved in LOS biosynthesis are clustered in the LOS biosynthesis gene locus (LOS locus). GBS-related *C. jejuni* strains are associated with a class A LOS locus, MFS-related *C. jejuni* strains with a class B LOS locus and strains of an uncomplicated gastroenteritis with a class C LOS locus.[67]

(iv) The putative LOS-modifying sialic acid transferase-II encoding gene *cst*II that is required for the synthesis of a GQ1b-like epitope is over-represented in GBS-related strains.[68]

(v) Immunization of mice with *C. jejuni* strains in which genes involved in sialylation of LOS (*cst*II and *orf*10/*orf*11) were knocked-out shows that these mice did not develop anti-GD1a antibodies, but only asialo-GM1 antibodies that are not associated with the development of GBS.[69]

These results indicate that genes involved in sialylation of LOS are crucial for the induction of cross-reactive antibodies. However, the above-mentioned associations are not absolute. Host factors are also involved in determining whether a person develops GBS after an encounter with a *C. jejuni* strain expressing ganglioside-mimicking epitopes. These host factors are the main topic of this thesis.

Host factors

Host factors are likely to (partially) confer susceptibility to the development of GBS or its clinical course upon infection, since:

(i) GBS rarely occurs after an infection with the agents frequently reported in GBS. Only 1 individual per 1000 infected persons develops GBS after a *C. jejuni* enteritis.[70] Although epidemics of *C. jejuni* have been reported, these outbreaks were not followed by an epidemic of GBS.[71]

(ii) One case report showed that in a family with an outbreak of *C. jejuni* enteritis, only one member developed GBS. This person was the only one who had high titers of anti-ganglioside antibodies.[72]

(iii) The estimated recurrence rate of GBS is about 1 to 5%, which shows a 10- to 50-fold increased risk compared to the risk for developing GBS for a first time.[73]

(iv) The clinical characteristics of GBS patients are even heterogeneous within subgroups of patients with the same type of antecedent infection.

(v) Although prognostic models of GBS have been reported, they have a limited predictive value. Independent predictors of poor outcome (inability to walk independently) are a preceding gastro-intestinal illness, older age (>50 years), a low MRC-sumscore and a recent CMV infection.[74]

Table 2. Population-association studies of immune-response genes in GBS other than HLA.*

polymorphism(s)	ethnic origin population	group size (n) (GBS vs controls)	reported association
TNF-α	Japanese	81 vs 85	TNFα 2-allele increased in *C. jejuni* + GBS patients vs controls (p=0.01)[99]
Fc-gamma receptors (Fc-gammaR-IIA, -IIIA, -IIIB)	Caucasian	31 vs 187	Fcγ-RIIA 131 H/H increased in GBS patients vs controls (OR 2.45, p=0.037) Fcγ-RIIA 131 H/H associated with severe disease (F-score 4 & 5; OR 18.6, p=0.007)[100]
	Caucasian	62 vs 89	Fcγ-RIIIb NA1/NA1 associated with a mild form of disease (p=0.027)[101]
IL-10 (-1082G/A, -819T/C, -592 A/C)	Caucasian	87 vs 87	CC homozygotes at position -819 and -592 significantly more frequent in GBS patients versus controls (p=0.027)[102]
KM (kappa light chain of Ig)	Caucasian	83 vs 196	KM3/KM3 increased in GBS patients vs controls (OR 2.3, p=0.01) KM1/KM3 decreased in GBS patients vs controls (OR 0.4, p=0.01)[103]
ApoE	Caucasian	91 vs 169/406	no association[104]

*Reports studying HLA-alleles in GBS are reviewed in Chapter 4.1. GBS: Guillain-Barré syndrome, TNF-α: tumor necrosis factor alpha, IL-10: interleukin 10, ApoE: apolipoprotein- E.

(vi) Seven previously published reports showed the occurrence of GBS within families.[75-81] Siblings were affected in three families, and parent and child were affected in four families. Clustering of a disease in families is generally suggestive for a genetic component in the development of the disease.

(vii) Several groups have studied the highly polymorphic human leucocyte antigen (HLA)-alleles in groups of patients with GBS to assess whether HLA-antigens confer susceptibility for developing GBS or the clinical pattern of a specific subgroup. Some studies indicated that certain HLA antigens are a susceptibility factor for developing GBS or are related to specific clinical or serological subgroups (reviewed and discussed in Chapter 4.1).[82-98] In recent years some groups studied other genetic polymorphisms involved in regulating the immune response and their results are encouraging (Table 2).[99-104]

This thesis focuses on the contribution of genetic susceptibility factors in the pathogenesis of GBS.

Genetic approaches

Generally, there are two different approaches to study whether there is a genetic susceptibility for the development of a disease; whole genome screens and candidate gene studies. Whole genome screens using a linkage analysis approach can be used to identify chromosomal regions that are shared in excess by affected members within families. The basic principle of this method is that loci that lie close together on a specific part of the genome are likely to be transmitted together. In this way, affected relatives are likely to more often share markers in the close proximity of a causal disease mutation than unaffected relatives. In the case of an increased frequency of a certain marker, further screening of the chromosomal region is required to identify the causative mutation. Candidate gene studies are based on association analyses, which can be conducted in population-based and family-based studies. In population-based genetic association studies, the frequency of allelic variants are compared between a group of unrelated patients and a group of healthy controls. In this case an association can be due to the biological consequences of the marker studied (causative relationship) or to a gene that is in close proximity and inherited together with the marker (linkage disequilibrium). In a family-based association study, the difference between the frequency of allelic variants transmitted from heterozygous parents to the affected offspring are compared with the frequency of markers not transmitted (transmission-disequilibrium test). Population-based genetic association studies are presently the only way to unravel the contribution of genetic factors in GBS, as no occurrence of large families has been reported.

Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) in candidate genes are most commonly studied in population-based genetic association studies. These SNPs are widely distributed throughout the genome and are by definition present in at least 1% of the general population.[105] The distribution of SNPs can be studied at allelic and genotypic level (Figure 2). Recently, the Human Genome Consortium reported a near complete sequence of the human genome, which covers 99% of the gene-rich portion of the genome consisting of $3x10^9$ basepairs.[106] About $10x10^6$ SNPs are expected to be distributed throughout the genome, as one SNP is approximately present per 300 base pairs. Within a protein-coding gene these SNPs can be located in (i) the promoter region, which is involved in the transcriptional regulation of the gene expression, (ii) the coding region which is translated to a protein, (iii), the intron which is not translated to a protein but is involved in splicing, (iv) 3' untranslated region (UTR) which affects the stability of RNA. In this manner a SNP can account for differences in protein levels, altered function of a protein or the absence of a protein.[107] An example is represented in Figure 3. In clinical terms SNPs can influence a person's susceptibility

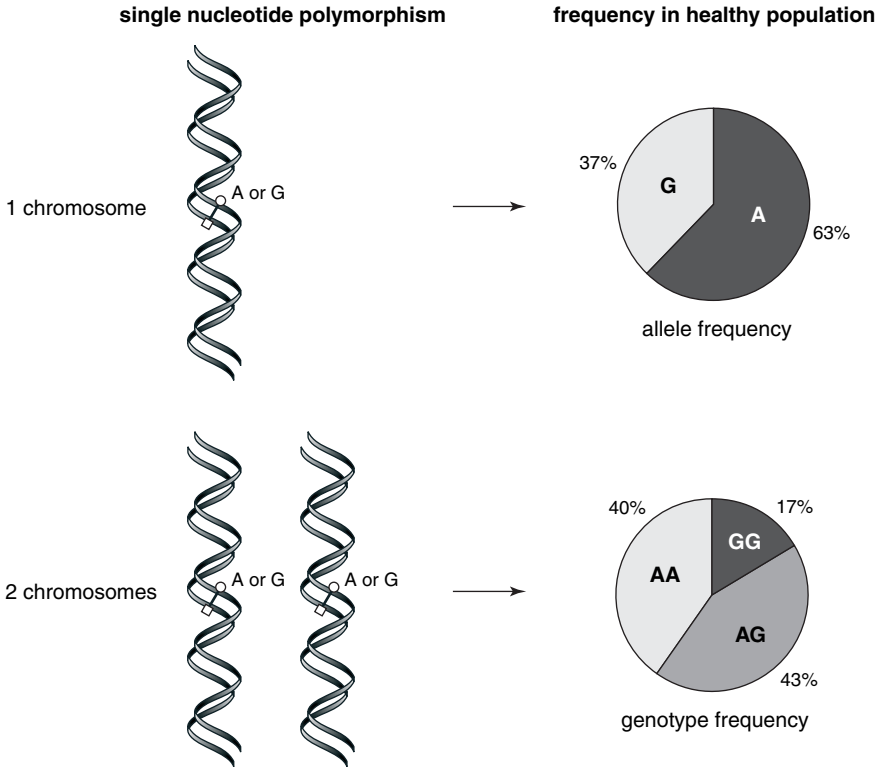

Figure 2. Single nucleotide polymorphism.

This figure represents an example of a distribution of the allele and genotype frequencies of a single SNP, which is located in the *CTLA4* gene (A(+49)G). When taking only one copy of DNA (1 chromosome) into account the SNP locus is referred to as an allele. An individual, however, carries two alleles as they inherit one of each parent. The combination of two alleles is called a genotype.

to develop GBS upon infection but can also cause differences in the severity of disease and outcome. Moreover, these SNPs can influence the way a patient responds to immunosuppressive therapy. Assessing the frequencies of SNPs in genes expected to be involved in the aberrant immune response in GBS patients can provide useful information about the pathogenesis, susceptibility, progression, response to specific types of treatment and outcome of GBS.

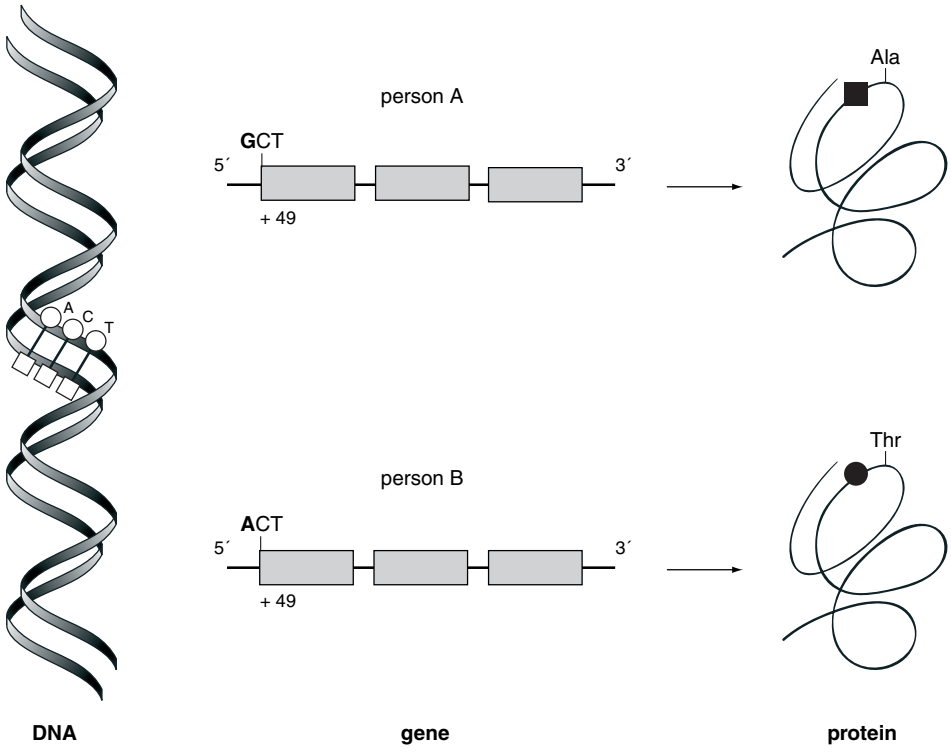

Figure 3. Functional effect of a single nucleotide polymorphism.

This figure represents an example of a single nucleotide polymorphism (SNP) that is located within in the peptide leader sequence of the human *CTLA4* gene. At position +49 some individuals have a guanine (G) as nucleotide incorporated whereas others have an adenosine (A). This change results in an amino acid substitution: Ala (alanine) versus Thr (threonine) in codon 17 affecting the peptide leader sequence. The GG genotype is associated with a lower expression of CTLA-4 and a higher proliferating T-cell response.[114]

Methodological considerations

As SNPs are abundantly present throughout the genome, a selection of specific SNPs was made, which was primarily based on our current understanding of the pathogenesis of GBS and on SNPs in candidate genes reported in literature. The selected SNPs were located within protein-encoding genes and fulfilled the following criteria: (i) immune response genes suspected to play a role in the pathways that are likely to be involved in the pathogenesis of GBS, (ii) assumed or proven to have a functional effect and (iii) reported to be associated with other autoimmune or infectious disorders.

Table 3. Serological and clinical subgrouping of Guillain-Barré syndrome.

serological subgroups	clinical subgroups	general characteristics
antecedent infection	severity based on MRC-sumscore*	age
C. jejuni	severity based on F-score#	sex
Cytomegalovirus	artificial respiration	
Epstein-Barr virus	cranial nerve deficits	
M. pneumoniae	sensory deficits	
	poor outcome (based on F-score at 6 months)	
anti-ganglioside antibodies		
anti-GM1		
anti-GQ1b		
anti-GD1a		
anti-GM2		

* Severely affected patients were defined as an MRC-sumscore < 40 and mildly affected patients as an MRC-sumscore ≥ 40. The MRC-sumscore ranges from 0 (tetraparalysis) to 60 (normal). # Severity of disease was based on the functional disability score (F-score) at nadir. Mildly affected patients are still capable of walking independently, while severely affected patients are not able to walk independently and some need artificial respiration.

Since GBS is a rare and heterogeneous disorder, it is to be expected that many genes are involved. Therefore each individual gene will only have a small effect on susceptibility or outcome.[108] To detect these small effects, which may only occur in subgroups, a large sample size of patients described in detail with respect to infections, anti-ganglioside antibodies, clinical, features and outcome is required to perform subgroup analyses. Performing multiple comparisons, however, might result in false positive associations.[109] It is still a matter of debate whether or not to correct for multiple testing, as too conservative corrections might result in false negative studies. One way to reduce the risk of false positive associations is to also study the functional effects of the observed association within the study population and to confirm an association in another cohort of GBS patients.

Outline of the thesis

Guillain-Barré syndrome is a heterogeneous post-infectious immune-mediated polyneuropathy in which pathogen-derived factors as well as host factors are likely to be involved in the pathogenesis. This thesis focuses on the contribution of genetic host factors in Guillain-Barré syndrome. To accommodate for the heterogeneity of GBS, the putative role of the SNPs was also studied in relation to defined clinical and immunological subgroups.

The presence of a familial form of GBS or multiplex families would be of great

advantage to unravel genetic susceptibility factors. Therefore, we actively searched for families with two or more affected family members. The results are described in Chapter 2.

Chapters 3-6 describe the results of several population-based genetic association studies, in which we assessed whether SNPs in immune-regulatory genes confer susceptibility or are related to clinical or serological subgroups of GBS. These studies were performed on a unique cohort of about 275 Dutch GBS patients. More than half of the patients had participated in one of the Dutch randomised controlled trials and are therefore documented in high detail with regard to antecedent infection, anti-ganglioside antibodies, severity of disease, outcome and residual deficits, thereby enabling subgroup analysis (Table 3). As a control group, 212 healthy Caucasian blood bank donors, who were representative for the Dutch population based on their HLA-typing, were used.

Each chapter represents the results based on genes that are involved in different phases of the immune response: pathogen-recognition (Chapter 3), antigen presentation and costimulation (Chapter 4), complement and macrophage mediated damage (Chapter 5) and apoptosis (Chapter 6). In Appendix 2 the complete list of SNPs studied is represented.

Chapter 7 discusses the significance of the results described in Chapters 2-6. Finally, directions for future investigations are put forward, mainly based on the results from the studies presented in this thesis.

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Chapter 2

Familial Guillain-Barré syndrome?

Chapter 2.1

The occurrence of Guillain-Barré syndrome within families

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Abstract

This report describes 12 Dutch families of which at least two members have had Guillain-Barré syndrome (GBS). We observed an earlier onset of GBS in successive generations. The occurrence of GBS within families suggests a role for genetic factors in the pathogenesis of GBS.

Introduction

Although Guillain-Barré syndrome (GBS) is the most common cause of a flaccid paralysis in the Western world, it is still a rare disease. In the Netherlands, the incidence rate is 1.18 cases per 100.000 persons per year.[1] It is not yet known why about 1 in 1000 persons develops GBS after a *Campylobacter jejuni* infection.[2] Genetic susceptibility and other host factors may play a role in the pathogenesis of GBS. Although generally a sporadic disease, a few familial occurrences of GBS have been reported (Table 1).[3-9] To examine the evidence for genetic susceptibility to develop GBS, we searched for families in the Netherlands in which at least two members have had GBS. Here we report the time and age at onset, antecedent clinical infections and other clinical characteristics of the patients within these families.

Table 1. Overview of previously published familiy reports of Guillain Barré syndrome.

Family	age at diagnosis	relation	time-interval	prodromal illness	clincial features
ref. 3	71 years	brother		pain radiating from lumbar region	gradually increasing weakness
	73 years	sister	4 years	pain radiating from lumbar region	paraesthesia, weakness
ref. 4	56 years	father		no comment	low back pain, paralysis
	24 years	daughter	2 years	"nettle rash"	paraesthesia
ref. 5	2 years	brother		febrile illness and cold	progressive weakness, hyporeflexia, dysphagia, death
	22 months	sister	4 years	pharyngitis	weakness, ataxic gait
	9 months	brother	7 years	fever and rinorrhea	tetraparalysis, ventilated
ref. 6	58 years	father		flu-like	weakness, paresthesia, bilateral facial palsy, ventilated
	43 years	son	9 years	URTI	weakness, paresthesia
ref. 7	50 years	father		haemoroidectomy followed	1 year later weakness and sensory loss, by right hand weakness AIDP
	24 years	daughter	4 years	EBV	lower limb weakness, AIDP
ref. 8	19 mnths	sister		Diarrhea (*C. jejuni)*	pure motore, dysphagia,nuchal weakness, opthalmoplegia
	3 1/2 years	sister	10 days	Diarrhea (*C. jejuni)*	pure motore, dysarthria, opthalmoplegia, ventilated
ref. 9	35 years	mother		headache and retro-orbital pain	weakness and sensory deficits, bilateral facial palsy, dysarthria
	6 years	son	7 years	general anaesthetic, sore throat	lethargy, neck pain and bulbar weakness

All selected family cases are reported as Guillain-Barré syndrome and written in English. Insufficient information is available whether all cases meet the diagnostic criteria for GBS. Most of the patients had increased CSF protein and loss of deep tendon reflexes, which are not listed in the table. *C. jejuni: Campylobacter jejuni*, EBV: Epstein-Barr virus, AIDP: acute inflammatory demyelinating polyneuropathy.

Methods

Study population

In this study we included families in which at least two members were diagnosed as GBS. To identify as many families with GBS patients as possible, we obtained patients in three ways:

1. One group of patients had already participated in one of the Dutch trials or surveys. In 2001-2002 the patients were asked to participate in a genetic population-association study. Those who agreed to take part were visited and asked to fill in a questionnaire, including the question whether or not they had affected relatives.
2. In 2003, the Dutch Society of Muscle Disorders (VSN) sent all of their 298 present members with GBS or chronic inflammatory demyelinating polyneuropathy (CIDP) a letter to inform them about the project and asked them whether they had a relative with GBS or CIDP.
3. In the period 2002-2003, GBS patients admitted to the Erasmus Medical Center were asked about any affected relatives.

Families were only included when the probands as well as their affected family members fulfilled the diagnostic criteria for GBS.[10] Parental consent was sought when patients were younger than 18 years old. Clinical characteristics of the patients were obtained from a letter of discharge or a standardized questionnaire. The protocol for this study was reviewed and approved by the Medical Ethical Committee of Erasmus Medical Center and all patients who participated gave their written informed consent.

Statistical approach

The standardized morbidity ratio was used to verify whether the incidence of GBS among siblings was higher than expected based on the general population data. The SMR was estimated using the data from the genetic population-association study.

Results

In total we managed to contact approximately 500 GBS patients. Within this group of patients, 20 families were initially contacted, but patients in two families suffered from a subacute GBS or CIDP, in two other families the diagnosis of one of the patients could not be verified and in four other families at least one of the patients refused participation. Finally, 12 families were included in this study of which the pedigrees and the age of onset of the affected members are shown in figure 1. Eleven women and 14 men were affected within these families.

Among siblings, the observed incidence was 2.6 fold increased compared to the expected incidence (SMR 2.6, 95% C.I. 0.4-18.5, P=0.32). With regard to the age of onset, we observed a trend over generations in a way that the age of onset decreased in younger generations. The median difference in age at onset between siblings was 6 years (ranging from 0 to 14 years) and between cousins 6.5 years (ranging from 1 to 17 years). The difference in age between two mothers and their sons was in both cases 33 year. The median difference in age between aunts or uncles and their

nieces or nephews was 27 years (ranging 26 from to 49). The age difference between grandmother and her grandson was 47 years. The year of diagnosis and the clinical characteristics are summarized in table 2. The median time-interval between the years of onset of GBS in affected relatives is 6.5 years (ranging from 2 months to 22 years). The prodromal illness, clinical features and severity of the disease varied between the affected members within the families. Since the estimated recurrence rate of GBS seems about 1-5%, it is of note that one of the familial patients had four times an episode of GBS (Family 8).

Discussion

In this study we describe 12 families in which at least two members had GBS. Until now, worldwide only 7 families with two or three probands have been reported (see table 1).[3-9] These reports described families in which siblings (n=3) or parents and their children were affected (n=4). In our report we further described families in which second and third degrees relatives are affected.

In our study, which is not an epidemiological survey, the number of reported families is insufficient to prove that there is a familial occurrence of GBS. The slightly more frequent occurrence of GBS within siblings, the observed earlier onset of GBS in successive generations and the patient having four times GBS might suggest a genetic susceptibility. However, more patients are needed to obtain a precise estimate of the SMR and to confirm the trend of a decrease in age at onset over generations. Retrospectively, this trend can also be observed in previously reported family-studies: the median difference in age of onset between affected parents and their child was 27.5 years and between siblings 1.25 years.[3-9] The earlier onset in successive generations could also merely reflect the lack of follow-up time, since the youngest generation has not lived long enough to be ascertained at later age.

Environmental factors like antecedent infections play an important role in the pathogenesis. In some of the families GBS was preceded by the same type of antecedent infection, while in other families and even in the patient who had four episodes of GBS, the prodromal illnesses were different. The observed large time-interval between the onsets of symptoms within the families excluded the possibility that GBS was triggered within the same period of antecedent infection. Besides the clinical symptoms related to a specific pathogen, no specific clinical variants within families are observed, which is in line with the other case-reports.[3-9]

This paper supports that GBS is rather a complex genetic disorder than a Mendelian inherited disorder, which outcome is determined by environmental and genetic factors. Investigation of the genealogy and molecular genetics of a large number of families with GBS may give more insight into host factors determining a subject's susceptibility to GBS.

Table 2. Clinical characteristics of familial Guillain-Barré reports.

Family	Relation	year at onset	prodromal illness	Clinical features	F-score at nadir*
1.	Brother	1986	flu	SM, bilateral facial palsy	4
	Cousin	1989	fatigue, fever (CMV)	SM	3
	Sister	2002	sinusitis	SM	1
2.	Son	1994	URTI	SM, bulbar weakness	3
	Mother	1999	diarrea, sore throat	M	4
3.	Grandmother	1975	flu	SM, ataxia	2
	Grandson	1997	none	M, pain	3
4.	Sister	2003	flu-like	Bulbar weakness, ataxia	2
	Brother	2003	URTI	SM, ataxia	2
5.	Cousin	1989	sore throat	M	4
	Cousin	2002	flu	SM, dysphagia, ptosis	4
6.	Sister	1988	diarrhea	M, bilateral facial palsy, bulbar weakness	5
	Brother	1996	diarrhea	M	1
7.	Mother	1996	pregnant (CMV)	SM, bilateral facial palsy	5
	Son	1996	none (CMV)	SM	5
8.	Nephew	1996	flu	M, bilateral nVII palsy	2
	Aunt	1999	URTI	M, opthalmoplegia	2
		1999	diarrhea	M, ptosis	2
		2000	sore throat	M, opthalmoplegia	4
		2004	sore throat	M, opthalmoplegia	3
9.	Niece	1985	none	SM	4
	Uncle	1995	diarrhea (*C. jejuni)*	SM	5
10.	Cousin	1999	diarrhea	SM, bilateral facial palsy	3
	Cousin	2001	diarrhea *(C. jejuni)*	M, bulbar weakness, autonomic dysfunction	5
11.	Uncle	1996	flu	SM, pain	4
	Niece	1998	diarrhea	M, pain	4
12.	Brother	1990	Diarrhea	M, facial palsy	5
	Sister	2004	None (*C. jejuni*)	M	3

*GBS disability score at nadir: 1= minor symptoms and capable of running, 2= able to walk 10m across an open space without assistence, 3= able to walk 10m with a walker or support, 4= bed or chairbound and unable to walk as in 3, 5= assisted ventilation required for at least part of the day or night.
SM: motor and sensory deficits, CMV: cytomegalovirus, URTI: upper respiratory tract infection, M: pure motor variant, *C. jejuni*: *Campylobacter jejuni*.

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Chapter 3

Pathogen recognition

Chapter 3.1

Functional polymorphisms in LPS-receptors CD14 and TLR4 are not associated with disease susceptibility or Campylobacter jejuni infection in Guillain-Barré patients

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Abstract

Guillain-Barré syndrome (GBS) is an acute immune-mediated polyneuropathy preceded by infections. *Campylobacter jejuni* is the most frequent pathogen and its lipopolysaccharide (LPS) induces antibodies cross-reactive with gangliosides. In this study we assessed whether known functional polymorphisms in the LPS receptors CD14 and Toll-like receptor 4 (TLR4) are associated with an increased susceptibility for GBS or with *C. jejuni* serology or *C. jejuni* related clinical and serological features. Comparison of the genotypes of 242 GBS patients and 210 healthy subjects showed that polymorphisms in *CD14* and *TLR4* did not confer disease susceptibility and were not associated with *C. jejuni* infection.

Introduction

Guillain-Barré syndrome (GBS) is an acute heterogeneous immune-mediated polyneuropathy, which is frequently preceded by an infection. *Campylobacter jejuni*, a Gram-negative bacterium of which LPS is the major component of the outer membrane, is the most commonly found pathogen in the Netherlands.[1] The currently favoured hypothesis on the immunopathogenesis of GBS indicates that molecular mimicry between *C. jejuni* LPS and gangliosides expressed on peripheral nerves leads to the production of pathogenic cross-reactive antibodies. This concept is supported by the observed association between antecedent *C. jejuni* infections and the presence of antibodies to GM1, GM1b, GD1a, GalNAc-GD1a and GQ1b, cross-reactivity of anti-LPS antibodies to these gangliosides, and molecular mimicry between LPS and gangliosides. Finally, the concept is supported by the induction of cross-reactive antibodies in animals after LPS immunization.[2-6] The fact that such a cross-reactive immune response to LPS and gangliosides is rare after a *C. jejuni* infection in humans, may explain why only 1:1000 persons develop GBS after a *C. jejuni* enteritis.[7] This would indicate that host factors determining the immune response to LPS play a crucial role in the etiology of GBS.

CD14 and Toll-like receptor 4 (TLR4) are important in the antigen presentation and intracellular signalling of LPS. CD14 is a 55kDa glycoprotein, which is mainly expressed on mature monocytes, macrophages and activated granulocytes.[8] Recently, a functional polymorphism in the promoter region of the *CD14* gene has been described. This C (-260) T polymorphism is located at one of the binding sites for the Specificity protein (Sp) transcription factors, which are involved in regulation of the *CD14* transcription and level of expression. The T-allele is associated with higher expression of CD14 on peripheral blood mononuclear cells, particularly in the case of TT homozygotes. Moreover, this T-allele is associated with Crohn's disease and an increased risk of a myocardial infarct after a Gram-negative infection.[9-12]

Since CD14 lacks a transmembrane domain, TLR4 is required for intracellular signalling. After CD14 has transferred LPS to the TLR4 complex, a cascade of intracellular events leads to the activation of NF-κB, which in turn induces the transcription of several cytokine genes such as IL-1, IL-6 and TNF-α resulting in a pro-inflammatory environment.[13, 14] In the *TLR4* gene, two co-segregating polymorphisms in the fourth exon have been reported: A(+896)G and C(+1196)T. Both polymorphisms result in an amino acid substitution and are usually referred to as Asp299Gly and Thr399Ile, which alter the structure of the extracellular domain of TLR4. The mutant allele of the Asp299Gly in the fourth exon of *TLR4* is associated with an attenuated immune response to LPS and secretion of lower levels of pro-inflammatory cytokines.[15, 16] Individuals having the Asp299Gly *TLR4* allele have an increased risk of Gram-negative infections, Gram-negative shock and systemic inflammatory response syndrome.[17-19]

Recent advances in the field of TLR-signaling have established their importance for the initiation of both innate and adaptive immune responses and their requirement

for maintaining immune tolerance.[20] Ligation of TLR on dendritic cells (DC) initiates a process of cell maturation and upregulation of the expression of MHC and co-stimulatory molecules like B7 and of the secretion of various cytokines and chemokines. In case of GBS the induction of cross-reactive antibodies to LPS and gangliosides may be caused by inappropriate activiation of the DCs via the CD14-TLR4 complex. Therefore, we hypothesized that *C. jejuni* infected GBS patients have an altered immune response to LPS due to polymorphisms in these LPS-signalling genes which affects the secretion of pro-inflammatory cytokines and influences the adaptive immune response. We investigated whether these polymorphisms occur more often in GBS patients and whether these single nucleotide polymorphisms (SNPs) are related to *C. jejuni* infection, anti-ganglioside antibodies and clinical subgroups.

Material and Methods

Study population

Peripheral blood samples were collected from 242 Caucasian GBS patients (median age at onset of disease 45 years, range 7-82 years, male/female ratio=1.03) who had participated in trials or other national survey studies coordinated by the Department of Neurology of Erasmus MC. All patients fulfilled the diagnostic criteria for GBS.[21] The protocol of this study was reviewed and approved by the Medical Ethical Committee of Erasmus MC, and all patients gave their written informed consent. Of these 242 patients, 137 had participated in one of the Dutch GBS trials and are therefore prospectively documented in high detail with regard to clinical and serological aspects of the disease.[22, 23] The patients enrolled in one of these trials were followed for six months after randomisation. At randomisation these patients were examined clinically and blood was drawn for serological screening for antibodies against the most common antecedent infections in GBS (*C. jejuni*, cytomegalovirus, Epstein-Barr virus and *Mycoplasma pneumoniae*), and for the presence of anti-ganglioside antibodies according to standard procedures.[1, 24] During follow-up, the patients were neurologically examined at 16 time points according to the protocol. The Medical Research Council (MRC)-sumscore[25], the GBS functional disability score[26], the involvement of sensory and cranial nerves were established at all these time-points. The severity of the disease was defined in two different ways. One was based on the MRC-sumscore (ranging from 0-60) at nadir; mildly affected patients were defined by an MRC-sumscore ≥ 40 and severely affected patients by an MRC-sumscore < 40.[27] The other was based on the GBS functional disability score at nadir: mild disease was defined as able to walk independently (F-score of 1 or 2) and severe disease as unable to walk independently (F-score of 3, 4 or 5).[28] Additionally, clinical and serological information was obtained from 35 patients who had participated in a prospective study: anti-ganglioside antibodies were determined in 28 of the patients, *C. jejuni* serology in 24 patients and F-score at nadir in 34 patients.[29] In the remaining 70 patients, additional information was only available on sex and age.

Genomic DNA of 210 Caucasian healthy subjects (median age 35 years, range 19-60

years, male/female-ratio=0.65) was provided by the Laboratory for Histocompatibility and Immunogenetics, Sanquin Bloodbank South West Region, The Netherlands. These controls were not serologically screened for the presence of antibodies to *Campylobacter jejuni*. All healthy subjects had given written informed consent.

Isolation of genomic DNA

Isolation of genomic DNA from EDTA anti-coagulated peripheral blood samples was performed with the use of the Invisorb® MaxiBlood kit (Invitek, Berlin, Germany) according to the manufacturer's instruction. At the end of the isolation procedure, the DNA samples were dissolved in 0.1 TE-buffer (1 mM Tris-HCl (pH 7.5) + 0.1 mM EDTA) and stored at -80°C. DNA samples for SNP detection were diluted in milli-Q water to a final concentration of 10 ng/µl and stored at –20°C.

RFLP analysis of the C(-260)T polymorphism in the promoter region of CD14

Amplification of a 367-bp fragment of the *CD14* promoter was performed using the Perkin Elmer DNA Thermal cycler. The polymerase chain reaction (PCR) was run in a 50 µl volume containing 200 ng of genomic DNA, 12.5 pmol of each primer (Table 1), dNTPs at 0.1 mM and 0.2 U of Ampli Taq® DNA polymerase (Perkin Elmer, Norwalk, CT) and 1x Ampli Taq Gold™ Gene Amp® 10x PCR Buffer II & MgCl2 (Perkin Elmer) and milliQ-water. The PCR profile included 1 cycle of 7 min at 95°C; 40 cycles of 45 sec at 92°C, 90 sec at 60°C and 2 min at 72°C; 10 min at 72°C and 10 min at 4°C. The PCR products were analysed by restriction fragment length polymorphism (RFLP) analysis. Five µl of PCR product and 5 µl of an internal control were digested in 2 units of restriction endonuclease *Hae*III (Roche Diagnostics, Mannheim, Germany) at 37°C for two h. Digests were analysed by 6% acrylamide gels + 0.5X TBE (45mmol/l Tris, 45 mmol/l boric acid, 1.25 mmol/l EDTA). The gels were stained with ethidium bromide and DNA was visualized with UV light. In every lane, the internal control was digested into a 479-bp and 97-bp fragment. Digestion of the samples yielded bands of 367 bp in TT homozygotes, 155-bp and 212-bp in CC homozygotes and all three restriction bands in heterozygotes.

LightCycler analysis of the Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene

In this study we detected the Asp299Gly and Thr399Ile polymorphism of the human *TLR4* gene in a single LightCycler assay with one set of primers and two sets of hybridisation probes (Table 1). The general principle of this PCR-based method have been previously described.[30] Primers and hybridisation probes for detection of the SNPs were designed by TibMol Biol (Mannheim, Germany). The PCR-reaction and the melting curve were performed in LightCycler capillaries (Roche Diagnostics) with a final volume of 20 µl, containing 10 ng genomic DNA, 0.5 µM of each primer, 0.15 µM of each hybridisation probe, 1x LightCycler DNA Master Hybridisation

Table 1. Primers and hybridization probes for detection of SNPs in CD14 and TLR4.

Gene bank accession number U00699	Gene bank accession number AF172171
Primers CD14	**Primers TLR4**
5'-CCTAAGGCACTGAGGATCATC-3' (forward)	5' GTTTAGAAGTCCATCGTTTG-3' (forward)
5' GCTTTAGCTTCTTTCCTACACAG-3'(reverse)	5' TAAGCCCAAGAAGTTTGAA-3' (reverse)
	Hybridisation probes Asp299Gly
	CTACCTCGATGATATTATTGACTT- fluorescein
Primers CD14 internal control	LC Red640-AATTGTTTGACAAATGTTTCTTCATTTTCC
5' CAAGACAAGGAAGAAGTCAAAGAA-3' (forward)	
5' CCCCATCCAACCCCTGT-3 (reverse)	**Hybridisation probes Thr399Ile**
	CTTGAGTTTCAAAGGTTGCTGTTCTCAAAG-fluorescein
	LC Red705-ATTTTGGGACAACCAGCCTAAAGTAT

Probes (Roche Molecular Biochemicals, Mannheim, Germany) and 3 mM $MgCl_2$. Sealed capillaries were centrifuged and placed in the rotor of the LightCycler instrument (Roche Diagnostics). The PCR thermocycling profile included 10 min at 95°C, followed by 45 cycles of 95°C for 0.1 sec, 56°C for 15 sec and 72°C for 20 sec. Next, the melting curve profile was performed, which included 1 cycle of 95°C for 0.1 sec, 40°C for 1 min after which the temperature was slowly increased (0.1°C/ sec) to 85°C under continuous detection of the emitted light. Finally, the cooling down was performed at 40°C for 30 sec. Due to the fact that in this experiment two differently labelled fluorophores were used, interference between the channels could occur. To compensate for this, an installed colour compensation set was used (Roche Molecular Biochemicals). Data were analysed using the melting curve program. In each experiment we used sequenced DNA from confirmed control donors for each genotype.

Statistical analysis

Verification of Hardy-Weinberg equilibrium was performed using the statistical method program of Ott. The genotype and allele-frequencies between patients and healthy subjects and between subgroups of patients were compared using Pearson's chi-square test. When one of the cells had an expected value less than five, the Fisher's exact test was used. Allelic odds ratios and confidence intervals were computed. *P*- values less than 0.05 were considered to be significant.

Results

Clinical and serological characteristics of the GBS patients

With regard to the clinical characteristics, 17.5% of the patients had a pure motor variant of GBS, 19% needed artificial respiration, 47.8% were severely impaired based on the MRC-sumscore (MRC-sumscore <40 out of 60) at nadir and 94.7% had a severe disease course based on the GBS functional disability score at nadir (F-score> 3). In the weeks prior to GBS, 28.1% of the patients had diarrhoea and 27.2% had a serologically confirmed *C. jejuni* infection. Anti-GM1 antibodies were present in 25.5% of the patients and anti-GQ1b antibodies in 12.7%. These clinical characteristics and the presence of anti-GM1 and anti-GQ1b antibodies are related to a *C. jejuni* positive serology and were present in a similar percentage of patients as reported in former publications. Furthermore, associations between a *C. jejuni* positive serology and anti-GM1 (p=0.001) and anti-GQ1b antibodies (p=0.02) were very similar to those described previously.[24]

No association between SNPs in LPS-signalling genes and Guillain-Barré syndrome

To determine whether *CD14* and *TLR4* polymorphisms confer susceptibility in GBS patients, we assessed the genotypes in 242 GBS patients and 210 healthy subjects. The genotype distribution of the C (-260) T polymorphism within the *CD14* gene and the

Asp299Gly polymorphism in the *TLR4* gene did not differ between GBS patients and controls (see Table 2). Moreover, these genotype distributions also did not differ between *C. jejuni* infected patients (n=41) and 110 GBS patients who did not have an antecedent *C. jejuni* infection (see Table 2).

Since the *TLR4* Thr399Ile mutation co-segregates with the *TLR4* Asp299Gly mutation, the genotype distribution of this polymorphism was almost identical to that found for Asp299Gly. Thr/Thr was present in 89.3% of the GBS patients and in 86.2% of the controls and Thr/Ile was present in 10.7% of the GBS patients and 13.3% of the controls. In the control group one Ile/Ile homozygote was detected. A few exceptions with regard to the co-segregation of the mutant alleles in the *TLR4* gene were noticed: in one patient and one control a Gly-allele was inherited with a Thr-allele and in one other patient an Asp-allele with an Ile-allele.

As the SNPs were in Hardy-Weinberg-equilibrium, the allele frequencies of the SNPs in *CD14* and *TLR4* could be compared between patients and controls. The T-allele of the SNP in *CD14* was present in 51.4% of the patients and in 53.8% of the controls and this difference was not significant (p=0.5, OR=0.9, 95% C.I. 0.7 to 1.1). The distribution of the Gly-allele did not differ between patients and controls (5.4 vs. 7.4%; p=0.2, OR=0.7, 95% C.I. 0.4 to 1.2).

Table 2. Genotype distribution of SNPs in CD14 and TLR4 in controls and GBS patients.

	controls		**GBS-patients**		**GBS-patients**			
					C. jejuni positive serology		*C. jejuni* negative serology	
	n=210	%	n=242	%	n=41	%	n=110	%
CD14								
C(-260) T								
CC	50	23.8	58	24.0	7	17.1	25	22.7
CT	94	44.8	119	49.2	23	56.1	52	47.3
TT	66	31.4	65	26.8	11	26.8	33	30.0
TLR4								
Asp299Gly								
Asp/Asp	180	85.7	216	89.3	38	92.7	97	88.2
Asp/Gly	29	13.7	26	10.7	3	7.3	13	11.8
Gly/Gly	1	0.5	0	0.0	0	0.0	0	0.0

The genotype distributions were compared between the GBS patients and controls by using the Pearson chi-square test. No significant differences for the SNPs in *CD14* and *TLR4* were found. Furthermore, no significant association between these SNPs and *C. jejuni* serology was observed. TLR4: Toll-like receptor 4.

No association between SNPs in the LPS-signalling genes and C. jejuni serology and related anti-ganglioside antibodies, neuroligcal deficits and prognosis

To test our hypothesis that SNPs in *CD14* and *TLR4* are involved in the altered immune response in *C. jejuni* infected GBS patients, we subdivided the patients into different groups according to clinical and serological characteristics of a *C. jejuni* infection. Since a pure motor variant of GBS, a severe course of disease, anti-GM1 and anti-GQ1b antibodies are more frequently present in the *C. jejuni* infected group of patients, we determined the association between these characteristics and the SNPs. The occurrence of any of the *C. jejuni* related characteristics was not associated with one or more of these SNPs (Table 3). Furthermore, we investigated whether there was an association between the SNPs and the severity of the disease at different time-points during follow-up. No relation between the severity, defined by MRC-sumscore or F-score, was found at randomisation, after four weeks or six months of follow-up.

Discussion

We postulated that the aberrant immune response to LPS in GBS patients, characterised by the production of high titers of cross-reactive anti-ganglioside antibodies was related to polymorphisms in the *CD14* and *TLR4* genes. In this study we have shown that in a large group of GBS patients, representative for the whole spectrum of GBS, the SNPs in *CD14* and *TLR4* were not related to the susceptibility to develop GBS. Furthermore, we showed that these SNPs were not related to *C. jejuni* serology, neurological deficits and anti-ganglioside antibodies associated with *C. jejuni* infection.

The SNPs we have studied have previously been shown to be functionally relevant and to be associated with disorders in which Gram-negative infections play a role.[9-12] As far as we know, no other functional polymorphisms in the *CD14* and *TLR4* genes have been reported in the literature. We detected the SNP in *CD14* with RFLP-analysis. In all experiments, an internal control was used to verify whether there had been a total enzymatic digestion. The SNPs in the *TLR4* gene were detected by using the LightCycler technique, which is highly sensitive and specific. The samples we sequenced were in accordance with the LightCycler results. Three sequenced samples with different genotypes were used in the experiments as an internal control. With both techniques, the genotypes of all samples could be determined. The genotype distributions of the *CD14* and *TLR4* polymorphisms in our large group of Caucasian controls were comparable with those found by others, suggesting that the control group and the technique we have used are representative.

The most frequent antecedent event related to GBS is a *C. jejuni* infection, which occurs in about 30% of the patients. This infection is very common in the general population of which most of the individuals suffer minor complaints and only less than 1:1000 subsequently develop GBS. To be able to accept or reject the hypothesis that the SNPs in *CD14* and *TLR4* do confer susceptibility of GBS after a *C. jejuni*

Table 3. Genotype distribution in C. jejuni related subgroups of GBS patients.

		TLR4				CD14					
	N	Asp/Asp		Asp/Gly		CC		CT		TT	
			%		%		%		%		%
Antecedent infection											
diarrhoea	42	37/134	27.6	5/15	33.3	6/32	18.8	23/72	31.9	13/45	28.9
Campylobacter jejuni (serology)	41	38/135	28.1	3/16	18.7	7/32	21.9	23/75	30.7	11/44	25.0
Anti-ganglioside antibodies											
anti-GM1 antibodies	42	40/147	27.2	2/18	11.1	9/35	25.7	23/84	27.4	10/46	21.7
anti-GQ1b antibodies	21	16/147	10.9	5/18	27.8	4/35	11.4	12/84	14.3	5/46	10.9
Clinical features											
MRC-sumscore <40/60	65	58/123	47.2	7/13	53.8	13/27	48.1	30/68	44.1	22/41	53.7
F-score ≥ 3	162	143/151	94.7	19/20	95.0	36/37	97.3	76/81	93.8	50/53	94.3
artificial respiration	30	27/137	19.7	3/19	15.8	7/37	18.9	14/72	19.4	9/47	19.1
pure motor	24	22/124	17.7	2/13	15.4	5/27	18.5	16/69	23.3	3/41	7.3

N: number of patients in whom the indicated characteristic is present. The genotype distribution within *C. jejuni* related subgroups is shown by the number of patients in which the particular characteristic and genotype is present divided by the total number of patients in which the genotype is present and the characteristic is tested. The Pearson chi-square test was used to compare the genotype distributions. No significant differences were found. MRC-sumscore: medical research council sum score (min=0 and max=60). F-score: GBS functional disability score (min=0 and max=6). TLR4: Toll-like receptor 4.

infection, it could be argued that *C. jejuni* enteritis patients who did not develop GBS should optimally have been used as control group. Based on the high incidence of *C. jejuni* infection, the majority of controls are expected to have had an infection at any point in life. However, we cannot exclude the possibility that some of the controls will develop GBS after a *C. jejuni* enteritis in the future. Although the controls were younger in age than the GBS patients, we want to stress that when considering different age strata the genotype and allele frequency in cases and controls did not vary over these strata. Based on our study, allelic ORs higher than 1.2 were rejected for both SNPs.

We also tested our hypothesis in another way. We therefore subdivided the patients into two groups based on *C. jejuni* serology, the ones with a positive *C. jejuni* serology and the ones with a negative serology. Additionally, we have taken into account the clinical symptoms that are related with a *C. jejuni* infection within the GBS patients. The *C. jejuni* related symptoms, such as diarrhoea, severity, pure motor variant and presence of anti-ganglioside antibodies also showed no association with the determined SNPs.

Given the central role of antecedent *C. jejuni* infections and cross-reactive immune response to *C. jejuni* LPS in GBS, other LPS receptors than CD14 and TLR4 may be involved. There is evidence that CD14 and TLR4 are part of a larger antigen recognition and signalling complex. In the initial step LPS is bound to CD14, which in turn catalyzes the transfer of LPS to a complex of receptors, including chemokine receptor 4 (CRCX4), heat shock protein (Hsp) 70 and 90, and growth differentiation-factor 5 (GDF5). The TLR4-MD-2 complex is subsequently recruited to achieve maximum responses upon LPS stimulation.[31, 32] Besides the membrane-bound receptors, molecules involved in the intracellular signalling pathway of TLR4 may also be involved.[33] Recently, two different mutations within the *IRAK4* gene have been reported in patients with pyogenic bacterial infections and an impaired response to LPS.[34] Moreover, B cells do express another TLR molecule, RP105, which co-regulates the humoral immune response to LPS. Polymorphisms in all the genes of above-mentioned molecules may be involved in conferring susceptibility to GBS after an infection with *C. jejuni*. Until now polymorphisms in the IgG-Fc receptor genes, immunoglobulin KM gene and IL-10 gene have been reported to be associated with disease susceptibility or severity of GBS.[35-38]

In this study we have shown that SNPs in *CD14* and *TLR4* neither conferred susceptibility to develop GBS nor were related to *C. jejuni* serology, anti-ganglioside antibodies or neurological deficits related to a *C. jejuni* infection. Since GBS is a heterogeneous disorder of multifactorial origin other SNPs are most likely involved in conferring susceptibility and severity of the disease. With regard to the *C. jejuni* infected GBS patients, SNPs in genes of other molecules involved in LPS responses may be involved in conferring the susceptibility and clinical pattern of GBS.

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Chapter 4

Antigen presentation and costimulation

Chapter 4.1

HLA class II alleles are not a general susceptibility factor in Guillain-Barré syndrome

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Abstract

Objective: To assess whether human leucocyte antigen (HLA)-DRB1 and HLA-DQB1 alleles confer susceptibility to Guillain-Barré syndrome (GBS) or are related to specific clinical or serological subgroups of GBS.

Methods: The HLA-DRB1 and -DQB1 loci were genotyped by PCR amplification with sequence-specific primers in 164 well-documented Dutch patients with GBS and 207 healthy Dutch control subjects. Patients with GBS were divided into subgroups based on clinical features, severity of disease, antecedent infection, and anti-ganglioside antibodies. Data were compared with those of all case-control HLA studies in GBS performed previously.

Results: In this case-control study, HLA-DRB1 and HLA-DQB1 alleles did not differ between GBS patients and control subjects. The frequency of HLA-DRB1*01 was significantly increased in patients who needed mechanical ventilation (OR 4.2, 95% CI 1.9-9.6; p_c=0.02). Multivariate logistic regression analysis showed that this association was independent of the severity of paresis and the presence of cranial nerve involvement (p-values <0.05). There was a tendency towards an association between certain HLA alleles and several anti-ganglioside antibodies.

Conclusion: Human leucocyte antigen (HLA) class II antigens are not a general susceptibility factor in Guillain-Barré syndrome (GBS). However, HLA-class II alleles may be a determinant in distinct subgroups of Guillain-Barré syndrome, indicating the need for further exploration in large-scale studies.

Introduction

Patients with Guillain-Barré syndrome (GBS) show considerable variation in the pattern and severity of neurological deficits, prognosis, type of antecedent infection, and the presence of anti-ganglioside antibodies. These antibodies are most likely evoked by molecular mimicry between microbial structures and gangliosides present on the peripheral nerves.[1] However, this transient humoral autoimmune response, only rarely occurs in response to common types of infections. The susceptibility to develop such an immune response as well as the clinical heterogeneity of GBS may at least partially be explained by immune modulating genetic host factors.

The highly polymorphic human leucocyte antigen (HLA) system is one of the prominent candidate gene clusters influencing GBS as it plays a central role in the immune responses to infection. Until now, inconsistent findings have been reported with regard to HLA-class II alleles as a susceptibility factor for GBS or its clinical and serological subgroups (Table 1; see also table E-1 on the *Neurology* Web site at www.neurology.org). The susceptibility to several autoimmune diseases is most closely linked with the class II HLA-DR and DQ-antigens.[2] Therefore, we assessed whether HLA-DRB1 and HLA-DQB1 alleles confer susceptibility to GBS or are related to specific clinical and serological subgroups in the largest group of well-documented GBS patients reported thus far (n=164). Furthermore, we compared our findings with previously published case-control studies.

Materials & Methods

Study population

One hundred and sixty-four Dutch Caucasian GBS patients, who had participated in one of the Dutch treatment trials or other national survey studies, were enrolled in this study.[3-5] All patients fulfilled the diagnostic criteria for GBS.[6] Clinical manifestations, infections and ganglioside serology were documented in detail.[4, 7, 8] Screening for antibodies against the most common antecedent infections in GBS (*Campylobacter jejuni*, Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and *Mycoplasma pneumoniae*), and for the presence of the most common anti-ganglioside antibodies were performed according to standard procedures.[7,9] During follow-up, the patients enrolled in the treatment trials were neurologically examined at 13 to 17 different time points (ranging from day of entry to six months of follow-up). The Medical Research Council (MRC)-sumscore of 6 bilateral muscle groups, ranging from 60 (normal) to 0 (tetraparalysis)[10], the GBS functional disability score[11] and the involvement of sensory and cranial nerves were also established at these time points. The severity of the disease was defined in two different ways according to previously reported criteria.[8, 12] One was based on the MRC-sumscore at nadir. The median value of this sumscore at nadir was used as a predefined cut-off point; mildly affected patients were defined by an MRC-sumscore ≥ 40 and severely affected patients by an MRC-sumscore < 40.[8] The other was based on the GBS functional disability score (F-score) at nadir: mild disease was predefined as the ability to walk independently (F-score of 1 or 2) and severe disease as unable to

Table 1. Overview of case-control studies and subgroup analyses in Guillain-Barré syndrome.

(Sub)groups	Significant association	No significant associations
GBS vs. healthy controls	DR3 increased in Mexican GBS patients (n=38) compared to 100 controls[18] A3-B8 increased in Egyptian GBS patients (n=32) compared to 234 controls[19]	GBS patients (n=993) and controls (n=5608) of various ethnic origin[16-17,20-32, *]
Subgroups in GBS patients		
C. jejuni infection	DQB1*03 increased in a Caucasian *C. jejuni*+ GBS group (n=30)[29] HLA-B54 and Cw1 increased in a Japanese *C. jejuni*+ GBS/MFS group (n=26)[22]	37 *C. jejuni* + Caucasian GBS patients (total GBS n=164)[*] 42 *C. jejuni* + Japanese GBS patients (total GBS n=81)[25] 2 Chinese case-control studies[24,26]
Anti-GM1 antibodies		3 studies[24,25, *]
Electrophysiological subgroups	DQ-beta RLD[55-57]/ED[70-71] and DR-beta $E^{9}V^{11}H^{13}$ epitopes associated with AIDP in Chinese patients (n=25)[26]	12 Chinese[27] and 67 Caucasian AIDP patients [*]
Severity of disease		3 studies[20,31, *]
Mechanical ventilation	HLA-DRB1*01 associated with mechanical ventilation[*]	

GBS: Guillain-Barré syndrome, MFS: Miller Fisher syndrome, *C. jejuni: Campylobacter jejuni*, HLA: human leucocyte antigens, AIDP: acute inflammatory demyelinating polyneuropathy, *: present study

walk independently (F-score of 3, 4 or 5).[12] The electrophysiological data of the GBS patients were classified as acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN), equivocal, normal or inexcitable according to previously described electrophysiological criteria.[13]

Two hundred and seven Dutch Caucasian healthy blood donors were used as controls. These control subjects were not age or sex matched. The protocol of this study was reviewed and approved by the Medical Ethical Committee of Erasmus MC, and all patients and healthy control subjects gave their written informed consent.

HLA-DRB1 and –DQB1 typing

Genomic DNA was extracted from ethylenediaminetetraacetate-anti-coagulated peripheral blood samples by a salting out procedure, which was performed with the use of the Invisorb® MaxiBlood kit (Invitek, Berlin, Germany) according to the manufacturer's instruction. Subsequently, HLA-DRB1and HLA-DQB1 typing was performed at the two-digit level by PCR–amplification with sequence-specific primers (PCR-SSP).[14]

Literature search

To compare our data with previously published case-control studies in GBS, we performed a literature search in the Medline® database (National Library of Medicine, Bethesda, MD) using the keywords 'Guillain-Barré' and 'HLA'. Publications written in English up to March 2004 were taken into account and all publications reporting case-control studies were analyzed. The reference list of these publications was screened for additional case-control studies.

Statistical analysis

The HLA-antigen frequencies of GBS patients and control subjects and between different subgroups of GBS patients were compared using the Fisher's exact test (uncorrected p-values (p_u)). To adjust for the number of alleles tested, we also calculated the corrected p-values (p_c). The strength of the association was estimated by calculating the odds ratio's using Woolf-Haldane's method. Multivariate logistic regression was used to further analyze the observed associations. The level of significance was set at $p_{(c)} < 0.05$.

Results

Characteristics of GBS patients and controls

In this study, 77 women and 87 men were included as GBS patients with a median age at onset of 47 years (ranging from 7 to 82 years). Twenty-seven percent of these patients had a gastro-enteritis as prodromal illness, while 39% had an upper-respiratory tract infection. *C. jejuni* serology was positive in 26%, CMV serology in 17%, EBV serology in 15% and *M. Pneumoniae* serology in 7% of the patients. Anti-GM1 antibodies were detected in 23%, anti-GQ1b antibodies in 14% and anti-GM2 antibodies in 9% of the patients. Furthermore, in this group of patients the associations between a *C. jejuni* positive serology and anti-GM1 (p=0.001) and anti-GQ1b antibodies (p=0.02) were

very similar to those described previously.[9] Mechanical ventilation was required in 21% and cranial nerve involvement was present in 56% of the patients. A severe form of disease, based on the MRC-sumscore at nadir, was found in 49% of the patients. Since an F-score ≥ 3 was an inclusion criterion for enrollment in the Dutch treatment trials, almost all of the patients (96%) were classified as having a severe form of GBS based on this score. The electrophysiological data were classified as AIDP in 67 patients (54%), AMAN in 4 patients (3%), equivocal in 51 patients (41%), inexcitable in 1 patient (1%), and normal in 1 patient (1%).

The control group consisted of 125 Dutch women and 82 men with a median age of 35 years (ranging from 19 to 60 years).

HLA-DRB1 and DQB1 allele distribution in GBS patients and controls

The HLA frequencies of the healthy control subjects were compared with the serologically typed HLA frequencies of a large cohort of healthy Dutch controls (n=2440) and proved to be representative for the general Dutch Caucasian population.[15] There was no significant difference in the frequencies of HLA antigens between GBS patients and controls (Table 2).

Table 2. HLA class II allele distribution in Guillain-Barré patients and healthy controls.

	GBS patients (n=164)	controls (n=207)	OR (95% CI)
	n (%)	n (%)	
DRB1*01	35 (21)	41 (20)	1.1 (0.7-1.8)
DRB1*03	38 (23)	63 (30)	0.7 (0.4-1.1)
DRB1*04	56 (34)	58 (28)	1.3 (0.9-2.1)
DRB1*07	33 (20)	42 (20)	1.0 (0.6-1.6)
DRB1*08	11 (7)	11 (5)	1.3 (0.6-3.0)
DRB1*09	6 (4)	5 (2)	1.5 (0.5-4.8)
DRB1*10	4 (2)	7 (3)	0.8 (0.2-2.5)
DRB1*11	20 (12)	35 (17)	0.7 (0.4-1.2)
DRB1*12	6 (4)	3 (1)	2.4 (0.6-8.9)
DRB1*13	42 (26)	46 (22)	1.2 (0.7-1.9)
DRB1*14	18 (11)	9 (4)	2.6 (1.2-5.9)
DRB1*15	38 (23)	64 (31)	0.7 (0.4-1.1)
DRB1*16	2 (1)	7 (3)	0.4 (0.1-1.7)
DQB1*02	55 (34)	84 (41)	0.7 (0.5-1.1)
DQB1*03	92 (56)	102 (49)	1.3 (0.9-2.0)
DQB1*04	9 (5)	10 (5)	1.1 (0.5-2.8)
DQB1*05	56 (34)	65 (31)	1.1 (0.7-1.8)
DQB1*06	74 (45)	96 (46)	1.0 (0.6-1.4)

GBS: Guillain-Barré syndrome, OR: odds ratio and 95% CI: 95% confidence interval.

HLA-DRB1 and –DQB1 alleles in relation to clinical and serological characteristics

When occurrence of HLA-DRB1 and DQB1 was compared to prodromal illness, antecedent infections (*C. jejuni*, CMV, EBV and *M. pneumoniae*), severity of disease, requirement of mechanical ventilation, cranial and sensory nerve deficits, prognosis and electrophysiological subgroups, there was a significant association between the presence of the HLA-DRB1*01 allele (HLA-DR1) and the requirement of mechanical ventilation. Forty-four percent of the ventilated patients were DR1 positive compared to only 16% of the non-ventilated patients (OR 4.2, 95% CI 1.9-9.6, p_u <0.001 and p_c=0.02; table 3). We also compared the difference in the percentage of patients requiring mechanical ventilation between the DR1$^+$ group and DR1$^-$ group of patients at different time points (Figure 1). A higher percentage of patients within the DR1$^+$ group needed mechanical ventilation 1 week after randomization compared to the DR1$^-$ group (p=0.001).

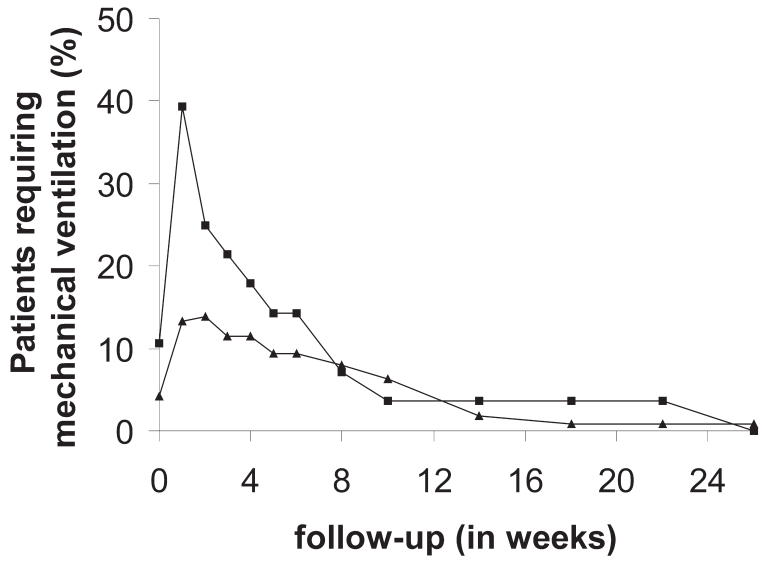

Figure 1.

HLA-DRB1*01 allele is associated with mechanical ventilation. Percentage of GBS patients requiring mechanical ventilation in the HLA-DR1$^+$ group (black square; n=28) versus HLA-DR1$^-$ group (black triangle; n=118) represented at 13 time points during a follow-up of 26 weeks.

* Significant difference between HLA-DR1$^+$ group versus HLA- DR1 $^-$ group (p=0.001).

DR1 was also associated with the presence of cranial nerve deficits, but this association did not reach significance after correction for multiple testing (see Table 3). To further evaluate the clinical relevance of the association between HLA-DRB1*01 allele and the need for mechanical ventilation, we performed a univariate logistic regression analysis on the following variables: age, sex, HLA-DR1, MRC-sumscore at entry, cranial nerve deficits and *C. jejuni* and CMV infection. The following variables showed a significant positive effect: HLA-DR1, MRC-sumscore at entry and cranial nerve deficits (all $p < 0.005$). We included these variables in a multivariate logistic regression model and all variables showed a p value of < 0.05, indicating that the association between HLA-DR1 and mechanical ventilation was independent of the severity of paresis and the presence of cranial nerve deficits.

The frequency of the HLA-DRB1*04 allele was increased in the group of patients who had diarrhea, but this association did not reach significance. We also observed several associations between the presence of anti-ganglioside antibodies and HLA-class II alleles, but these associations did not reach significance (Table 3).

Review of previous case-control studies on HLA-distribution in GBS

To our knowledge 17 case-control studies investigating HLA-distribution in GBS patients have been reported. The ethnic origin, number of patients and controls studied, subgroups and associations are listed in table 1 (see also Table E-1 on the Neurology Web site). In the Discussion, these data are compared with the results of our current study.

Table 3. HLA class II allele distribution in clinical and serological subgroups of GBS.

	HLA-association*	HLA-allele frequency †		p_c-value ‡
		positive (%)	negative (%)	
Antecedent infection				
diarrhea	DRB1*04	19/48 (40)	20/94 (21)	0.49
Campylobacter jejuni	none			
Anti-ganglioside antibodies				
anti-GM1,GQ1b, GM2 or GD1a	DRB1*03	16/28 (57)	37/114 (32)	0.65
anti-GM1	DRB1*04	6/49 (12)	26/93 (28)	0.57
anti-GQ1b	DRB1*04	12/49 (24)	8/93 (9)	0.31
anti-GM2	DRB1*08	3/8 (38)	9/134 (7)	0.38
Clinical features				
mechanical ventilation	DRB1*01	15/34 (44)	19/122 (16)	0.02
MRC-sumscore < 40/60	none			
F-score ≥ 3	none			
cranial nerve deficits	DRB1*01	24/31 (77)	56/111 (50)	0.14

* An uncorrected p-value <0.05 is used to identify possible HLA-associations. † The frequency of the selected HLA-allele within a subgroup of GBS patients is compared between patients with or without the mentioned characteristic. ‡ P_c-value: p-value corrected for testing multiple alleles.

Discussion

In the last three decades, 17 case-control studies have investigated whether there is an association between HLA-class I or II antigens and GBS susceptibility and subgroups (Table 1 and Table E-1 on the Neurology Web site).[16-32] With regard to disease susceptibility, most of the studies did not find any association or at most only a weak association that could not be confirmed by other studies. In our study, involving the largest group of Caucasian GBS patients evaluated thus far, we could not find an association between HLA-DR and HLA-DQ molecules and disease susceptibility either. Thus, we can conclude that the HLA-system probably does not play a general role in the susceptibility to develop GBS.

However, as GBS is a very heterogeneous disorder in which the underlying immunological mechanisms could be different in the different clinical subgroups, analysis of these subgroups is potentially a better approach than studying the group as a whole. Within our well-documented cohort of GBS patients we report an association between the presence of the HLA-DRB1*01 allele and the requirement of mechanical ventilation, which has not previously been reported. This association was independent of the severity of paresis and cranial nerve deficits, this last was used as a variable in the multiple regression analysis, since cranial nerve involvement was weakly associated with HLA-DRB1*01. Moreover, deficits of the bulbar nerves could cause dysphagia leading to a pneumonia and mechanical ventilation. HLA-DR1 has not previously been demonstrated to be associated with artificial respiration or respiratory distress. The association between HLA-DRB1*01 and mechanical ventilation might be related to the presentation of a pathogen-derived peptide in the pocket of the HLA-DR1 molecule itself. Alternatively, this association might be indirectly due to other immune-regulatory genes located within the HLA system, such as TNF-alpha and complement factors.[33] In theory, the association could also be merely due to multiple-testing bias and it therefore requires independent confirmation in another well-defined and large group of Caucasian GBS patients.

Inconsistent HLA associations have been reported for *C. jejuni* infected GBS patients, the presence of certain anti-ganglioside antibodies and electrophysiological characteristics (Table 3 and (E)T-1). Briefly, GBS after a *C. jejuni* infection was associated with HLA-DQB1*03 in a Caucasian group[29], while in a Japanese group it was associated with HLA-B54 and Cw1.[22] The frequency of HLA-DRB1*0803 was increased in Japanese GBS patients with anti-GM1 antibodies[25], while in Chinese patients HLA-DQA1*0301 was increased, but neither significantly.[24] We could not confirm any of these associations within our large subgroups of GBS patients. The data obtained from Japanese or Chinese patients may reflect differences in ethnic origin, or the occurrence of AMAN, which is common in Japan and China and rare in The Netherlands. In our study, the frequency of HLA-DRB1*04 was increased in the anti-GQ1b positive subgroup and decreased in the anti-GM1 positive subgroup, but this difference did not reach significance after correction for testing multiple alleles. The tendency towards an association between certain HLA-class II alleles and anti-

ganglioside antibodies suggests that the production of anti-ganglioside antibodies may be partly dependent on the presence or absence of certain HLA-antigens.

In Chinese AIDP patients (n=25) the frequencies of the HLA peptide motifs DQβ ED^{70-71} and DRβ $E^{9}V^{11}H^{13}$ were higher, while that of DQβ RPD^{55-57} was lower compared with the control group.[26] Although we did not specifically analyze the HLA-peptide motifs, we were able to directly compare our results with these results since the peptide motif DQβ ED^{70-71} in our population is predominantly present in HLA-DQB1*04 alleles and the DRβ $E^{9}V^{11}H^{13}$ peptide motif in HLA-DRB1*04 alleles. The frequencies of these alleles were not increased in our large group of AIDP patients (n=67) compared to the controls. In general, the results of all HLA studies and GBS subgroup analyses showed only weak associations, if any, and the results are inconsistent. This inconsistency could be based on the small numbers of patients studied, methods used to detect *C. jejuni* infections and anti-ganglioside antibodies, differences in defining clinical patient subgroups and differences in susceptibility due to ethnic origin.

Another explanation could be that presentation of antigens by HLA molecules may not be involved in the predisposition to develop GBS after a common infection.

HLA-molecules bind pathogen-derived peptides and present these to T cells, which in turn are activated in an HLA-restricted manner. In the pathogenesis of GBS there is no convincing evidence as yet that peptide epitopes of either the host or pathogen play a major role. In an animal model for GBS, experimental autoimmune neuritis, the peripheral nerve myelin proteins P_0, P_2, PMP-22, myelin-associated glycoprotein and myelin basic protein initiate an inflammatory demyelinating response.[34] However, the pathogenic role of these proteins in GBS is a matter of debate.[35] In contrast, the antibody response in GBS patients is directed against glycolipids instead of proteins. In the case of *C. jejuni* infected GBS patients, antibodies are directed to lipo-oligosaccharide present on the outer membrane of *C. jejuni* and to gangliosides in the peripheral nerves.[36, 37] Gangliosides may be presented by CD1b, a nonpolymorphic HLA class I-like molecule instead of classical HLA-class II molecules.[38, 39]

As some pockets of the HLA molecules can bind large hydrophobic residues, we cannot exclude the possibility that besides CD1b-molecules, HLA molecules also present relevant glyco-conjugates and thereby modulate the immune response in GBS. This might explain the association between HLA class II allele and requirement of mechanical ventilation and the tendency towards an association with antibody responses against individual gangliosides.

The finding that there is no clear association between HLA-class II and GBS is remarkable, considering the well-known HLA associations in classical autoimmune diseases. However, it is consistent with the fact that GBS does not have the demographic characteristics which are typical for other autoimmune disorders like multiple sclerosis and rheumatoid arthritis: (i) in GBS women are not more frequently affected than men, (ii) the incidence of GBS is not highest during the fertile period, but instead slightly increases with age, (iii) GBS is a post-infectious disorder, (iv) instead

of a relapsing-remitting course, GBS patients have a monophasic course of disease, and (v) GBS is not associated with other autoimmune disorders. These differences further indicate that the pathogenesis of GBS differs considerably with that of HLA II-related autoimmune diseases.

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Chapter 4.2

Genetic polymorphisms in costimulatory molecules in relation to clinical and serological subgroups of Guillain-Barré syndrome

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Abstract

Objectives: Guillain-Barré syndrome (GBS) is a heterogeneous immune-mediated polyneuropathy preceded by common types of infection. This post-infectious autoimmune response is rarely seen suggesting a role for variations in genes encoding immune regulatory molecules. In this study we assessed whether single nucleotide polymorphisms (SNPs) in the costimulatory genes *CTLA4*, *CD28*, *CD86*, *CD40*, *CD40L* and *ICAM1* are a general susceptibility factor for developing GBS or are related to antecedent infection, anti-ganglioside antibodies, neurological deficits, severity of disease and recovery rate.

Methods: 274 Dutch GBS patients, of whom 154 participated in one of the Dutch treatment trials, and 212 healthy Dutch controls were recruited and their genotypes were determined.

Results: The genotype frequencies did not differ between GBS patients and controls. However, the T-allele at position –318 of the promoter region of *CTLA4* and its $T^{-318}A^{+49}$ haplotype were associated with a mild form of disease (F-score $\leq$ 2). The T-allele at position –1 of *CD40* was associated with the presence of anti-ganglioside antibodies.

Conclusion: These associations suggest that genetic variations in costimulatory molecules are not a general susceptibility factor, but might modulate the immune response in specific subgroups of GBS.

Introduction

Guillain-Barré syndrome (GBS) is the most common form of acute flaccid paralysis in the Western world with a mortality rate of about 5%. Crucial in the pathogenesis of GBS is the breakdown of self-tolerance after common types of infection in which molecular mimicry plays an important role.[1] The antecedent infections and anti-ganglioside antibodies are related to specific clinical patterns of neurological deficits and disease progression.[2] This transient autoimmune response after infection occurs not generally. Host susceptibility may be explained by genetic polymorphisms regulating the immune response.

Costimulatory molecules play a central role in the activation, maturation, longevity and apoptosis of antigen presenting cells (APC), B-cells and T-cells, and in the maintenance of self-tolerance. An aberrant immune response after a common type of infection could be the result of an imbalance in the interplay between APC, B and T-cells between costimulatory signals (CD86-CD28 and CD40-CD40L) and down-regulatory signals (CD86-CTLA-4 interaction).[3, 4] The effectiveness of these interactions is promoted by the adhesion molecule ICAM-1. Due to their central role in the immune response, some of these costimulatory molecules are targets for new anti-inflammatory strategies in autoimmune disorders and transplantation.[3, 4]

Single nucleotide polymorphisms (SNPs) within *CTLA4* are associated with several immune-mediated disorders (reviewed by Kristiansen *et al.*).[5] A SNP in the promoter region of *CTLA4*, C(-318)T, is associated with a higher promoter activity.[6] The A(+49)G polymorphism within the first exon of *CTLA4* leads to a reduced expression of CTLA-4 and a more pronounced T-cell proliferation.[7] Other SNPs within the genes encoding costimulatory molecules are the G(+1057)A polymorphism in exon 8 of CD86,[8] the polymorphisms G241R in exon 4 and E469K in exon 6 of *ICAM1*[9] all three resulting in an amino acid substitution, a C/T transition in the Kozak sequence (at position –1) of *CD40* [10] and the third intron of *CD28* [11] and a C(-726)T and C(+220)T polymorphisms in *CD40L*.[12] We postulated that these SNPs might be a susceptibility factors for the development of GBS or for specific clinically or immunologically defined subgroups of GBS.

In this study, we assessed whether these SNPs are a general susceptibility factor for GBS or are related to specific antecedent infection, anti-ganglioside antibodies, neurological deficits, severity of disease and outcome.

Patients and methods

Two hundred and seventy-four Dutch GBS patients (median age at disease onset 46 years, range 7-82 years, male/female ratio=1.08) fulfilling the diagnostic criteria for GBS were included. This group of patients consisted of 154 well-documented GBS patients who participated in one of the Dutch GBS trials[13, 14], 35 patients who participated in an open prospective study[15] and 85 patients who have not participated in a trial or survey. Detailed information about clinical and serological characteristics of these patients has been described previously. Severe (versus mild)

GBS was defined as (1) an MRC-sumscore < 40/60 at nadir or (2) the inability to walk independently (F-score of 3, 4 or 5).[16] Poor outcome was defined as the inability to walk independently after six months of follow-up. Caucasian healthy blood bank donors were used as control group (n=212, median age 35 years, range 19-60 years, male/female-ratio=0.66).

Genotyping was performed using the LightCycler® technique (Roche Diagnostics, Mannheim, Germany). The set of primers and hybridisation probes were designed by Tib MolBiol (Berlin, Germany). Sequence-verified control donors for each genotype were used in every experiment.

Verification of Hardy-Weinberg equilibrium and genotype distribution and allele frequencies were compared using the Pearson's Chi-square test. When one of the cells had an expected value less than five, the Fisher's exact test was used. An expectation-maximization algorithm was used to test for linkage disequilibrium and to compare the estimated haplotype distributions (http://www.mrc-bsu.cam.uk/personal/adrian/welcome.shtml). P-values < 0.05 were considered to be statistically significant.

Results

All SNPs were in Hardy-Weinberg equilibrium (p>0.2) and the SNPs in *CTLA4*, *ICAM1* and *CD40L* were in linkage disequilibrium (p<0.001). The genotype distributions, allele frequencies and the haplotype distributions from the total group of GBS patients did not differ from the healthy controls (Figure 1).

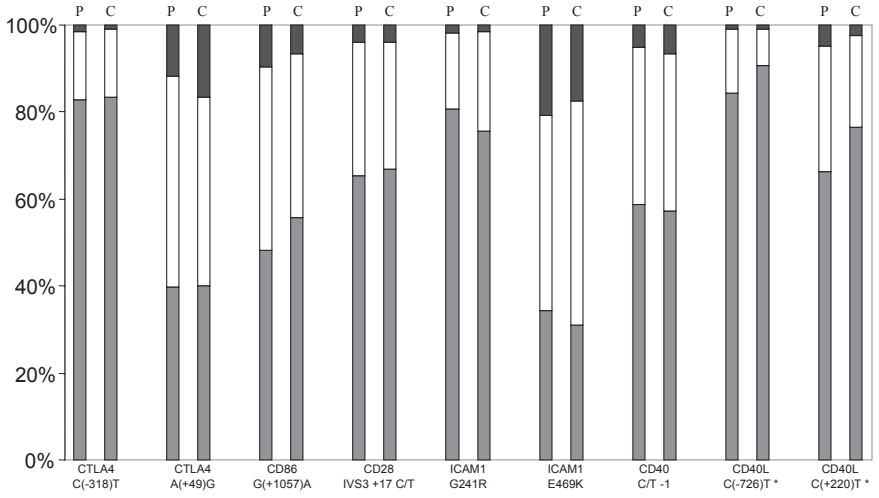

Figure 1. Genotype distribution of single nucleotide polymorphisms within costimulatory genes in GBS patients and healthy controls.

No differences in genotype distributions were found between patients (P) and controls (C): homozygotes wild type-allele, heterozygotes and homozygotes variant allele. CTLA-4: Cytotoxic T-lymphocyte antigen-4. * The CD40L gene is located on the X-chromosome. Since men have only one allele, only the genotype distribution of women is represented.

When GBS patients were subdivided with regard to the severity of disease based on the F-score at nadir, 14 patients were mildly affected and 180 patients were severely affected. In the group of mildly affected patients the T-allele at position –318 of the promoter region of *CTLA4* was more frequently found compared to the severely affected patients (OR = 4.0, 95% CI =1.6 to 10.0, p = 0.001). The haplotype analysis showed that in this group of mildly affected GBS patients the $T^{-319}A^{+49}$ was more frequently found (Table 1). Based on the MRC-sumscore, we did not observe any differences in genotype distribution between mildly and severely affected patients.

In the group of patients whose serum was screened for the presence of anti-ganglioside antibodies, 74 patients had detectable levels of anti-ganglioside antibodies (directed to GM1, GQ1b, GD1a and/ or GM2) and 108 patients did not have detectable of these antibodies. The T-allele at position –1 of the *CD40* was more frequently found in patients with anti-ganglioside antibodies compared with those without these antibodies (29% vs. 18.9%, OR (95% CI = 1.8 (1.1-3.0); p=0.02). The genotype distribution between these subgroups, however, was not significantly different (p=0.065).

No other differences were found between the studied SNPs and age, sex, antecedent infection and outcome.

Table 1. CTLA4 polymorphisms in mildly versus severely affected GBS patients.

SNP	mildly affected (n=14)	severely affected (n=180)	OR (95% CI)	p-value
	F-score < 3 %	F-score ≥ 3 %		
CTLA4 C(-318)T				
CC	50.0	82.8	reference	0.007
CT	42.9	16.1	4.4 (1.3-14.3)	
TT	7.1	1.1	2.4 (0.2-32.3)	
CTLA4 A(+49)G				
AA	42.9	36.1	reference	NS
AG	42.9	50.6	0.7 (0.2-2.4)	
GG	14.3	13.3	0.9 (0.2-4.9)	
CTLA4 -318/+49				
CA	35.7	52.2	reference	0.04
CG	35.7	38.6	1.4 (0.6-3.5)	
TA	28.6	9.2	1.1 (0.4-2.9)	

Severity of disease was based on the functional disability score (F-score) at nadir. Mildly affected patients are still capable of walking independently, while severely affected patients are not able to walk independently and some need artificial respiration. Fisher's exact test was used to compare genotype distributions. OR: Odds ratio, CI: confidence interval. CTLA-4: cytotoxic T-lymphocyte antigen-4.

Discussion

This study shows that none of the selected SNPs in the costimulatory molecules is a general susceptibility factor for developing GBS after a common infection. This lack of association could be due to the heterogeneity of GBS and therefore we also performed subgroup analysis to assess whether the SNPs contribute to specific subgroups of GBS. We did not observe any associations between any of the SNPs studied and age, sex, antecedent infection, neurological deficits or outcome.

However, the T-allele at position –1 of the *CD40* was more frequently found in patients with anti-ganglioside antibodies compared with those without these antibodies. Although the functional effect of this SNP, *CD40* C/T_{-1},has yet not been established, it may affect the translational efficiency of *CD40* and thereby influencing the CD40-CD40L interaction, which is involved in B-cell, T-cell and macrophage activation.[10]

In the subgroup analysis, we also showed that the T-allele at position –318 of the promoter region of *CTLA4* and the $T^{-318}A^{+49}$ haplotype are associated with mildly affected patients based on the GBS functional disability score (F-score < 3; Table 1). This association is mainly due to the presence of a T-allele at position –318, which is associated with a higher promoter activity.[6] This allele may therefore contribute to an increased expression of CTLA-4 upon T-cell activation, thereby promoting down-regulation of the T-cell response, which may favor a mild form of disease. An A-allele at position + 49 might have the same protective effect, since only the GG genotype at this position is associated with a reduced expression of CTLA-4 and an increased T-cell proliferation.[7] Interestingly, results from a mouse model for GBS (experimental autoimmune neuritis) showed that blockade of CTLA-4 resulted in enhanced incidence and more severe course.[17]

Both associations reported could theoretically be false positive results due to multiple testing biases or in the case of CTLA-4 and severity of disease also due to the relative small number of mildly affected patients we studied. Confirmation of these associations in larger subgroups of patients, especially also in mildly affected patients are required. However, given the clear role of CD40 in B-cell activation, antibody production of different subclasses and augmentation of T-cell responses, and of CTLA-4 in down-regulation of T-cell responses, these results encouraging functional studies.

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Chapter 5

Complement and macrophage-mediated nerve damage

Chapter 5.1

Mannose-binding lectin contributes to the severity of Guillain-Barré syndrome

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Abstract

Complement activation plays an important role in the pathogenesis of Guillain-Barré syndrome (GBS). Mannose-binding lectin (MBL) activates the complement system via the lectin pathway after recognition of repetitive sugar groups on pathogens. We investigated whether *MBL2* genotype, serum MBL level and MBL complex activity are associated with the development and severity of GBS. Single nucleotide polymorphisms (SNPs) in the promoter region (-550 H/L and –221 X/Y) and exon 1 (A/O) of the *MBL2* gene were determined in 271 GBS patients and 212 healthy controls. The frequencies of the H-allele, the HY promoter haplotype and the HYA haplotype, which are associated with an increased MBL-production, were all increased in GBS patients compared to healthy controls ($P \leq 0.03$). These associations at genomic level were particularly pronounced in severely affected GBS patients (MRC-sumscore < 40) ($P \leq 0.02$). Moreover, severe muscle weakness at entry was significantly associated with high MBL concentrations and high MBL complex activity in sera from GBS patients ($P < 0.01$). These results favour the hypothesis that complement activation mediated by MBL contributes to the extent of nerve damage in GBS, which is co-determined by the *MBL2* haplotype.

Introduction

Guillain-Barré syndrome (GBS) is a post-infectious immune-mediated polyneuropathy characterized by a monophasic subacute paresis and sensory deficits. It has been hypothesized that antecedent infections in susceptible hosts induce cross-reactive antibodies to peripheral nerve gangliosides, resulting in antibody-mediated complement activation and subsequent nerve damage.[1]

Complement activation seems to play a crucial role in the pathogenesis of GBS. In GBS patients, complement activation products are elevated in serum and cerebrospinal fluid.[2-4] Deposits of activated complement factors, C3d and C5b-9, are found on the outer membrane of Schwann cells in acute inflammatory demyelinating polyneuropathy (AIDP)[5], and on the axolemma of motor fibres in acute motor axonal neuropathy (AMAN).[6] In the mouse diaphragm-phrenic nerve model in which the pathophysiological effects of antibodies can be studied, complement activation is crucial for induction of electrophysiological alpha-latrotoxin-like effects by anti-GQ1b-antibodies and for nerve cytoskeletal damage.[7, 8] Recently, the complement dependency of the pathogenic effect of anti-ganglioside antibodies was confirmed in an *in vitro* assay with neuronal cells expressing gangliosides.[9] Furthermore, the beneficial effect of intravenous immunoglobulin (IVIg) treatment in GBS is likely to be partly due to the prevention of complement activation.[10]

The complement system can be activated via three different pathways: the classical, alternative and lectin pathway. The lectin pathway can be activated via mannose-binding lectin (MBL), a C-type lectin that is primarily synthesized in the liver and that recognizes repetitive sugar groups such as mannose and N-acetyl-glucosamine on the surface of bacteria and viruses.[11, 12] Binding of these sugar groups leads to complement activation and opsonization of the pathogens with C4b and C3b, thereby facilitating phagocytosis. Furthermore, MBL recognizes structures on apoptotic cells and facilitates the clearance of these cells by macrophages.[13, 14]

Serum levels and the functional activity of MBL are highly variable in the human population. This variation is mainly determined by single nucleotide polymorphisms (SNPs) located in the promoter region and in exon 1 of the *MBL2* gene.[15-17] SNPs in exon 1 affect the polymeric structure of the MBL molecule, resulting in low serum levels of MBL and an impaired ability to activate the complement system.[18] Furthermore, two SNPs in the promoter region affect the levels of MBL by regulating the transcriptional activity of the *MBL2* gene: the -550 H/L polymorphism and -221 X/Y polymorphism.[19, 20]

Given the role of MBL in pathogen recognition and opsonization, complement activation and clearance of apoptotic cells, we hypothesized that the functional activity of the lectin pathway partly determines the susceptibility to develop GBS and/or the extent of nerve damage in GBS. Therefore, we assessed the association of the *MBL2* genotype, MBL serum levels and MBL functional activity with GBS susceptibility and severity.

Patients & Methods

Study population

In this study peripheral blood samples were obtained from 271 Dutch Caucasian GBS patients (median age at onset of disease 46 years, range 7-82 years, male/female ratio=1.06) who participated in one of the Dutch trials or national survey studies.[21-24] All patients fulfilled the diagnostic criteria for GBS.[25] The protocol of this study was reviewed and approved by the Medical Ethical Committee of the Erasmus MC, and all patients gave their written informed consent. Of these patients 153 participated in one of the Dutch GBS trials and are therefore documented in detail.[22, 23] At randomisation these patients were clinically examined and pre-treatment blood samples were obtained for screening for the most common antecedent infections in GBS (*Campylobacter jejuni*, cytomegalovirus, Epstein-Barr virus and *Mycoplasma pneumoniae*) and the presence of anti-GM1 and anti-GD1a antibodies.[26, 27] Sera were all tested in 1:100 dilutions in ELISA and were considered to be positive according to criteria defined previously.[27] During a follow-up of six months, the patients were neurologically examined on 13 to 16 time points according to the protocol. The Medical Research Council (MRC)-sumscore (ranging from 0-60),[28] the GBS disability F-score,[29] and the involvement of sensory and cranial nerves were assessed at these time points. Severe (versus mild) GBS was defined as (1) an MRC-sumscore < 40 at nadir[30] or (2) the inability to walk independently (F-score of 3, 4 or 5).[31]

Genomic DNA of 212 Dutch Caucasian healthy subjects (median age 35 years, range 19-60 years, male/female-ratio=0.65) was provided by the Laboratory for Histocompatibility and Immunogenetics, Sanquin Bloodbank South West Region, Rotterdam, The Netherlands. All healthy subjects had given a written informed consent.

Isolation of genomic DNA

Isolation of genomic DNA from EDTA anti-coagulated blood samples was performed using the Invisorb® MaxiBlood kit (Invitek, Berlin, Germany) according to the manufacturer's instruction. The DNA samples were dissolved in 0.1x TE-buffer and stored at -80°C.

Detection of polymorphisms in the promoter region and exon 1 of the MBL2 gene

In this study we detected the (-550) H/L and (-221) X/Y polymorphism in the promoter region of the human *MBL2* gene in a single LightCycler® (Roche Diagnostics, Mannheim, Germany) assay with two sets of hybridisation probes (TibMolBiol, Berlin, Germany). The forward primer 5'-GCCAGAAAGTAGAGA GGTATTTAGC-3' and the reverse primer 5' TGTGACATGCGTGACTAGTAC-3' were used to amplify a fragment spanning the two SNPs in the promoter region. For genotyping the H/L polymorphism at position –550, we used the fluorescein-labelled detection probe 5'-TTTTAGACAGGCTTGCCTGGGT-

3'FLU which is complementary to the L allele and the 5'-LC Red 705- AGCATTT TCTCTGGAAATTTCTTACTACGTTGG-3'-phosphorylated anchor probe. The 5'-CTATAAACATGCTTTCGGTGGCAGT-3'FLU detection probe and the 5'-LC Red 640- AACAAATGGGACCGTGCATTGCCA-3'-p anchor probe were used for genotyping the X/Y SNP.

In another assay, the three SNPs in codon 52, 54 and 57 in exon 1 were detected using one set of hybridisation probes. The area of the *MBL2* gene spanning exon 1 was amplified by using the forward primer 5' TGAGTATGGTCAGCGTCTTA-3' and the reverse primer 5' TGGGCTGGCAAGACAACTATTAG-3'. The 5'-LC-Red 640-TTCTTCCTTGGTGCCATCACACCCA-3'-p detection probe and the fluorescein-labelled anchor probe 5'-CAGCCCAACACGTACCTGGTTCCCCCT-3'FLU were used to detect the three SNPs in exon 1 (TibMolBiol). The "A"-allele represents the wild type, while the "O" allele represents the variant alleles B, C, and D together.

The PCR-reaction and the melting curve were performed in LightCycler capillaries (Roche Diagnostics) with a final volume of 20 µl, containing 10ng genomic DNA, 0.5 µM of each primer, 0.15 µM of each hybridisation probe, 1x LightCycler DNA Master Hybridisation Probes (Roche Molecular Biochemicals, Mannheim, Germany) and 2 mM $MgCl_2$. The PCR amplification profile of both experiments consisted of 10 min at 95°C, followed by 45 cycles of 95°C for 3 sec, 60°C (63°C in case of detection of the *MBL* exon 1 polymorphism) for 15 sec and 72°C for 10 sec. Next, the melting curve profile was performed, which consisted of 1 cycle of 95°C for 3 min, 55°C for 1 min, 45°C for 30 sec and 40°C for 3 min after which the temperature was slowly increased (0.1°C/sec) to 80°C under continuous detection of the emitted light. Data were analysed using the melting curve program. In each experiment we used sequence-verified control donors for each genotype.

Detection of MBL concentration in serum

MBL concentration levels were measured in pre-treatment serum samples from 82 randomly selected GBS patients. Fifty-one of these patients were mildly and 31 severely affected based on the MRC-sumscore at nadir. Anti-ganglioside antibodies directed to GM1 and/or GD1a were present in 58 patients and absent in 23 of these patients. Additionally, in eight randomly selected GBS patients MBL concentrations were measured in follow-up serum samples obtained at 2 weeks, 4 weeks, 3 months and 6 months after randomisation. Since IVIg is the standard therapy for GBS patients, an IVIg solution preparation (Gammagard SD, Baxter Healthcare, Hyland Division) was tested for the possible presence of MBL. MBL concentrations were determined using a human MBL oligomer ELISA kit (Antibodyshop, Gentofte, Denmark), which specifically detects oligomerized forms of MBL. The ELISA and the calculation of the concentrations were performed according to the manufacturer's instruction.

Detection of MBL complex activity

To assess the MBL complex activity we determined the specific C4b-depositing capacity of the MBL-pathway in serum from the randomly selected GBS patients (n=78; serum from 2 mildly and 2 severely affected patients was not available anymore) by using an ELISA-based method as described before[32] with slight modifications. In short, Maxisorb microtiter wells (Nunc, Kamstrup, Denmark) were coated with 10 μg mannan (Sigma, St. Louis, Missouri) in 100 μl of coating buffer (100mM $NaHCO_3$, 100 mM Na_2CO_3, pH 9.6) and incubated overnight at room temperature (RT). After this coating step plates were washed three times with PBS/0.05 % Tween 20. To avoid non-specific binding, plates were blocked with PBS-1% BSA during 1 h at 37°C. All samples were diluted in cold GVB^{++} buffer (veronal buffered saline, 0.08% gelatine, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.05% Tween 20) containing 1M NaCl, overnight at 4°C. Plates were washed with PBS/0.05% Tween 20 containing 5 mM $CaCl_2$, followed by incubation with purified C4 (1 μg/ml), diluted in GVB^{++}, for 1 h at 37°C. Activation of C4 was assessed as described.[18]

Statistical analyses

Pearson's chi-square test was used to verify whether the SNPs were in Hardy-Weinberg equilibrium and to compare the genotype, allele and haplotype frequencies between patients and healthy subjects. Conditional logistic regression was used to determine the independent contribution of each SNP. The Mann-Whitney *U* test was used to compare MBL concentrations and complex activities in mildly versus severely affected GBS patients. The Spearman rank correlation coefficient was used for correlation analyses. The Friedman test was used to compare the MBL levels in follow-up serum samples from individual patients. The Wilcoxon sign rank test was used to compare MBL-concentrations in follow-up serum samples at two different time points. Probability (*P)* values ≤ 0.05 were considered to be statistically significant.

Results

Genotype, allele and haplotype frequencies in GBS patients and controls: MBL2 gene polymorphism is a susceptibility factor for GBS

In this study we determined the polymorphisms in the promoter region and exon 1 of the *MBL2* gene in 271 GBS patients and 212 healthy subjects. Since all SNPs were in Hardy-Weinberg equilibrium ($P > 0.3$) and in linkage disequilibrium ($P < 0.001$), we were able to compare the genotype, allele and haplotype frequencies between GBS patients and healthy controls. The genotype distribution of the H/L SNP at position –550 of the promoter region and the haplotype distributions were different between GBS patients and healthy controls ($P = 0.004$ and $P = 0.02$ respectively; Table 1). GBS patients had significantly higher frequencies of the H-allele, HH genotype, HY promoter haplotype and the HYA haplotype (all *P*-values ≤ 0.03). The differences in haplotype distributions were mainly due to the presence of an H-allele at position

Table 1. Genotype and haplotype distribution of MBL2 polymorphisms in GBS patients and healthy controls.

	GBS patients (n=271) n (%)	Controls (n=212) n (%)	OR (95% C.I.)	*P*-value	severely affected patients (n=71)* n (%)	mildly affected patients (n=82) n (%)	OR (95% CI)	*P*-value
promoter SNP -550H/L								
genotype frequency								
L/L	95 (35.1)	101 (47.6)	reference	0.004	18 (25.4)	39 (47.6)	reference	0.02
H/L	127 (46.9)	91 (42.9)	1.5 (1.0-2.2)		36 (50.7)	31 (37.8)	2.5 (1.2-5.3)	
H/H	49 (18.1)	20 (9.4)	2.6 (1.4-4.7)		17 (23.9)	12 (14.6)	3.1(1.2-8.0)	
MBL2 haplotype								
allele-frequency								
LYA	131 (24.3)	124 (29.5)	reference	0.02	36 (25.4)	44 (26.8)	reference	0.04
HYA	176 (32.7)	103 (24.5)	1.6 (1.1-2.3)		55 (38.6)	44 (26.8)	1.5 (0.8-2.7)	
LXA	106 (19.7)	94 (22.4)	1.1 (0.8-1.6)		20 (14.1)	31 (18.9)	0.8 (0.4-1.7)	
LYO	78 (14.5)	73 (17.4)	1.0 (0.7-1.5)		16 (11.3)	34 (20.7)	0.6 (0.3-1.3)	
HYO	47 (8.8)	26 (6.2)	1.7 (0.9-1.2)		15 (10.6)	11 (6.8)	1.6 (0.6-3.9)	
genotype frequency								
no HYA-allel present	126 (46.8)	122 (58.1)	reference	0.03	29 (40.8)	47 (57.3)	reference	0.1
HYA heterozygotes	110 (40.9)	73 (34.8)	1.5 (1.0-2.2)		31 (43.7)	26 (31.7)	1.9 (0.9-3.9)	
HYA homozygotes	33 (12.3)	15 (7.1)	2.1 (1.1-4.1)		11 (15.5)	9 (11.0)	2.0 (0.7-5.4)	

The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated in comparison with the reference (most frequent allele, genotype or haplotype in controls). Since two MBL2 haplotypes in GBS patients contained missing values and two LXO haplotypes were present in one of the controls, the haplotype analysis compares 269 GBS patients to 210 healthy controls.

* Severely affected patients were defined as an MRC-sumscore < 40 and mildly affected patients as an MRC-sumscore ≥ 40. The MRC-sumscore ranges from 0 (tetraparalysis) to 60 (normal). The "A" represents the wild-type alleles for the exon 1 SNPs and the "O" the variant alleles (either B, C or D).

–550 of the promoter region, which was independent of the SNPs in exon 1 ($P = 0.001$). The relation between the number of H-alleles or HYA haplotypes and disease susceptibility is best described by a multiplicative model: the presence of one H-allele or HYA haplotype was associated with an increased risk for GBS, but the presence of two H-alleles or HYA haplotypes increased this risk even more ($P \leq 0.03$). Together, these data indicate that the susceptibility for GBS is associated with *MBL2* genotypes that promote high production of MBL.

The distribution of exon 1 variant alleles did not significantly differ between patients and controls. In the control group, the frequencies of the four different exon 1 alleles were: 75.7% (A-allele = wild-type), 15.6% (B-allele), 1.9% (C-allele) and 6.8% (D-allele). In GBS patients these frequencies were 76.6 % (A-allele), 13.2% (B-allele), 2.0% (C-allele) and 8.2% (D-allele). The X/Y promoter SNP at position –221 also did not significantly differ in genotype distribution between GBS patients and controls. The Y-allele was present in 77.1% of the controls and in 80.4% of the GBS patients.

Genotype, allele and haplotype frequencies are associated with a severe form of GBS

Considering the significant differences in genotype and haplotype frequencies between GBS patients and controls and the heterogeneity of GBS, we searched for correlations between *MBL2* SNPs and clinical and serological subgroups of GBS patients. The genotype distribution of the H/L SNP at position –550 of the promoter region and the haplotype distributions were different between severely affected (MRC-sumscore < 40) and mildly affected (MRC-sumscore ≥ 40) GBS patients ($P = 0.02$ and $P = 0.04$ respectively; Table 1). The frequencies of the H-allele, HH and HL genotype and HY promoter haplotype (all P-values ≤ 0.02) were significantly increased in severely affected GBS patients. The *MBL2* haplotype frequencies did not differ between severely and mildly affected patients.

The frequencies of these genotypes and haplotypes did not differ between the mildly affected GBS patients and healthy controls. However, the genotype distribution ($P < 0.001$) and haplotype distributions ($P \leq 0.02$) were significantly different between severely affected GBS patients and healthy controls. The frequencies of the H-allele, HH genotype, HY promoter haplotype and HYA haplotype were significantly increased in severely affected GBS patients (all P-values ≤ 0.02). The observed differences in haplotype distribution were mainly due to the presence of an H-allele at position –550 of the promoter region, which effect was independent of the SNPs in exon 1 ($P < 0.001$). To describe the relation between the number of H-alleles and HYA-alleles and the severity of GBS, a multiplicative model was the best-fitted model ($P < 0.02$). These data indicate that the *MBL2* haplotypes that promote high production of MBL are mainly a susceptibility factor for getting a severe form of GBS.

In further subgroup analyses, the O-allele was more frequently found in the group of patients with a positive serology for either *C. jejuni*, CMV, EBV or *M. pneumoniae* (30.4% versus 19.2%, $P = 0.02$), but not with a positive serology for one antecedent

infection in particular. The SNPs in the *MBL2* promoter and exon 1 were not associated with sex, age, sensory and cranial nerve deficits, severity of disease based on the GBS functional disability score, need for artificial respiration and the presence of antibodies to GM1 and/or GD1a.

MBL serum levels and MBL complex activity in relation to genotypes

In order to establish the functionality of the SNPs we studied the MBL2 genotype in relation to the serum MBL concentration and functional activity of MBL in GBS patients. The MBL complex activity was highly correlated with MBL concentrations in serum samples from 78 GBS patients (Figure 1A). The MBL concentrations were significantly different when comparing the genotypes of exon 1. Highest concentrations were found in A/A homozygotes (median level 2950 ng/ml), intermediate concentrations in A/O heterozygotes (median level 620 ng/ml) and lowest concentrations in O/O homozygotes (median level 30 ng/ml; $P < 0.0001$, data not shown). Similar differences were observed for the median level of MBL complex activity: highest in A/A homozygotes (386 U/ml), intermediate in A/O heterozygotes (66 U/ml) and undetectable in O/O homozygotes (Figure 1B). As previously described [19], also the polymorphism at position –550 of the promoter region affected the MBL concentration. The median concentration of MBL was higher in H/H homozygotes (3450 ng/ml) compared to L/L homozygotes (1160 ng/ml; $P = 0.02$, data not shown). A similar difference was found with respect to MBL function: median complex activity of 372U/ml in H/H homozygotes versus 133 U/ml in L/L homozygotes (Figure 1C).

High MBL concentrations and MBL complex activity are associated with severe form of GBS

Since we observed an association between *MBL2* haplotypes that promote high MBL production and a severe form of GBS, we examined the relation between MBL serum concentration and complex activity and the severity of GBS. High MBL concentrations were associated with low MRC-sumscores at entry ($R = -0.27$; $P = 0.01$, data not shown). The same trend was found between high MBL concentration and low MRC-sumscores at nadir, although this association was not significant ($R = -0.20$; $P = 0.07$, data not shown). Furthermore, high MBL complex activity was associated with low MRC-sumscores at entry ($P = 0.009$; Figure 2A) and at nadir ($P = 0.04$; Figure 2B). The MBL-complex activity was also significantly higher in the severely affected patients compared to the mildly affected GBS patients (Table 2).

Since the presence of antibodies directed to GM1 or GD1a is also associated with a severe weakness in GBS, we compared the MBL concentrations and complex activity in patients with or without these antibodies. The MBL concentration and MBL complex activity were significantly higher in the group of patients with these antibodies than patients lacking these antibodies (Table 2).

Table 2. Serum MBL concentration and MBL complex activity in subgroups of patients.

	severity of disease		*P*-value	anti-ganglioside antibodies		*P*-value
	severe (n=31)*	mild (n=51)		present (n=23)*	absent (n=58)	
MBL concentration (ng/ml)	3324.9 (+/- 522.7)	2506.4 (+/- 418.0)	0.08	4088.3 (+/- 792.0)	2308.9 (+/-322.1)	0.04
MBL complex activity (U/ml)	336.8 (+/- 40.6)	222.7 (+/- 32.4)	0.01	373.2 (+/-54.3)	214.4 (27.7)	0.02

MBL concentration (ng/ml) and MBL complex activity (U/ml) are represented by mean value and the standard error of the mean between brackets. Severely weakness defined as having an MRC-sumscore < 40 and mild weakness as an MRC-sumscore ≥ 40. The presence of anti-ganglioside antibodies was defined as the presence of IgM or IgG antibodies directed to GM1 or GD1a. *MBL-complex activity was determined in 78 GBS patients: 29 severely and 49 mildly affected GBS patients and 23 GBS patients with and 54 patients without anti-ganglioside antibodies.

MBL serum levels during the disease course

The kinetics of MBL concentrations was determined in serum samples from eight randomly selected GBS patients during a follow-up of six months. Nadir of MRC-sumscore was reached at entry in six patients and at 14 days after entry in two patients. The median MRC-sumscore at nadir was 44.5, ranging from 13 to 50. The median MRC-sumscore after six months of follow-up was 57, ranging from 45 to 60. The MBL levels changed significantly during follow-up ($P < 0.001$; Figure 3). Compared to pre-treatment samples, MBL levels were significantly higher in serum obtained at 2 weeks (1.2-fold increase, $P = 0.02$) and significantly lower in serum obtained at six months (1.8-fold decrease, $P = 0.03$). IVIg preparations contained no detectable levels of MBL (data not shown).

Discussion

Antecedent infection in combination with genetic host factors most likely precipitate the cross-reactive immune response and deposition of activated complement factors at peripheral nerves in GBS. MBL plays a crucial role in both pathogen recognition and activation of the complement system and may be one of the host factors in the susceptibility to develop GBS. In the present study we show that the frequencies of *MBL2* haplotypes that promote high levels of MBL are increased in GBS patients, in particular in those patients with a severe paresis. The patients with a severe paresis also had high serum MBL levels and complex activity. MBL activity determined by *MBL2* gene polymorphisms may therefore contribute to the extent of peripheral nerve damage in GBS.

MBL serum levels and complex activity are mainly determined by *MBL2* polymorphisms. In this study we studied in a large group of Dutch Caucasoid GBS patients the SNPs in the *MBL2* gene which are known to influence the serum MBL

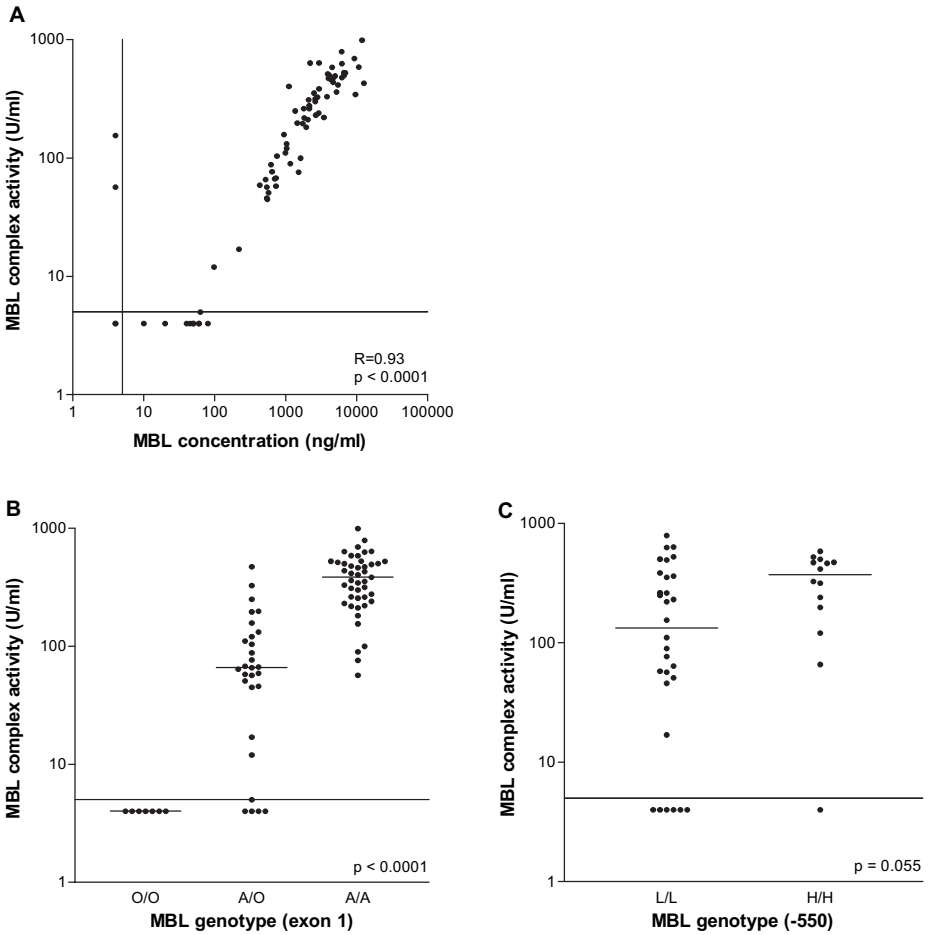

Figure 1.

MBL serum concentration in relation to MBL complex activity in GBS patients (N=78) (A). The two outliers with no detectable MBL concentrations despite the presence of MBL complex activity represent two patients with a LXA/LXA haplotypes. *MBL2* exon 1 genotypes in relation to MBL complex activity (B) in GBS patients. *MBL2* promoter genotype at position –550 in relation to MBL complex activity (C). The thick black line represents the median MBL serum level and complex activity. The dotted lines represent the detection limits of the assays.

levels and functional activity. We did not investigate the +4 P/Q SNP at the 5' untranslated region of exon 1, since the LYPA haplotype is almost absent in Caucasoid individuals and the Q-variant is only present in combination with the LY promoter haplotype.[19] A recent paper reported other SNPs in a haplotype block at the 3' end of

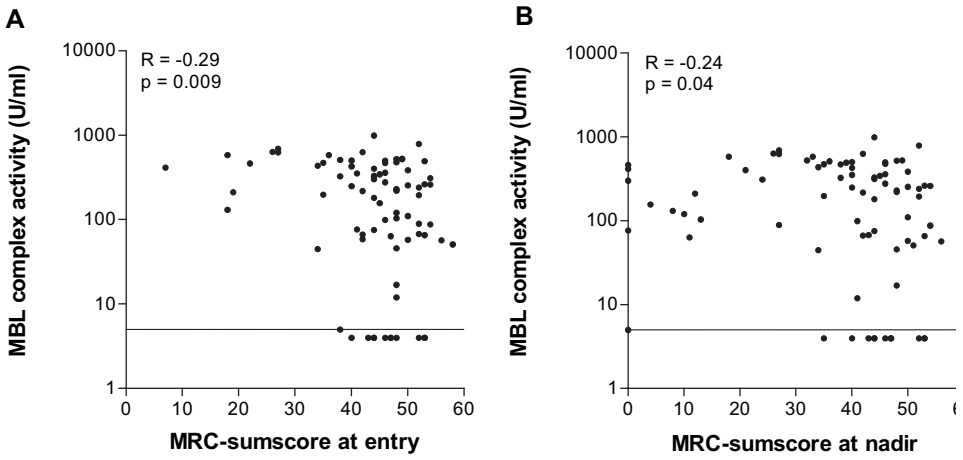

Figure 2.

MBL complex activity in relation to the extent of muscle weakness (MRC-sumscore) measured at entry (A) or nadir (B) in GBS patients (N=78). The MRC-sumscore ranges from 0 (tetraparalysis) to 60 (normal). The dotted lines represent the detection limits of the assays.

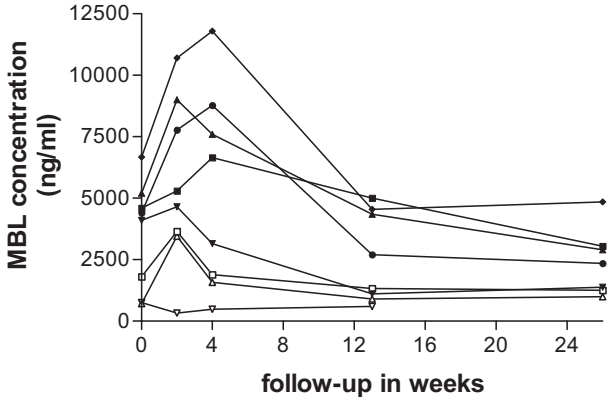

Figure 3.

MBL serum levels during a follow-up of six months in eight GBS patients.

the *MBL2* gene of which the functional effects on MBL have not been established.[33]

The observed genotype frequencies in our healthy control group and the high associations between genotype/haplotype and serum MBL levels and complex activity in GBS patients are consistent with the findings of others in healthy controls, indicating that our data on genomic, serum and functional level are accurate and representative.[18, 19, 34, 35]

The high frequencies of the HH genotype and HY and HYA haplotypes in GBS patients, particular in severely affected GBS patients, were largely determined by the presence of an H-allele at position -550 of the promoter region and not by the other polymorphisms. The H/L and X/Y alleles have a direct effect on *MBL2* gene expression by regulating the transcriptional activity. The strong association between the H-allele, HYA haplotype and severe disease in GBS patients most likely reflects a role for high production of functional MBL, which was confirmed at serum protein and functional level.

Our data showed that patients with MBL deficiency, characterized by undetectable MBL complex activity, show a mild weakness, with MRC-sumscores above 40 at entry. The inverse correlation between MRC-sumscores and MBL functional activity may indicate that patients only develop a severe form of GBS when a certain threshold level of MBL is present. MBL levels usually increase upon infection, since MBL is an acute phase protein. This acute monophasic response was also found in follow-up serum samples from GBS patients, which showed an initial rise in MBL in the acute phase and a decline to baseline 6 month after onset of disease. This transient rise in MBL most likely is related to an acute phase response after infection, but could not explain the relation between high MBL levels and GBS or the extent of peripheral nerve damage. During acute phase reactions MBL levels may rise up to 3-fold. The more than 1000 fold differences in MBL levels found in the pretreatment serum samples from the GBS patients could only be explained by genetic variation.[12]

High levels of MBL may exert its effects both at the afferent and efferent part of the immune response leading to peripheral nerve damage in patients with GBS.

(i) MBL binds to repetitive sugar residues on a broad range of pathogens, thereby possibly enhancing the immune response to antecedent bacterial and viral infections associated with GBS. Activation of the complement system facilitates phagocytosis by opsonization and enhances antigen presentation and antibody production.[12, 36] This may increase the susceptibility to develop a cross-reactive immune response to peripheral nerves after infection. Accordingly an association between high MBL levels and the presence of antibodies to GM1 and GD1a was found, which are known to be frequently cross-reactive with infectious agents and are associated with severe GBS.

(ii) MBL may also be involved in the efferent part of the immune response by inducing or enhancing tissue damage. MBL can activate the lectin pathway upon recognition of antibodies of the IgA subclass[37] and of non-galactosylated IgG antibodies[38] or might activate this pathway upon recognition of carbohydrate clusters formed via cross-linking of gangliosides by anti-ganglioside antibodies. Moreover,

MBL is able to bind directly to apoptotic and necrotic cells[14]. Binding of MBL to damaged endothelium following hypoxia is involved in complement activation and tissue damage following ischemia/reperfusion injury.[39] Recently, a role for MBL in tissue injury has been suggested for several inflammatory diseases such as rheumatoid arthritis,[40] ulcerative colitis[41] and diabetic vasculopathy.[42] Moreover, a recent report demonstrated an association between high levels of MBL and chronic rheumatic heart disease in patients previously diagnosed with rheumatic fever.[43] GBS and rheumatic fever share the important role of antecedent infections, molecular mimicry and cross-reactive antibodies in the pathogenesis. High MBL levels may further trigger these post-infectious complications further as indicated by this paper and our manuscript. Therefore, the association between MBL and the severity of GBS shown in the present study might be explained by binding of MBL to initially damaged nerve tissue, followed by complement activation, attraction of inflammatory cells, and aggravation of tissue injury.

The overlap in MBL levels between mildly and severely affected GBS patients illustrates the complexity and heterogeneity of GBS. If complement activation indeed plays such an important role in the pathogenesis, the extent of peripheral nerve damage and motor deficits will also depend on local regulatory components, such as complement-inhibitory molecules like the decay-accelerating factor (CD55). Moreover, other host factors like higher age at onset and environmental factors such as *C. jejuni* infection have already been shown to be prognostic factors for the development of a severe weakness.[26, 30]

In conclusion, our study demonstrates an association between *MBL2* gene polymorphisms and severity of GBS. The higher levels of MBL protein and complex activity in patients with severe GBS associated with these polymorphisms further suggests that complement activation mediated by MBL contributes to the extent of nerve damage in GBS. Since the lectin pathway can be involved in several phases of the immune response, further studies are warranted to elucidate the underlying mechanism.

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Chapter 5.2

Genetic polymorphisms of macrophage-mediators in Guillain-Barré syndrome

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Abstract

In this study we assess in a large cohort of Guillain-Barré patients (n=262) whether eight SNPs in genes encoding macrophage related inflammatory mediators are related to the development of GBS or to specific clinical or immunological subgroups. A SNP in TNF-α and a SNP in MMP-9 were associated with severe weakness (MRC-sumscore < 40) and poor outcome (not able to walk unaided after 6 months), suggesting that these SNPs might be one of the factors contributing to a severe form of GBS.

Introduction

Macrophages play a crucial role in demyelination and axonal degeneration in respectively the acute inflammatory demyelinating neuropathy (AIDP) and acute motor axonal neuropathy (AMAN) variants of Guillain-Barré syndrome (GBS).[1] Macrophages are guided to their target sites by complement-receptors, Fcγ-receptors and complement chemotaxis, and exert their functions by the release of cytokines and other inflammatory mediators. Tumor necrosis factor-alpha (TNF-α), matrix metalloproteinase 9 (MMP-9) and nitric oxide synthetase (iNOS) induced nitric oxide (NO) play a role in leucocyte attraction, extracellular matrix degradation, demyelination and axonal degeneration. Other mediators like urokinase-type plasminogen-activator (uPA) and its inhibitor plasminogen activator inhibitor 1 (PAI-1) may also be involved in blood-nerve breaching. IL-10, another mediator released by macrophages, may exert pro-inflammatory as well as anti-inflammatory effects, since it promotes antibody production as well as the termination of inflammatory response and tissue destruction.[2] Interactions between macrophages, these mediators and blood-nerve barrier may contribute to the extent of nerve damage.

Single nucleotide polymorphisms (SNPs) in genes encoding these mediators and affecting its levels or functions may be susceptibility factors for macrophage-induced nerve damage in GBS. Previous studies showed that elevated levels of TNF-α and MMP-9 are associated with disease severity and electrophysiological changes in GBS patients.[3,4] Another report showed that a SNP in the promoter region of IL-10 associated with high IL-10 production was a susceptibility factor for the onset of GBS.[5]

In this study we assess whether SNPs in the promoter region of TNF-α, MMP-9, IL-10, PAI-1 and in the coding region of iNOS are susceptibility factors for the development of GBS and whether they are related to the severity and outcome of the disease.

Methods

Study population

In this study 262 GBS patients (median age at disease onset 45.5 years, range 7-82 years, male/female ratio=1.07), who fulfilled the diagnostic criteria for GBS were included. Caucasian healthy blood bank donors (n=201, median age 35 years, range 19-60 years, male/female-ratio=0.62) served as controls. Of the patients, 149 had participated in one of the Dutch trials and 30 in a national prospective study coordinated by the Department of Neurology of Erasmus MC and were clinically and serologically documented in detail.[6] Severe (versus mild) GBS was defined as (1) an MRC-sumscore < 40/60 at nadir or (2) the inability to walk independently (F-score of 3, 4 or 5). Poor outcome was defined as the inability to walk independently after six months of follow-up.

DNA isolation

Isolation of genomic DNA from EDTA anti-coagulated peripheral blood samples was performed with the use of the Invisorb® MaxiBlood kit (Invitek, Berlin, Germany) according to the manufacturer's instruction.

SNP detection

The C(-1562)T polymorphism in MMP-9 was genotyped with the restriction fragment length polymorphism (RFLP)-technique using the restriction enzyme Sph1. Genotyping of the SNPs in the promoter region of TNF-α (C(-863)T, G(-308)A, G(-238)A), two SNPs in the promoter region of IL-10 (G(-1082)A and C(-819)T), a 4G/5G insertion deletion polymorphism at –675 in the promoter region of PAI-1 and one SNP in the coding region of iNOS (S608L) were performed by using multiplex single base extension reactions. Primers and conditions for the conventional PCR-reactions and multiplex SBE reactions are listed in supplementary data ((E)T-1, (E)T-2).

Statistical analysis

Verification of Hardy-Weinberg equilibrium and comparison of genotype and allele-frequencies were performed using Pearson's chi-square test or Fisher's exact test when appropriate. An expectation-maximization algorithm was used to test for linkage disequilibrium and to compare the estimated haplotype distributions (http://www.mrc-bsu.cam.uk/personal/adrian/welcome.shtml). P-values < 0.05 were considered to be statistically significant.

Results

In this study all SNPs were in Hardy-Weinberg equilibrium and the genotype distributions and allele frequencies did not differ between GBS patients and healthy controls (Table 1). The three SNPs in the promoter region of TNF-α and the two SNPs in the promoter region of IL-10 were in linkage disequilibrium ($p < 0.001$). Therefore, the haplotype distributions between GBS patients and controls could be compared, but this did not significantly differ between patients and controls. Since GBS is a heterogeneous disorder we determined whether the SNPs were associated with clinical or serological defined subgroups. The variant allele of the SNP at position –863 of the TNF-α gene and of the SNP at position –1562 of the MMP-9 gene were more frequently found in the severely affected GBS patients (MRC-sumscore $< 40/60$) compared to the mildly affected patients (Table 2). The genotype distribution of the SNP at position –863 of TNF-α did also differ significantly between these subgroups ($p = 0.02$), but the genotype distribution of the SNP at position -1562 of the MMP-9 gene did not ($p = 0.06$). The haplotype distribution of the three SNPs in the promoter region of TNF-α did not significantly differ between mildly and severely affected patients. The variant allele of the SNP at position –863 of TNF-α was also more frequently found in the subgroup of patients who were not able to walk unaided after six months compared to a large group of patients who were able to walk

Table 1. Genotype distribution in Guillain-Barré patients versus healthy controls.

SNP	Guillain-Barré patients	Healthy controls
	n =262 %	n=201 %
MMP-9 C(-1562)T		
CC	78.2	78.1
CT	21.4	21.9
TT	0.4	0
TNF-α C(-863)A		
CC	70.6	70.6
CA	26.0	26.9
AA	3.4	2.5
TNF-α G(-308)A		
GG	73.7	65.2
AG	22.1	32.8
AA	4.2	2.0
TNF-α G(-238)A		
GG	87.4	86.1
AG	11.8	13.9
AA	0.8	0
IL-10 G(-1082)A		
GG	27.1	26.9
AG	46.2	44.3
AA	26.7	28.9
IL-10 C(-819)T		
CC	61.8	62.2
CT	32.4	30.8
TT	5.7	7.0
PAI-1 4G/5G		
AA	31.7	27.9
GA	46.9	53.2
GG	21.4	18.9
NOS2 S608L		
GG	63.7	60.2
AG	32.4	34.3
AA	3.8	5.5

No significant differences were found.

Table 2. Allele frequencies in severely versus mildly affected GBS patients.

	severity of disease		OR (95% CI)	p-value
	severely affected (n=70) %	mildly affected (n=78) %		
TNF-α -863				
C	76.4	87.2	reference	0.02
A	23.6	12.8	2.1 (1.1-3.9)	
MMP-9 -1562				
C	86.4	94.3	reference	0.02
T	13.6	5.7	2.6 (1.1-6.0)	

Severity of disease was based on the Medical Research Council-sumscore at nadir ranging from tetraparalysis (score 0) to normal muscle strenght (score 60). Patients with a MRC-sumscore at nadir of < 40 were defined as severely affected. GBS: Guillain-Barré syndrome, OR :Odds ratio, CI: confidence interval, TNF-α: tumor necrosis factor-alpha, MMP-9: matrix metalloproteinase 9.

independently after six months (Table 3). No associations were found between the SNPs in TNF-α, MMP-9, PAI-1, NOS and IL-10 and age, sex, antecedent infection and anti-ganglioside antibodies.

Discussion

Although the selected SNPs in TNF-α, MMP-9, iNOS, PAI-1 and IL-10 do not confer susceptibility in GBS patients, the variant alleles of a SNP at position –863 of the TNF-α gene and at position –1562 of the MMP-9 gene were associated with the severity of disease based on the MRC-sumscore. Moreover, the variant allele of TNF-α was also associated with poor outcome in a small subgroup of patients. Therefore, these SNPs may contribute to the extent of nerve damage mediated by macrophages. Previous reports showed that high serum levels of MMP-9 as well as TNF-α were associated with severity of disease.[2-5] Whether these high levels of TNF-α

Table 3. Genotype distribution of TNF-α C(-863)A polymorphism in GBS patients with poor or good outcome.

	Walk independently after 6 months		OR (95% CI)	p-value
	% not reached (n=15)	% reached (n=133)		
TNF-α -863				
CC	40.0	71.4	reference	0.02
CA	46.7	25.6	3.3 (0.3-10.7)	
AA	13.3	3.0	7.9 (1.2-53.9)	

The GBS functional disability score at six months was used to define the clinical outcome. Patients who were not able to walk independently after six months of follow-up were defined as having a poor outcome. GBS: Guillain-Barré syndrome, OR: Odds ratio, CI: confidence interval, TNF-α: tumor necrosis factor-alpha, MMP-9: matrix metalloproteinase 9.

and MMP-9 are directly correlated to the SNPs studied is not clear. One study showed that the A-allele at position –863 was associated with higher TNF-α concentration due to an inadequate down regulation of its production.[7] In the case of the SNP at position –1562 of MMP-9, the T-allele is associated with an increased promoter activity that might result in higher MMP-9 levels.[8] These results favor a possible genetic contribution of these promoter SNPs for developing a severe form of GBS.

Interestingly, administration of an inhibitor of MMP-9 and TNF-α release (BB-1101) in a rat experimental autoimmune neuritis model for GBS prevented the development of neurological deficits and conduction abnormalities or markedly reduced the severity of disease.[9] This finding supports the important role of MMP-9 and TNF-α in the pathogenesis of GBS.

In this study we did not confirm the previously reported associations between the SNP at position –819 in the promoter region of IL-10 and disease susceptibility,[5] and between a TNF-α microsatellite marker and *Campylobacter jejuni*-positive GBS patients.[10] This could be caused by chance, the higher sample sizes we studied, selection of patients, the heterogeneity of disease and population stratification.

Although the association between the promoter SNPs in the MMP-9 and TNF-α genes and disease severity or outcome need to be confirmed in another large cohort of GBS patients, it warrants further study to assess the genetic vulnerability in relation to severity of disease and its therapeutic consequences.

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Chapter 6

Apoptosis

Chapter 6.1

Fas polymorphisms are associated with the presence of anti-ganglioside antibodies in Guillain-Barré syndrome

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Abstract

Polymorphisms in genes involved in regulation of immune homeostasis may be a susceptibility factor in the induction of cross-reactive anti-ganglioside antibodies after infection in patients with Guillain-Barré syndrome (GBS). In this study we assessed whether polymorphisms in the promoter region of *Fas* and *FasL* and sFas and sFasL are related to GBS or its distinct clinical or serological subgroups. We show that the A(-670)G SNP in the promoter region of *Fas* and high levels of sFas are associated with the presence of anti-ganglioside antibodies, suggesting that Fas-FasL interaction is involved in the production of cross-reactive antibodies in GBS.

Introduction

Guillain-Barré syndrome is characterized by an acute self-limiting immune-mediated flaccid paralysis occurring after common infections. Autoantibodies generated by molecular mimicry between infectious agents and peripheral nerve structures play an important role in the immunopathogenesis of GBS.[1] The presence of some of these anti-ganglioside antibodies is likely to cause nerve tissue destruction in a macrophage-mediated and complement-dependent way.[2] As only 1 in approximately 1.000 to 100.000 individuals develop GBS after a *Campylobacter jejuni* infection, apparently the occurrence of a cross-reactive pathogenic antibody response is very rare.[3] Therefore, genetic host factors regulating the immune response may be involved in the aberrant antibody response directed against self.

One of the receptor-ligand pairs that are critically involved in the regulation of immune homeostasis by inducing apoptosis is Fas (CD95) and its ligand FasL (CD95L). Fas-FasL interactions play an important role in the elimination of autoreactive B and T-cells.[4, 5] We postulated that single nucleotide polymorphisms (SNPs) affecting the expression levels may be a susceptibility factor to develop an aberrant immune response leading to GBS.

Mutations in *Fas* (TNFRSF6) and *FasL* (TNFSF6) have been associated with lymphoproliferative syndromes and SNPs in their promoter region with several autoimmune disorders.[6-8] These promoter SNPs, G(-1377)A and A(-670)G in the case of *Fas* and C(-843)T in the case of *FasL*, are located at transcription factor binding sites, thereby affecting transcriptional regulation of these genes.[9, 10]

In addition to the interactions between the membrane-bound forms of Fas and FasL, soluble forms of Fas (sFas) and FasL (sFasL) also interfere with the induction of apoptosis. sFas is an alternative splice variant lacking the transmembrane domain of the membrane-bound Fas molecule and it inhibits the induction of apoptosis by binding to FasL.[11] sFasL is cleaved by metallo-proteases from the membrane-bound FasL upon activation and it induces apoptosis.[12] High levels of these soluble forms could therefore also be involved in an aberrant immune response.

In this study, we determined whether SNPs in the promoter of *Fas* and *FasL* are susceptibility factors in the development of GBS or are related with clinical outcome or immunological subgroups. Furthermore, we assessed whether the serum levels of sFas and sFasL are related to clinical outcome or immunological subgroups.

Materials & Methods

Study population

Peripheral blood samples were obtained from 272 Caucasian GBS patients (median age at disease onset 46 years, range 7-82 years, male/female ratio=1.05) who had participated in trials or other national survey studies coordinated by the Department of Neurology of Erasmus MC.[13-16] All patients fulfilled the diagnostic criteria for GBS.[17] The protocol of this study was reviewed and approved by the Medical Ethical Committee of Erasmus MC, and all patients gave their written informed consent. Of

these patients, 153 had participated in one of the Dutch GBS trials and are therefore prospectively documented in high detail with regard to clinical and serological aspects of the disease.[13, 15] The clinical and serological assessment of this group of patients has been described earlier.[18] In addition, clinical and serological information was obtained from 35 patients who had participated in a prospective study.[19] In all patients, anti-ganglioside antibodies were determined in 180 patients (66.2%), *C. jejuni* serology in 167 patients (61.4%), Medical Research Council (MRC)-sumscore at nadir in 153 patients (56.3%) and the GBS disability score (F-score) at nadir in 187 patients (68.8%). In the remaining 84 patients, additional information was only available regarding sex and age.

Genomic DNA of 212 Caucasian healthy subjects (median age 35 years, range 19-60 years, male/female-ratio=0.66) was provided by the Laboratory for Histocompatibility and Immunogenetics, Sanquin Bloodbank South West Region, The Netherlands. All healthy subjects had given written informed consent.

Isolation of genomic DNA

Isolation of genomic DNA from EDTA anti-coagulated peripheral blood samples was performed with the use of the Invisorb® MaxiBlood kit (Invitek, Berlin, Germany) according to the manufacturer's instruction. At the end of the isolation procedure, the DNA samples were dissolved in 0.1x TE-buffer (1mM Tris-HCl (pH 7.5) + 0.1 mM EDTA) and stored at -80°C. DNA samples for SNP detection were diluted in milli-Q to a final concentration of 10 ng/µl and stored at –20° C.

Detection of the SNPs in the promoter region of Fas and FasL

The SNPs in the promoter regions of *Fas* (G(-1377)A and A(-670)G) and *FasL*(C(-843)T) were detected using the LightCycler® technique (Roche Diagnostics, Mannheim, Germany). The SNPs located in one gene were detected in a single assay. The different sets of primers and hybridization probes were designed by TibMol-Biol (Berlin, Germany) (Table 1). The PCR-reaction and the melting curve of all assays were performed in LightCycler capillaries (Roche Diagnostics) with a final volume of 20 µl, containing 10 ng genomic DNA, 0.5 µM of each primer, 0.15 µM of each hybridisation probe, 1x LightCycler DNA Master Hybridisation Probes (Roche Molecular Biochemicals, Mannheim, Germany) and 3 mM $MgCl_2$. The PCR thermocycling profile for *Fas* consisted of 10 min at 95°C, followed by 45 cycles of 95 °C for 0.1 sec, 60°C for 15 sec and 72°C for 10 sec. The PCR-thermocycling profile for *Fas*L was 10 min at 95°C, followed by 45 cycles of 95°C for 0.1 sec, 56°C for 15 sec and 72°C for 15 sec. After amplification, the melting curve profile was performed and data were analysed using the melting curve program. To compensate for the interference between channels in the *Fas* assay, we installed a colour compensation set (Roche Molecular Biochemicals). In each experiment we used sequence-verified control donors for each genotype.

Table 1. Primers and hybridization probes for detection of SNPs in promoter region of Fas and FasL.

	primers	hybridization probes
Fas	GATAATACAGAGAATGCCCATATAC	ACAAGGCTGGCACACCCA-Fl
G(-1377)A	CCTCCTGAGGGCTTTCCA	LC Red640-GTCTTCCTCATGGCACTAACAGTCTACTG-p
A(-670)G	ATAGCTGGGGCTATGCGAT	ATCTGTACTTTTTCATATGGTTAACTGTCC-Fl
	GTCCATGTTGTGGCTGCAA	LC Red705-TTCCAGAAACGTCTGTGAGC-p
FasL	TGAGCCCAGGAGTTTGAG	TGAAAACATTGCGAAATACAAA-Fl
C(-843)T	CTCTTCCCCACACACACCTAC	LC Red640-CAGCTCTGTGGGTTCCACTGGTTT-p

Fl: fluorescein, p: phosphorylated, SNP: single nucleotide polymorphism

Detection of soluble Fas and FasL in serum

We determined the levels of sFas and sFasL using a commercially available sandwich ELISA kit (Diaclone Research, Besançon, France). The levels were determined in pre-treatment serum samples from 104 randomly selected GBS patients (20 mildly and 84 severely affected) and serum samples from 41 healthy individuals. In addition, follow-up samples from 20 severely affected GBS patients at 2 weeks and 6 months post-treatment were tested.

Statistical analysis

Verification of Hardy-Weinberg equilibrium was performed using the program of Ott (http://linkage.rockefeller.edu/ott/linkutil.htm#HWE). The genotype and allele-frequencies between patients and healthy subjects were compared using Pearson's chi-square test. To assess the association between the haplotypes of the Fas gene and GBS, several loglinear models were fitted by maximizing the log-likelihood function using the hapipf program of Mander in STATA (http://www.mrcbsu.cam.uk/personal/adrian/welcome.shtml). This program uses the expectation-maximization algorithm to adjust for unknown phase for double heterozygosity and tests for linkage disequilibrium. From the allelic and estimated haplotype frequencies, odds ratios were computed. Logistic regression was used to further analyze the observed associations. The levels of sFas and sFasL in serum from patients and controls were compared using the Mann-Whitney U test. The Friedman test was used to compare the levels of sFas and sFasL in related serum samples drawn at different time-points from individual patients. The Wilcoxon sign rank test was used to compare these levels in related follow-up serum samples at two different time points. P-values less than 0.05 were considered to be significant.

Table 2. Genotype and haplotype distribution in GBS patients and healthy controls.

SNP	GBS patients (n=272) %	healthy controls (n=212) %
Fas -1377		
GG	76.5	80.6
AG	22.4	18.9
AA	1.1	0.5
Fas -670		
AA	27.9	26.4
AG	47.5	53.8
GG	24.6	19.8
Fas haplotype (-1377/-670)		
GA	51.7	53.3
GG	36.0	36.8
AG	12.3	9.9
FasL		
CC	43.8	39.6
CT	42.6	49.5
TT	13.6	10.9

No significant differences were found. GBS: Guillain-Barré syndrome, SNP: single nucleotide polymorphism

Results

Frequencies of SNPs in Fas and FasL genes in GBS patients versus controls

In this study we determined SNPs in the promoter region of the *Fas* and *FasL* in DNA from 272 GBS patients and 212 healthy subjects. Since the genotype distributions of all three SNPs were in Hardy-Weinberg equilibrium and the two SNPs in the promoter region of *Fas* were also in linkage disequilibrium, we were able to compare the genotype, allele and haplotype frequencies between GBS patients and healthy controls (Table 2). In summary, the genotype and allele frequencies of the detected SNPs in *Fas* and *FasL* and the haplotype frequencies of *Fas* did not differ between GBS patients and controls.

Frequencies of SNPs in Fas and FasL genes in subgroups of GBS patients

GBS is a clinically defined entity, which can be subdivided based on antecedent infection, anti-ganglioside antibodies and neurological deficits. Therefore, we compared the genotype, allele- and haplotype-frequencies within clinical and serological subgroups of GBS patients. We observed a significant difference in the genotype distribution of the SNP at position –670 of the *Fas* gene (p=0.003) and the *Fas* promoter haplotype distribution in patients with and those without anti-ganglioside antibodies (GM1, GM2, GD1a or GQ1b) (Table 3). The frequencies of

Table 3. Allele and haplotype frequencies of Fas promoter polymorphisms in immunological subgroups of GBS patients.

SNP	anti-ganglioside antibodies		OR (95% CI)	p-value
	present (n=74) %	absent (n=106) %		
Fas -1377				
G-allele	81.8	89.1	reference	0.05
A-allele	18.2	10.9	1.8 (1.0-3.3)	
Fas -670				
A-allele	39.2	57.6	reference	0.001
G-allele	60.8	42.4	2.1 (1.4-3.2)	
Fas haplotype (-1377/-670)				
GA	39.2	57.5	reference	0.006
GG	42.6	31.6	2.0 (1.2-3.2)	
AG	18.2	10.9	2.5 (1.3-4.8)	

Guillain-Barré patients were subdivided into those with anti-GM1, anti-GQ1b, anti-GM2 and/or anti-GD1a antibodies and those without these anti-ganglioside antibodies.

the G-allele at position –670 and the A-allele at position –1377, the GG genotype at position -670, the GG (-1377/-670) and AG promoter haplotype were increased in the patients with anti-ganglioside antibodies.

To further evaluate the relevance of the association between SNPs and the presence of anti-ganglioside antibodies, we performed a univariate logistic regression analysis on the following variables: age, sex, infection (*C. jejuni,* CMV, EBV, or *M. pneumoniae*) and two SNPs in the *Fas* promoter region. The following variables showed a significant positive effect: infection (p=0.003), A(-670)G SNP (p=0.001) and G(-1377)A SNP (p=0.04) in *Fas*. We included these three variables in a multivariate logistic regression model and only infection and the SNP in *Fas* at position –670 showed a *p*-value < 0.05. This indicates that the association between the GG genotype at –670 and the presence of anti-ganglioside antibodies was independent of the presence of an antecedent infection. No associations were found between any of the SNP and age, sex, antecedent infection, severity of disease based on MRC-sumscore and GBS disability score and recovery.

Serum levels of sFas and sFasL

Since soluble forms of Fas and FasL may be involved in the dysregulation of the immune response in GBS patients, we determined the levels of sFas and sFasL in GBS patients and healthy controls. Levels of sFas (median levels 134 pg/ml in GBS vs. 55 pg/ml in controls) and sFasL (median levels 126 pg/ml in GBS vs. 25 pg/ml in controls) were significantly elevated in GBS patients compared to controls (p<0.005) (Figure 1A and 1B). When comparing the levels of sFas between different time-points of follow-up within 20 GBS patients, levels of sFas were significantly higher two weeks after start of treatment compared to pre-treatment or six months after

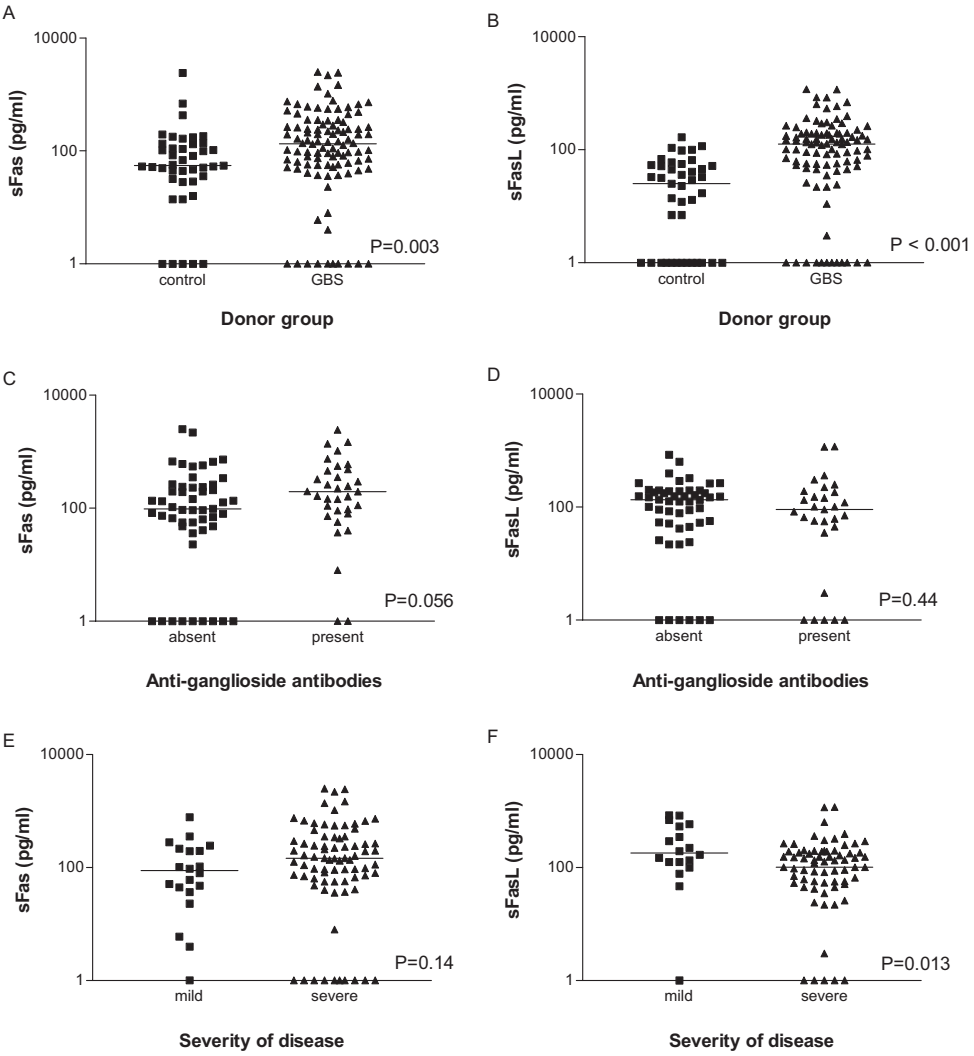

Figure 1. Levels of sFas and sFasL in serum from GBS patients in relation to the presence of anti-ganglioside antibodies and severity.

Levels of soluble Fas (A) and FasL (B) in serum from GBS patients (n=104) versus controls (n=41). In panel C and D, patients were subdivided into those with anti-GM1, anti-GQ1b, anti-GM2 or anti-GD1a antibodies (titre ≥ 100, n=33) and those without these antibodies (titre <100, n=51). In panel E and F, patients were subdivided based on the GBS disability score (F-score). Patients who were not able to walk independently (F-score ≥ 3) were defined as severely affected patients (n=78) and those who were able to walk independently (F-score ≤2) as mildly affected (n=20). The thick black line represents the median level of sFas and sFasL per group.

treatment (p=0.02); median levels pre-treatment were 116.5 pg/ml, 2 weeks post-treatment 429 pg/ml and six months post-treatment 104 pg/ml. The levels of sFasL did not change significantly in GBS patients during follow-up.

In subgroups of patients, levels of sFas tended to be elevated in the patients with anti-ganglioside antibodies (median level 195 pg/ml) compared with patients without these antibodies (median level 97 pg/ml; p=0.056; Figure 1C), but fell short of reaching significance. Levels of sFasL were not significantly different in these subgroups (Figure 1D). The levels of sFasL were significantly higher in mildly affected patients (median level 181.5 pg/ml vs. 101 pg/ml; p=0.01). There was no association between disease severity and sFas levels (Figure 1E and 1F).

The frequencies of the different genotypes within the group of randomly selected patients (n=104) at position –670 were 25.6% GG, 50.0% AG and 24.4% AA, at position –1377 32.6% AG and 67.4% GG and at position –843 of FasL 14.0% TT, 46.5% CT and 39.5% CC, which do not differ from the total group of patients. Since the soluble levels of Fas and FasL are not correlated with the SNPs we do not show the genotypes in figure 1.

Discussion

Fas-FasL interactions are crucial in the elimination of autoreactive B and T cells, and several other celltypes. SNPs in genes coding for these immunoregulatory molecules are associated with several antibody related autoimmune diseases, such as Sjögren's syndrome and systemic lupus erythematosus.[6-8] In this study we showed that these SNPs are not a general susceptibility factor in GBS. However, SNPs in the promoter region of *Fas*, in particular the SNP at position –670, and sFas were associated with the presence of anti-ganglioside antibodies in GBS patients. These SNPs as well as sFas may inhibit apoptosis via different mechanisms, which are discussed below.

The subgroup of patients with anti-ganglioside antibodies was defined by the presence of at least one of the most commonly found anti-ganglioside antibodies in GBS: anti-GM1, -GM2, -GD1a and -GQ1b antibodies. Within these subgroup of patient higher frequencies of the G-allele and GG genotype at position –670, the A-allele at position -1377 and the GG(-1377/-670) and AG promoter haplotypes of *Fas* were found compared to those patients without these antibodies. The SNPs at positions -670 and -1377 are both located at transcription factor binding sites and likely regulate the transcriptional activity of the *Fas* gene. The G-allele at position –670 is associated with a lower transcriptional activity compared to the A-allele.[20] In the case of the SNP at position -1377, the A-allele is associated with a reduced binding of the transcription factor and therefore postulated to result in reduced Fas expression.[21] The increased frequencies of the promoter haplotypes associated with lower Fas expression might indicate a diminished induction of apoptosis in B-cells in these GBS patients, which may contribute to the production of cross-reactive antibodies after infection.

GBS has been reported in two of 23 patients with the very rare autoimmune lymphoproliferative syndrome (ALPS), which is caused by mutations in *Fas* or

FasL.[22-24]Autoimmune disorders characterized by the presence of autoantibodies frequently occur in patients with ALPS. Most likely this association is due to impaired apoptosis caused by the mutations in *Fas* and *FasL*[22] As far as we know no SNPs in the coding regions resulting in an amino acid substitution have been reported in *Fas* and *FasL*. The effects of the promoter SNPs in the current study will be small and less detrimental than the mutations in the *Fas or FasL* in ALPS. Nevertheless these SNPs might be one of the genetic susceptibility factors involved in the production of autoantibodies.

In this study levels of sFas and sFasL were increased in pre-treatment serum samples from GBS patients compared to healthy controls. These increased levels of sFas and sFasL most likely reflect disease activity as has been reported for several immune-mediated disorders.[25-27] The initial increase of sFas in GBS patients is not likely to be due to their treatment with plasma exchange, intravenous immunoglobulins (IVIg) or IVIg plus methylprednisolone, since none of these affect the levels of sFas.[28, 29] Since sFas inhibits apoptosis by binding to membrane-bound FasL, high levels of this molecule in the group of patients with anti-ganglioside antibodies might act in conjunction with the observed higher frequency of the *Fas* promoter haplotype associated with lower expression of Fas. Both factors might be associated with diminished apoptosis of B-cells that are responsible for the production of these antibodies. Reduced apoptosis of antigen presenting cells and T cells, which may also be involved in the anti-ganglioside antibody production, may also have contributed to the aberrant immune response in GBS.

We also observed higher levels of sFasL in the mildly affected group of patients, who were still able to walk independently at nadir. Besides a role in eliminating of autoreactive B- and T-cells, Fas-FasL interactions may also cause tissue destruction or be involved in axonal regeneration. Schwann cells express high levels of both Fas and FasL.[30] This Fas expression may play a role in apoptosis of Schwann cells. On the other hand FasL expression on Schwann cells may induce apoptosis of Fas-bearing invading cells. Soluble FasL may also interfere with these Fas-bearing cells and favour the elimination of invading cells. In addition, sFasL may promote axonal regeneration.[31] These anti-inflammatory and recovery effects of sFasL may possibly explain the observed high sFasL in mildly affected GBS patients compared to severely affected GBS patients.

In conclusion, SNPs in the promoter region of *Fas* and *FasL* are not a general susceptibility factor for GBS. Subgroup analysis showed that the GG genotype at position –670 and the GG and AG promoter haplotypes of *Fas* and high levels of sFas are associated with the presence of anti-ganglioside antibodies in GBS patients, suggesting that Fas-FasL interactions are involved in the induction of a cross-reactive immune response after infection. The associations at genomic level could theoretically be merely due to multiple-testing bias and hence require confirmation in another well-documented and large group of Caucasian GBS patients. Further functional studies

are warranted to elucidate the role of these SNPs and sFas in the immunopathogenesis of GBS.

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Chapter 7

General discussion

Guillain-Barré syndrome is a rare immune-mediated polyneuropathy that occurs after common types of infection. Several lines of evidence show that this antecedent infection triggers the induction of anti-ganglioside antibodies and onset of GBS.[1, 2] However, since only 1 per 1000 infected persons develops GBS after such a common infection[3], host factors are also likely to be involved. This thesis focuses on the contribution of genetic factors in patients with GBS. Two different approaches were used to assess whether there is a genetic susceptibility for the development of GBS or for its specific clinical or serological subgroups, including (1) a search for a substantial number of families with GBS and (2) population-based genetic association studies. Several SNPs in candidate genes were selected for these studies based on their central role in the immune response and their reported effect on protein levels or protein function. The molecules and their interactions are schematically represented in Figure 1. In this section, the methodological issues of the studies performed will be discussed first. Hereafter, a short overview of the results will be presented and discussed. Finally, some future directions will be put forward.

Methodological issues

GBS cohort

The population-based genetic association studies were performed in one of the worlds largest group of well-documented GBS patients. The majority of these patients had participated in one of the Dutch treatment trials or surveys and were therefore documented in high detail with regard to antecedent infections, anti-ganglioside antibodies and clinical characteristics at standardized time points during a six-month follow-up.

Since we have detailed clinical and serological information, this enabled us to perform subgroup analyses. These subgroup analyses are of importance as the underlying immunological mechanisms might be different per antecedent infection and therefore also the genes that confer susceptibility. The patients included in the genetic studies did not differ from the whole group of GBS patients with respect to the clinical and serological characteristics. Most of the patients participated in one of the trials and were severely affected based on the functional disability score (F-score ≥ 3) as this was a primary inclusion criterion.[4, 5] Therefore, the number of patients who were mildly affected is relatively low in these genetic studies. Another way of defining severity of disease is based on the MRC-sumscore (ranging from 0-60) by using its median value as cut-off point: patients with an MRC-sumscore < 40 at nadir are defined as severely affected GBS patients.[6] In the genetic association studies both ways of defining severity of disease were used to assess whether SNPs were associated with disease severity. Performing subgroup analysis based on serological characteristics is limited by the sensitivity and specificity of the assays used for the detection of infections and anti-ganglioside antibodies. In addition patients with negative serology may have had other types of infections or antibodies directed against other gangliosides or ganglioside complexes as has been recently reported.[7]

In this view, subgroup analysis relies on the possibility to define subgroups based on clinical, infectious and immunological characteristics, which were largely present in our cohort of patients.

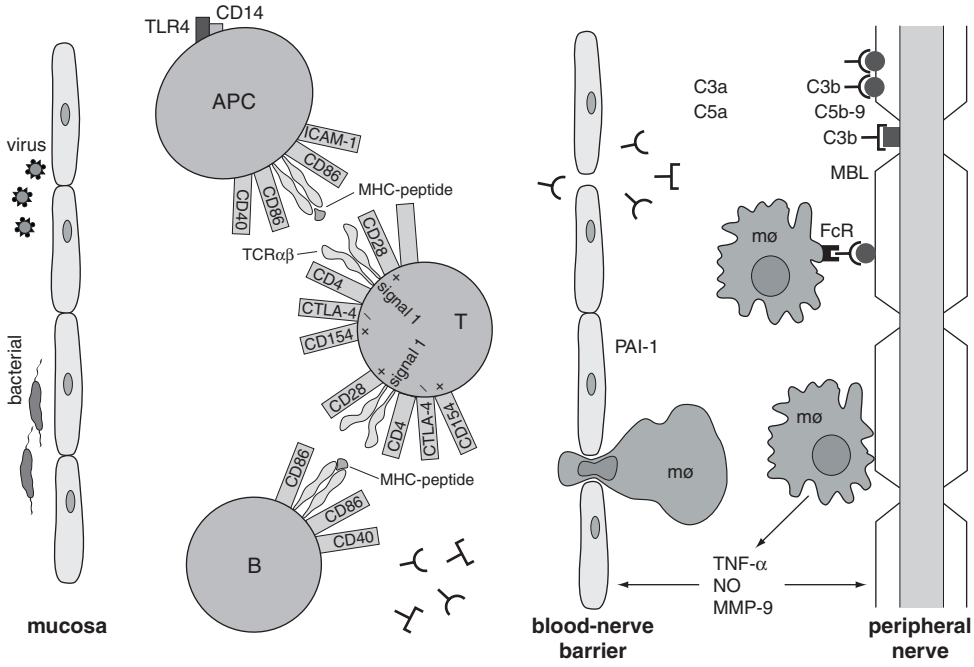

Figure 1.

The pattern recognition receptors CD14 and Toll-like receptor 4 (TLR-4) are involved in the recognition of LPS and in inducing the synthesis of pro-inflammatory cytokines and co-stimulatory molecules favoring an adaptive immune response.[41, 42] The polymorphic HLA-system[10] and costimulatory molecules (CD28, CD86, CTLA4, CD40, CD40L and ICAM1) provide the first and secondary signals to activate naive T-cells and B-cells.[28] Activation of the complement system by mannose-binding lectin (MBL), the release of tumor necrosis factor-alpha (TNF-α), matrix metalloproteinse-9 (MMP-9), nitric oxide (NO), plasminogen-activator inhibitor 1 (PAI-1) and Fc receptor (FcR)-mediated antibody dependent cell-cytotoxicity are likely to contribute to the extent of nerve damage in GBS patients.[18, 19, 43] Fas and its ligand (FasL) play an important role in eliminating autoreactive T and B-cells (not shown).

Control group

Two hundred and twelve Dutch healthy blood bank donors were used as controls in the population-based genetic association studies. GBS patients as well as the healthy subjects were all Caucasians. The HLA-allele frequencies of the control group were compared with the frequencies of a large Dutch cohort of healthy controls (n=2440) and proved to be representative for the general Dutch Caucasian population.[8] The distribution of the other polymorphism studied did also not differ between our control group and other Caucasian control groups, indicating that the control group was representative for Caucasian populations.

The controls had not suffered from GBS in the past, but it cannot be excluded that they will develop GBS in the future. Based on the lifetime risk (approximately 1: 1000) to develop GBS and the number of controls (n=212), however, this chance is very small and will not influence the conclusions based on the comparisons between GBS and controls.

No data are available on the infection status of these controls. To identify genetic host factors that determine the susceptibility to develop GBS after a specific type of infection our cohort of patients preferentially should have been compared with a group of infection matched controls. Based on the high incidence of *C. jejuni* infection and CMV-infection in the general population, the majority of controls, however, are expected to have had one of these infections at any point during life.

Genotyping methods

A highly sensitive and specific PCR-based method, the LightCycler®, was used to determine most of the SNPs in patients and controls.[9] As an internal quality control for this assay, sequence verified controls of each genotype per SNP were used. DNA was of high quality since all samples could be determined by using the LightCycler® technique. When it was impossible to develop appropriate hybridization probes to detect a SNP, the LightCycler® method could not be used. This was the case for the C(-1562)T polymorphism in the *MMP9* gene and the C(-260)T polymorphism in the *CD14* gene. In these cases, the restriction fragment length polymorphism analysis was used. Sequence verified controls with an additional restriction site were also used in these assays as internal quality controls.

Population-based genetic study design

Population-based genetic association studies are a way to study the contribution of genetic factors in the development or severity of a disease. One major issue in reporting the results of these kinds of studies generally is the inability to replicate many of these results. Population stratification, misclassification of outcome and allelic heterogeneity has been suggested as underlying factors, but publication bias, failure to attribute results to chance and inadequate sample sizes seem to be the most important factors underlying inability to replicate.

Publication bias. Negative studies are underreported in international journals.

Table 1. Overview of associations between SNPs studied and (sub)groups of GBS patients.

SNP	Comparison	p-value[#]
Pattern-recognition receptor and complement mediated nerve destruction		
MBL2 H(-550)L	GBS (n=271) versus controls (n=212)	< 0.004
	severely (n=71) versus mildly affected (n=82) GBS patients	0.02
	severely (n=71) versus controls (n=212)	< 0.001
MBL2 haplotype	GBS (n=271) versus controls (n=212)	0.03
	severely (n=71) versus mildly affected (n=82) GBS patients	0.03
	severely (n=71) versus controls (n=212)	0.001
Initiating adaptive immunity		
Antigen-presentation		
HLA-DR1	ventilated (n=34) versus non-ventilated GBS patients (n=122)	0.001/0.02*
Co-stimulation		
CTLA4 C(-318)T	mildly (n=14) versus severely (n=180) affected GBS patients	0.007
CTLA4 haplotype	mildly (n=14) versus severely (n=180) affected GBS patients	0.04
CD40 -1C/T	patients with (n= 74) versus patients without (n=106) anti-ganglioside Abs	0.02[a]/0.07
Macrophage mediated damage		
cytokines		
TNF-α C(-863)A	severely (n=70) versus mildly affected (n=78) GBS patients	0.02[a]/0.06
TNF-α C(-863)A	poor (n=15) versus good (n=133) outcome after six month	0.02
pro-inflammatory mediators		
MMP-9 C(-1562)T	severely (n=70) versus mildly affected (n=78) GBS patients	0.02
Fcγ receptors[‡]		
FcR-IIa R131H	ventilated (n=27) versus non-ventilated GBS patients (n=79)	0.049
FcR-IIIb NA1/NA2	ventilated (n=27) versus non-ventilated GBS patients (n=79)	0.007
Apoptosis		
Fas G(-1377)A	patients with (n= 74) versus patients without (n=106) anti-ganglioside Abs	0.05[a]
Fas A(-670)G	patients with (n= 74) versus patients without (n=106) anti-ganglioside Abs	0.001
Fas haplotype	patients with (n= 74) versus patients without (n=106) anti-ganglioside Abs	0.006

Generally, p-values are based on genotype distribution comparisons using the Pearson chi-square test. [a]P-value is based on the comparison of allele-frequencies. *P-value is corrected for testing multiple alleles. [‡]The results of these comparisons are not reported in this thesis nor reported in a separate article. GBS: Guillain-Barré syndrome. Abs: antibodies.

Systematic reviews of published results are therefore skewed to positive studies, and conclusions based on these results may be too optimistic.

Multiple testing. One of the major reasons for difficulties in replication is the failure to exclude chance. Hundreds of genes are possible candidate genes and several SNPs will be screened in one specific disorder. In addition subgroup-analysis or haplotype-analyses will be performed after initial tests of association that did not reach significance, thereby increasing the chance of finding an association.[10] For example, in this thesis we performed analysis on 18 different subgroups per SNP. The probability of finding at least one significant association per SNP studied with a p-value <0.05 in one of these subgroups that is based on chance alone is 60%. One strategy to reduce false positive associations is to apply corrections for multiple testing. A classic approach is to adjust the significance level by applying the Bonferroni correction (p-value divided by subgroups 0.05/18= 0.0027), which will ensure that when null hypotheses are true, the probability of obtaining at least one result more extreme than threshold will be about 5%. However, this situation is an oversimplification since the probability of a given association being true or false is not only dependent on significance level, but also on number of hypotheses being tested for which the null hypothesis is true. Significance thresholds of $5x10^{-5}$ have been proposed for association studies to adjust for 1000 SNPs being worldwide tested.[10] However, by using such stringent p-values, assessing the contribution of a SNP in the development or severity of a disease will be very hard since the contribution of an individual SNP is rather small. To increase the prior probability of a true association we selected polymorphisms in genes encoding molecules that are likely to be involved in the pathogenesis of GBS and which have a functional effect at protein level. In the case of *MBL2* we additionally performed functional assays to support the relation at genetic level. Another way to prove that the observed association is correctly is to confirm the association in a larger cohort of GBS patients and controls, which is one of our recommendations.

Insufficient power. In complex disorders the effect of an individual SNP will be small, since several SNPs and other factors will determine disease susceptibility. To be able to detect such a small effect, a large cohort of patients and controls is required. In this study one of the largest group of GBS patients was studied. By comparing this group of patients with 212 healthy controls, a standardized difference of 0.26 could be detected at a significance level of 5% and a power of 80%. For example, in the case of an allele frequency of 30% in the healthy controls, an odds-ratio of 1.4 could have been detected. When applying correction for the multiple tests performed larger sample sizes would be required to detect such a small difference.

Genetic host factors in Guillain-Barré syndrome

In Chapter 2 of this thesis 12 Dutch families are presented in which two or more GBS patients were identified. We observed a slightly more frequent occurrence of GBS within siblings, but this did not reach significance due to inadequate power of the study. We observed an earlier onset of GBS in successive generations, which

retrospectively could also be observed in other family-reports. Moreover, one of the patients had four times GBS. Collectively, these results suggest a genetic contribution to the pathogenesis of GBS. Larger numbers of families are required to establish familial aggregation in GBS. The results of the population-based genetic association studies are described in Chapters 3-6 and the main findings are listed in Table 1.

Do genetic variants confer susceptibility to GBS?

In this thesis none of the immunogenetic polymorphisms studied conferred disease susceptibility in GBS patients upon infection. Three previous reports showed that SNPs in the immune-response genes encoding IL-10, Fcγ receptors and the kappa light chains of immunoglobulins (KM genes) conferred disease susceptibility.[11-13] However, in case of IL-10 the results could not be replicated in our large cohort of GBS patients (Chapter 5.2). In the case of the Fc-receptors, a meta-analysis of a Dutch, English and Norwegian GBS population did not confirm the association between the polymorphisms and disease susceptibility.[14]

Although most of these results do not support a role of SNPs in conferring disease susceptibility, the rare occurrence of GBS after common types of infection is still a strong argument favoring a genetic contribution to develop GBS. However, identifying genetic variants that contributes to disease susceptibility might be a daunting task, due to the evident role of common antecedent infections and pathogen-related factors, the small effect of each individual SNP, the large number of SNPs involved, the gene-gene interactions and gene-environment interactions that have to be taken into account.

Do genetic variants contribute to the production of cross-reactive antibodies?

The presence of the cross-reactive anti-ganglioside antibodies in serum from GBS patients is one of the immunological hallmarks in GBS patients. These antibodies are initially evoked against structures present on the outer membrane of a pathogen and cross-react with gangliosides that are present on peripheral or cranial nerves.[1] Genes encoding proteins involved in antigen presentation, costimulation and life and death of B-cells are good candidate genes to assess whether the production of these cross-reactive antibodies is genetically determined. In this thesis, associations between (i) the *Fas* haplotype associated with a lower Fas expression[15, 16] (Chapter 6), (ii) the variant allele of *CD40* at position –1 that might affect the expression levels of CD40[17] (Chapter 4.2) and the production of anti-ganglioside antibodies are reported. The presence of the *Fas* promoter haplotype associated with a lower expression of Fas might results in a diminished induction of apoptosis in B-cell, which may contribute to the production of cross-reactive antibodies after infection. In addition, CD40-CD40L interactions are essential for activation, differentiation and isotype switching of the B-cell and also for rescuing the B-cell from apoptosis.[18] Overexpression of CD40 and CD40L has been suggested to play an important role in humoral autoimmunity due to excessive B-cell activation, class switching and autoantibody production.[19, 20] So,

Fas-FasL and CD40-CD40L interactions are both involved in the decision whether B-cells survive selection in the germinal centres.[19] Collectively, our results suggest that there are, besides the pathogen-related factors, genetic factors that partially determine whether a person produces cross-reactive antibodies.

Do genetic variants contribute to the severity of GBS?

The severity of GBS depends on the extent of damage of peripheral or cranial nerve fibres. SNPs may also influence this damage with respect to (i) the extent of immune activation, (ii) the production of antibodies, and (iii) complement and macrophage-mediated nerve damage.

In this thesis SNPs in the *MBL2*, *TNF-α* and *MMP-9* genes, encoding molecules that are all involved in complement and macrophage-mediated nerve damage, were associated with the severity of disease based on the MRC-sumscore at nadir. The MRC-sumscore assesses the muscle strength in 6 bilateral muscle groups, ranging from 60 (normal) to 0 (tetraparalysis).[21] This sumscore reflects the extent of motor fiber damage. The frequency of the *MBL2* haplotype associated with the highest MBL concentration and complex activity (HYA haplotype) was increased in GBS patients with a severe weakness compared with patients who were mildly affected. This association is further supported at serological and functional level; high MBL concentration and MBL complex activity are also associated with severe weakness. The correlation between high MBL concentration and complex activity, co-determined by the HYA haplotype, and severe weakness may indicate that patients only develop a severe form of GBS when a certain threshold level of MBL is present (Chapter 5.1). As GBS is a very complex and heterogeneous disorder other factors inhibiting the complement-factors like decay accelerating factor (DAF/CD55) and CD59, will probably co-determine the extent of nerve damage.

The variant alleles of SNPs at position –863 of the TNF-α gene and at position –1562 of the *MMP-9* gene, both probably resulting in higher levels of the encoded molecules are also associated with severe weakness (Chapter 5.2). Interestingly, previous reports showed that high serum levels of MMP-9 as well as TNF-α were associated with severity of disease.[22, 23] In addition administration of an inhibitor of MMP-9 and TNF-α release (BB-1101) in a rat experimental autoimmune neuritis model for GBS prevented the development of neurological deficits and conduction abnormalities or markedly reduced the severity of disease.[24]

FcR polymorphisms have also been reported to be associated with severity of disease based on the functional disability score or an expanded clinical grading scale and in a meta-analysis.[12, 14, 25] In the F-score also the extent of sensory nerve damage is important. We observed an association between a promoter haplotype in the *CTLA4* gene and a mild course of GBS based on this F-score (F-score $\leq$ 2; Chapter 4.2). This promoter haplotype has been reported to be associated with a higher expression of CTLA-4.[26, 27] CTLA-4 down-regulates the activation of T-cells, suggesting that a higher expression of this molecule might favors a mild course of disease.[28] Since our

group of mildly affected GBS patients based on the F-score is small (n=14), a larger group of mildly affected patients is required to draw any conclusion.

Collectively, these results favor a possible genetic contribution of SNPs in developing a severe form of GBS.

Do genetic variants contribute to the outcome of GBS?

Data obtained from large randomized clinical trials have indicated some prognostic factors for predicting poor outcome:

a) older age (> 50 years)
b) low MRC-sumscore at entry
c) high F-score at entry or nadir
d) rapid onset of muscle weakness (time between onset and day of randomization < 4 days)
e) preceding (*C. jejuni*) enteritis
f) recent CMV infection
g) low or absent muscle action potential amplitudes[6, 29]

Genetic variants in genes encoding molecules involved in the extent of nerve damage are also likely to be of prognostic value. Indeed, the variant allele at position -863 of TNF-α gene is also associated with a poor outcome, which was defined as not being able to walk unaided after six months of follow-up. No other studies assessed whether SNPs are associated with poor outcome. Our result encourages further searches of candidate genes in determining the outcome of GBS patients. To assess the contribution of SNPs in determining outcome, larger group of GBS patients with a poor outcome based on the F-score at six months are required.

Genetic variants contribute to GBS; a summary

The results described in this thesis favor a contribution of genetic factors in GBS, since:

a) at least 12 families with GBS in The Netherlands are present and GBS occurred a slightly more frequent in siblings (Chapter 2).
b) SNPs in genes encoding molecules involved in life and death of B-cells are associated with the presence of cross-reactive antibodies in serum of GBS patients (Chapter 4.2 and Chapter 6).
c) SNPs in genes encoding molecules involved in nerve damage are associated with the severity of disease and outcome (Chapter 5.1 and Chapter 5.2).

One of these genetic associations (the *MBL2* HYA haplotype and severe weakness) has been supported by functional assays, which strengthen its genetic contribution in the pathogenesis of GBS. The other reported associations have to be confirmed in another large GBS cohort to exclude the possibility that these associations are based on chance alone. To further assess the effects of these SNPs in the production of antibodies, disease severity and outcome, functional assays are warranted.

The aberrant immune response in GBS is initially evoked by different antecedent

infections, which might be recognized by different pattern recognition receptors present on immune cells, thereby activating different downstream pathways leading eventually to a cross-reactive antibody response. Since this complexity in the afferent phase of the immune response in which infections play an important role and the effect of individual genes will be small and might be different per infection, unravelling genetic factors involved in this process will be hard or even inconceivable. This conclusion might be daunting, however, genetic screening may be useful in predicting the severity or outcome in GBS patients, which will deal with great uncertainties of patients, their relatives and physicians as well. The severity of disease and outcome is determined by several factors (described above), including the administered treatment. In this thesis only immune-response genes have been studied to assess whether genetic factors are related to disease severity or outcome. However, other genes might also be involved in conferring disease severity and predicting outcome. Some candidate genes are suggested below.

Candidate genes to assess a genetic contribution to disease severity and outcome

Genes encoding Schwann-cell related molecules

Peripheral myelin protein 22 (PMP22), myelin protein zero (P0) and connexin 32 (CX32) are Schwann-cell related molecules, which are involved in the promotion of myelin formation and the maintenance and compaction of myelin. Mutations in the genes encoding these molecules are associated with hereditary neuropathies.[30] Polymorphisms in these genes might be partially determining the extent of nerve damage in GBS patients and could be therefore of prognostic value.

Genes encoding neurotrophic factors

Neurotrophic factors, like glia-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and insulin-like growth factor (IGF) and their receptors play an important role in protecting and preventing motor-neuron death and in the regeneration of nerves after injury.[31, 32] Polymorphisms in these genes that affect the levels and/or functions of these factors might be genetic factors related to the severity of GBS.

Pharmocogenetics

The outcome of GBS patients is partly determined by the therapy administered. SNPs in genes influencing interindividual variations in drug response might partly determine the effect of a treatment and thereby the outcome. In GBS patients the response to IVIg may be influenced by Fcγ-receptor polymorphisms[33] and the response to methylprednisolone might be less effective when the human glucorticoid receptor β variant is present. This variant allele is associated with a reduced sensitivity to glucocorticoids.[34] Other drug-response genes might also be involved. Recently, a set of tagging genes involved in absorption, distribution, metabolism and excretion of drugs has been identified, which might be useful in identifying the patients most likely

to respond positively to a certain medication. In addition, drugs likely to give negative side effects can be avoided.[35]

Ways to study the genetic contribution of GBS in future

It will be a major challenge to find genes or pattern of genes predicting the outcome of GBS at onset of disease, as this might have consequences to decide whether additional therapy is required. In this thesis we assessed whether individual SNPs were associated with disease severity, anti-ganglioside antibody production, disease severity and outcome. Since several SNPs and epistasis are likely to contribute to the pathogenesis of GBS, assessing the effect of only one SNP a time might not detect the contribution of this SNP to the pathogenesis of GBS. Recently, several appropriate methods have been developed which could be applied to our dataset to capture the contribution from multiple susceptibility loci located on different chromosomes.[36]

In future, a large European or even worldwide collaborative study is required to collect a DNA database from patients and controls that is large enough to deal with more stringent p-values to ascertain that the ratio of true positive and false positive associations will be 20:1.[10] For example, in the case we want to detect an odds ratio of 1.15 at $p=5x\ 10^{-5}$ by 80% power and an allele frequency of 30% in healthy controls, 4800 cases and 4800 controls would be required. A problem that might arise from a worldwide collaborative study is population stratification. However, a few strategies can be applied to overcome population stratification: i) carefully matching of cases and controls by demographic background, ii) screening for anonymous genetic markers (null loci) to detect population substructures and when these are present, association of the SNP with disease should be performed in each subpopulation[37] and iii) performing a transmission disequilibrium test (TDT) in family-based association studies.[38]

In this collaborative study it is of great importance to include GBS patients only as they are well documented with regard to antecedent infections, anti-ganglioside antibodies as well as clinical characteristics enabling the comparison of homogeneous groups of GBS patients.

In this thesis individual SNPs in candidate genes were determined in single assays. Other approaches are to assess tag SNPs within one gene, which is a reduced set of SNPs that can provide information about the presence of other alleles at different loci. This approach is likely to result in gain of efficiency and lower costs and is suitable in the case of common disease-common variant approach.[39] However, in the case of searching for rare alleles a considerable loss in power of association test is likely to be the result. Another approach is to perform genome-wide studies by using commercially available micro-array chips containing up to 100.000 SNPs. These chips contain SNPs with known and unknown effects. The main advantages of these SNP-chips are the high-throughput, low costs per SNP and increased power. A main disadvantage of these SNPs is the increased number of SNPs being tested

irrespectively of whether the SNPs are good candidate genes or not for an effect on disease risk, thereby reducing the prior probability of a true association.[40]

In conclusion

The results described in this thesis are based on one of world largest cohorts of well-documented GBS patients and favor some contribution of genetic factors in the pathogenesis of GBS. Although genetic factors could be involved in disease susceptibility, most evidence for a genetic contribution has been found in relation to disease severity. Identifying general genetic factors related to disease susceptibility of GBS seems rather a daunting task, since susceptibility genes may be so numerous, their interactions so divers and dependent on the type of antecedent infection. Based on the results described in this thesis, studying SNPs in relation to severity of disease or outcome is promising. An European or worldwide collaborative study would enable us to collect an extended cohort of well-documented GBS patients and to assess the contribution of SNPs in determining disease severity and outcome, which in turn might give valuable information to direction and intensity of treatment required.

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Summary

Guillain-Barré syndrome is a rare but potentially severe disease in which the peripheral nerves are affected by the immune system. Signaling from brain to muscles is impaired by the immune attack directed to the peripheral nerves, resulting in muscle weakness ranging from a mild decrease of muscle strength to a complete paralysis.

GBS is often preceded by an infection with a bacterium (*Campylobacter jejuni*, *Mycoplasma pneumoniae,* or *Haemophilus influenzae)* or a virus (cytomegalovirus, Epstein-Barr virus). These infections commonly occur in the general population. Our immune system is activated to eradicate these invading pathogens. However, in GBS the immune response is not only directed to the pathogen, but also to the peripheral nerve tissue (cross-reactive immune response). Previous reports showed that the bacterium or virus bears structures on its surface that resemble structures present on the peripheral nerves (molecular mimicry). This molecular mimicry is probably crucial for the induction of anti-ganglioside antibodies. However, the production of these antibodies is also dependent of other factors.

This thesis focuses on the contribution of genetic host factors in GBS. Two different approaches were used to assess the contribution of these genetic factors: 1. To identify families in which two or more members are affected. 2. To perform genetic population-based association studies comparing large groups of unrelated GBS patients and healthy controls to assess whether candidate immune response genes are susceptibility factors for conferring GBS or associated with clinical and serological subgroups of GBS.

In **Chapter 2**, 12 Dutch families in which more than one case of GBS is present are described. The number of reported families is insufficient to prove that there is a familial occurrence of GBS. A genetic susceptibility is suggested by the slightly more frequent occurrence of GBS within siblings, the observed earlier onset of GBS in successive generations and the single patient who developed GBS at four different occasions. Chapters 3-6 describe the results of the population-based genetic association studies performed in one of world largest group of well-documented GBS patients (n=272) and a group of healthy Dutch blood bank donors (n=212) as controls. Polymorphisms in several immune-response genes have been studied (listed in Appendix 2). In **Chapter 3**, we assessed whether known functional polymorphisms in the LPS receptors CD14 and Toll-like receptor 4 (TLR4) are associated with an increased susceptibility for GBS or *C. jejuni* related clinical and serological features. The C(-260)T polymorphisms in CD14 and the A(+896)G and C(+1196) in TLR4 did not confer disease susceptibility and were not associated with a preceding *C. jejuni* infection or its related clinical or serological subgroups, suggesting that these SNPs are not genetic host factors.

The distribution of the HLA-DRB1 and HLA-DQB1 antigens, involved in antigen presentation for B-cell responses, were compared between GBS patients and healthy controls in **Chapter 4.1**. No differences in distribution between these two groups were observed. However, the frequency of the HLA-DR1 antigen was increased in

the group of patients who required artificial respiration compared to those who did not. We reviewed all HLA-studies performed in GBS patients and conclude that the polymorphic HLA system is not a general susceptibility factor to develop GBS. Given the association between HLA-DR1 and artificial ventilation in GBS patients, the HLA-system might play a modulatory role in certain subgroups of GBS patients.

Besides the presentation of antigens to naive lymphocytes, signals provided via co-stimulatory molecules are also required to activate these cells. In **Chapter 4.2** we assessed whether immunogenetic polymorphisms in the genes encoding these molecules conferred disease susceptibility or are related to specific subgroups of GBS patients. The T-allele at position –318 of the promoter region of *CTLA4* and its $T^{-318}A^{+49}$ haplotype, probably resulting in a higher expression of CTLA-4 (which exerts an inhibitory role on activated T-cells) were associated with a mild form of disease (F-score $\leq$ 2). The T-allele at position –1 of *CD40*, a molecule involved in B-cell activation, was associated with the presence of anti-ganglioside antibodies, suggesting a role of this SNP in the production of anti-ganglioside antibodies.

The studies described in **Chapter 5** focus on SNPs in genes encoding molecules involved in complement and macrophage mediated nerve damage. Mannose-binding lectin recognizes sugar groups on pathogens, thereby activating the lectin pathway of the complement system and facilitating phagocytosis. In **Chapter 5.1** we showed that the frequency of the HYA haplotype, which is associated with highest MBL concentration, was increased in GBS patients compared to controls, especially in patients who had severe weakness. Highest MBL concentration and MBL complex activity were also associated with severe weakness, suggesting that high levels of functional MBL predispose for developing a severe form of GBS.

In **Chapter 5.2** it is shown that the variant alleles of a SNP in the promoter region of TNF-α (-863) and a SNP in the promoter region of MMP-9 (-1562) were also associated with severe weakness. These molecules are released by macrophages and may be involved in blood-nerve barrier breaching and aggravating tissue damage.

Fas-FasL interactions play an important role in inducing apoptosis in the Fas-bearing cells, thereby eliminating autoreactive T- and B-cells. The frequency of a promoter haplotype in the Fas gene associated with lowest Fas expression was increased in patients with detectable levels of anti-ganglioside antibodies in their serum (**Chapter 6**). Soluble levels of Fas that inhibits apoptosis by binding to membrane-bound FasL, were also higher in patients with detectable levels of anti-ganglioside antibodies. These results suggest that factors associated with a diminished apoptosis at least partly determine the induction of cross-reactive antibodies.

Finally, **Chapter 7** provides a discussion of the results and their contribution to understanding disease susceptibility, anti-ganglioside production, severity and outcome of GBS. Based on the results in this thesis, we conclude that there is some evidence that genetic factors play a role in the pathogenesis of GBS. Most of the reported associations were found between SNPs and disease severity or outcome and not the onset of GBS. These results are important for a better understanding of the factors

that are involved in determining severity of disease. Future research could focus on other candidate genes involved in determining disease severity, which in turn might give valuable information to the direction and intensity of treatment required.

Samenvatting voor niet-ingewijden

Het Guillain-Barré syndroom (GBS) is een zeldzame, maar potentieel ernstige ziekte waarbij de perifere zenuwen (door het eigen afweersysteem) worden aangetast. Door de aantasting van de zenuw worden de signalen vanuit de hersenen minder goed of helemaal niet meer doorgegeven aan de spieren, waardoor de patiënten met GBS minder spierkracht hebben of zelfs totaal verlamd kunnen zijn.

De ziekte wordt vaak voorafgegaan door een infectie met een bacterie (*Campylobacter jejuni*, *Mycoplasma pneumoniae*, Haemophilus influenzae) of een virus (Cytomegalovirus, Epstein-Barr virus). Deze infecties komen veel voor in de algemene bevolking. Deze infecties worden door ons lichaam bestreden door een afweerreactie in gang te zetten met als doel de bacterie of virus te doden. Bij GBS patiënten is deze afweerreactie niet alleen gericht tegen de bacterie of het virus, maar ook tegen het eigen zenuwweefsel (kruisreagerende afweerreactie). Eerder onderzoek heeft aangetoond dat de bacterie of het virus bepaalde structuren op het oppervlak draagt die lijken op structuren die voorkomen op ons zenuwweefsel (moleculaire mimicry).

Deze moleculaire mimicry is waarschijnlijk cruciaal voor de inductie van anti-ganglioside antistoffen. Of deze kruisreagerende antistoffen worden geproduceerd is echter ook afhankelijk van andere factoren.

Mogelijk spelen erfelijke (genetische) factoren hierbij een rol. In dit proefschrift is onderzocht of bepaalde genetische factoren een rol spelen bij het onstaan van GBS (susceptibiliteit) of bij bepaalde klinische of immunologische subgroepen van GBS patiënten. Het bestuderen van genetische factoren kan op twee manieren worden aangepakt: 1. Het bestuderen van families waarin meerdere leden GBS hebben doorgemaakt. 2. Het bestuderen van genetische variaties (polymorfismen) in genen die betrokken zijn bij de afweerreacties en het voorkomen van deze variaties te vergelijken tussen een groep GBS patiënten en gezonde controles (populatie associatie studies).

In **Hoofdstuk 2** worden 12 Nederlandse families beschreven waarin minstens 2 gevallen zijn van GBS. Het aantal gevonden families is te klein om te bewijzen dat GBS familiaal voorkomt. Het wat vaker voorkomen van GBS bij broers en zussen, het op jongere leeftijd ontstaan van GBS in opeenvolgende generaties en de ene patiënt die vier keer GBS heeft gehad, zijn wel suggestief voor een bijdrage van genetische factoren aan het ontstaan van GBS.

In de Hoofdstukken 3-6 worden de genetische associatie studies beschreven die uitgevoerd zijn in een van werelds grootste groepen van goed gedocumenteerde GBS patiënten (n=272) waarbij een groep van gezonde bloedbank donoren (n=212) als controle groep fungeerde. Polymorfismen in diverse genen die een rol spelen in verschillende fasen van de afweerreactie zijn bestudeerd (in Appendix 2 staan alle bestudeerde polymorfismen opgesomd).

In **Hoofdstuk 3** wordt beschreven of functionele polymorfismen in de LPS receptoren *CD14* en *TLR4* geassocieerd zijn met een verhoogde kans op het krijgen van GBS of met *C. jejuni* gerelateerde klinische en serologische kenmerken. Het C(-260)T polymorfisme in *CD14* en de A(+896)G en C(+1196)T polymorfismen

in *TLR4* bleken niet geassocieerd te zijn met een verhoogde kans op GBS of met een verhoogde kans op het ontwikkelen van GBS na een *C. jejuni* infectie. Bovendien zijn deze polymorfismen niet geassocieerd met *C. jejuni* gerelateerde klinische en serologische subgroepen van GBS patiënten. Deze bevindingen duiden erop dat deze SNPs geen genetische gastheerfactoren zijn.

De distributie van HLA-DRB1 en HLA-DQB1 antigenen, die betrokken zijn bij antigeen presentatie voor antistof responsen, werden vergeleken tussen GBS patiënten en controles (**Hoofdstuk 4.1**). De frequentie van deze antigenen in deze twee groepen verschilde niet van elkaar. Echter, de frequentie van het HLA-DR1 antigeen was verhoogd in de groep van beademende patiënten ten opzichte van de andere patiënten. In dit hoofdstuk hebben we alle eerder gepubliceerde HLA manuscripten onder de loupe genomen en geconcludeerd dat het polymorfe HLA systeem geen factor is die de kans op het krijgen van GBS bij alle patiënten verhoogd. Gegeven de gevonden associatie tussen HLA-DR1 en beademing bij GBS patiënten, zou het HLA systeem wel een modulerende rol kunnen spelen bij bepaalde subgroepen van GBS.

Naast de presentatie van antigenen aan naïeve lymfocyten zijn ook co-stimulatoire signalen nodig om deze cellen te activeren. In **Hoofdstuk 4.2** wordt bekeken of polymorfismen in genen coderend voor deze moleculen geassocieerd zijn met het krijgen van GBS of met bepaalde subgroepen van GBS patiënten. Signalering via het CTLA-4 molecuul, één van de co-stimulatoire moleculen, oefent een remmende invloed uit op geactiveerde T-cellen. In de groep van mild aangedane GBS patiënten (F-score ≤ 2) werd een verhoogde frequentie van het T-allel op positie –318 in the promoter regio van *CTLA4* en het $T^{-318}A^{+49}$ haplotype gevonden. Dit haplotype is waarschijnlijk geassocieerd met een hogere expressie van CTLA-4 en heeft daardoor mogelijk een remmende werking op het afweersysteem. Het T-allel op positie –1 van *CD40*, een molecuul betrokken bij B-cel activatie, is geassocieerd met de aanwezigheid van anti-ganglioside antistoffen en speelt mogelijk een rol bij de productie van deze antistoffen.

Hoofdstuk 5 wijdt zich aan SNPs in genen die coderen voor moleculen die betrokken zijn bij complement- en macrofaag gemedieerde zenuwschade. Het mannose-bindend lectine (MBL) herkent suiker groepen op het oppervlak van pathogenen en activeert daarna de lectine route van het complement systeem hetgeen leidt tot een verbeterde fagocytose. In **Hoofdstuk 5.1** laten we zien dat de frequentie van het HYA haplotype dat geassocieerd is met de hoogste MBL concentraties, verhoogd is in de groep GBS patiënten, met name in de groep van patiënten met ernstige spierzwakte. De hoogste MBL concentraties en MBL complex activiteit zijn tevens geassocieerd met ernstige spierzwakte. Dit wijst erop dat hoge niveaus van functioneel MBL predisponeren voor het ontwikkelen van een ernstige vorm van GBS.

In **Hoofdstuk 5.2** werd aangetoond dat de variant allelen van een SNP in de promoter regio van *TNF*-α (-863) en een SNP in de promoter regio van *MMP-9* (-1562) geassocieerd zijn met ernstige spierzwakte. Deze moleculen worden uitgescheiden door macrofagen en zijn mogelijk betrokken bij de aantasting van de bloed-zenuw

barrière en het verergeren van weefselschade.

Fas-FasL interacties spelen een belangrijke rol bij het induceren van apoptose, waardoor autoreactieve T en B cellen geëlimineerd kunnen worden. Bij patiënten met detecteerbare hoeveelheid anti-ganglioside antistoffen in het bloed komt het Fas promoter haplotype dat geassocieerd is met de laagste Fas expressie vaker voor **(Hoofdstuk 6)**. Deze resultaten suggereren dat factoren die geassocieerd zijn met een verminderde apoptose tenminste deels de inductie van kruisreagerende antistoffen bepalen.

Tenslotte worden in **Hoofdstuk 7** de resultaten en hun bijdrage aan ziekte susceptibiliteit, antistof productie, ernst en uitkomst van GBS bediscussieerd. Op basis van het onderzoek beschreven in dit proefschrift kan worden geconcludeerd dat er aanwijzingen zijn voor een rol van immuun respons genen in de pathogenese van GBS. Opvallend is dat er vooral een associatie is gevonden tussen bepaalde SNPs en de klinische manifestatie van GBS en niet ten aanzien van het ontstaan van GBS. Deze bevindingen zijn van belang om beter te kunnen begrijpen welke factoren bijdragen aan de ontwikkeling van een ernstige vorm van GBS. Toekomstig onderzoek zou zich kunnen richten op andere kandidaat-genen die bij kunnen dragen aan de ernst van de spierzwakte. Dit kan uiteindelijk leiden tot een betere voorspelling van de ernst van de ziekte, hetgeen mogelijk consequenties heeft voor de therapiekeuze en intensiteit van de in te stellen therapie.

List of abbreviations

AIDP	acute inflammatory demyelinating polyneuropathy
AMAN	acute motor axonal neuropathy
AMSAN	acute motor sensory axonal neuropathy
APC	antigen presenting cell
CD	cluster of differentiation
C. jejuni	*Campylobacter jejuni*
CMV	cytomegalovirus
CSF	cerebrospinal fluid
CTLA-4	cytotxic T-lymphocyte antigen 4
EBV	Epstein-Barr virus
EMG	electrodiagnostic examination
FcγR	Fc gamma receptor
GBS	Guillain-Barré syndrome
HLA	human leucocyte antigen
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-10	interleukin 10
iNOS	inducible nitric oxide synthetase
IVIg	intravenous immunoglobulins
LOS	lipo-oligosaccharide
LPS	lipopolysaccharide
MFS	Miller-Fisher syndrome
MMP-9	matrix metalloproteinase-9
M. pneumoniae	*Mycoplasma pneumoniae*
MRC	Medical Research Council
NO	nitric oxide
PAI-1	plasminogen activator inhibitor 1
PRR	pathogen recognition receptors
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
TLR-4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha
URTI	upper respiratory tract infection
UTR	untranslated region

Appendix 1: Diagnostic criteria of Guillain-Barré syndrome

Features required for the diagnosis

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

Features strongly supportive of the diagnosis

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

Features that rule out the diagnosis

- A current history of hexacarbon use
- Abnormal porphyrin metabolism
- A history or finding of recent diphtheric infection
- Lead intoxication
- The occurrence of a purely sensory syndrome
- Diagnosis of poliomyelitis, botulism, hysterical paralysis or toxic neuropathy

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

Appendix 2. List of polymorphisms studied.

Gene	SNP	chromosome region	functional effect on
pattern recognition receptors			
CD14	C(-260)T	5q31.1	CD14 expression
TLR4	A (+896)G	9q32-q33	amino acid substitution extracellular region
	C (+1196)T		
MBL2	H (-550)L	10q11.2-q21	transcriptional regulation
	X (-221)Y		transcriptional regulation
	codon 52 (D)		protein function
	codon 54 (B)		protein function
	codon 57 (C)		protein function
Antigen presentation and costimulation			
HLA	HLA-DRB1	6p21.3	binding peptide
	HLA-DQB1	6p21.3	
CD28	IVS3+ 17T/C	2q33	splicing?
CD86	G(+864)A	3q21	intracellular signaling?
CTLA4	C(-318)T	2q33	transcriptional activation
	A(+49)G		amino acid substitution in peptide leader sequence
CD40	-1 C/T	20q12-q13.2	efficiency translation
CD40L	C(-726)T	Xq26	not known
	C (+221)T		not known
ICAM1	G241R	19p13.3-p13.2	binding capacity ligand
	E469K		cell adhesion and costimulation
Complement and macrophage mediated damage complement activation			
MBL2	H (-550)L	10q11.2-q21	transcriptional regulation
	X (-221)Y		transcriptional regulation
	codon 52 (D)		protein function
	codon 54 (B)		protein function
	codon 57 (C)		protein function
cytokines			
TNF-α	C(-863)A	6p21.3	transcriptional regulation
	G(-308)A		
	G(-238)A		
IL-10	G(-1082)A	1q31-q32	transcritpional regulation
	C(-819)T		
pro-inflammatory mediators			
MMP-9	C(-1562)T	20q11.2-q13.1	transcritpional regulation
PAI-1	4G/5G (-504)	7q21.3-q22	PAI-1 production
iNOS	S(608)L	17cen-q11.2	amino acid substitution
Apoptosis			
Fas	G(-1377)A	10q24.1	transcriptional regulation
	A(-670)G		
FasL	C(-843)T	1q23	transcriptional regulation

TLR4: Toll-like receptor 4, *MBL2*: Mannose-binding lectin, *HLA*: Human Leucocyte antigen, *CTLA4*: Cytotxic T lymphocyte antigen-4, *TNF-α:* Tumor necrosis factor-alpha, *IL-10*; interleukin 1, *MMP-9*: matrix metalloproteinase 9, *PAI-1*: plasminogen activator inhibitor-1, *iNOS*: inducible nitric oxide synthetase

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About the author

Karin Geleijns (Cornelia Pieternella Wilhelmina) was born on the 6th August 1973 in Zevenbergen. She attended secondary school (VWO) at "de Nassau Scholengemeenschap" in Breda and finished it in 1993. In the same year she started studying Biomedical Sciences at the University of Leiden. From 1995 on, she also started Medical School at the same University. During these studies she conducted research on the role of hormones in impulsive obsessive disorders (Dr. M.R. Kruk, Leiden/Amsterdam Center for Drug Research) and on muscle cell antigens in inclusion body myositis (Dr. J.J.G.M. Verschuuren, Dept. of Neurology, Leiden University Medical Center and Dr. J.M. van Noort, TNO Prevention and Health, Leiden). In 2000 she obtained her Medical Degree (cum laude). In that year she started her PhD project at the departments of Neurology and Immunology of the Erasmus MC. The first year of her PhD project was partially used to finish her study Biomedical Sciences (cum laude, 2001). December 1st 2004, she started her training as neurologist at the department of Neurology of Erasmus MC (Prof. Dr. P.A.E. Sillevis Smitt).

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