T cell reactivity in inflammatory neuropathy

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Cover: Back, from left to right: human sural nerve, human Schwann cells on a layer of fibroblasts, 96-well plate for T cell culture. Front, from left to right: treehopper, FACS analysis of T cell culture, *C. jejuni* (photograph by Marc Wösten).

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T cel reactiviteit in inflammatoire neuropathie

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Contents

Chapter 1 General introduction **1**

Chapter 2 Expression of accessory molecules for T cell activation in peripheral nerve of patients with CIDP and vasculitic neuropathy **9**

Chapter 3 *Ex vivo* T cell stimulations with peripheral nerve myelin **25**

Chapter 4 Vigorous and oligoclonal expansion of human blood γδ T cells after *in vitro* stimulation with *Campylobacter jejuni* **35**

Chapter 5 Long-lasting γδ T cell non-responsiveness in patients with *Campylobacter jejuni*-associated Guillain-Barré syndrome **51**

Chapter 6 *Campylobacter* DNA is present in circulating myelomonocytic cells of healthy individuals and patients with Guillain-Barré syndrome **63**

Chapter 7 General discussion **71**

Reference List **79**

Summary **97**

Samenvatting **100**

Dankwoord **103**

Curriculum vitae **105**

Chapter 1

General introduction

Scope of the project "T lymphocytes in inflammatory neuropathies"

This thesis deals with three subjects: inflammatory neuropathies, the (human) immune system, and microbial pathogens. In the broadest sense, the goal of the work described in this thesis is to reveil how activation of the immune system causes inflammatory neuropathy. This is a very general description, but the work presented here is mainly focussed on one neuropathy, Guillain-Barré syndrome (GBS), which is often induced by a bacterium called *Campylobacter jejuni*. The part of the immune system that was concentrated on was restricted to the T cell response, which resulted in a description of the presence of T cell-stimulatory molecules in neuropathy-affected nerve, the T cell response against myelin, and the γδ T cell response to *C. jejuni* in healthy subjects and GBS patients. The choice for the study of T cells in peripheral neuropathy made it an unusual project in the field, since most work on the pathogenesis of GBS is concentrated on the role of antibodies. The molecular mimicry hypothesis, which is based on structural resemblances between *Campylobacter* and peripheral nerve constituents, and is supported by the presence of cross-reactive antibodies in the serum of GBS, is currently dominating experimental GBS research. Although one of the most sophisticated hypotheses in the field, there is no evidence that it accurately describes the mechanism of pathogenesis of GBS. Since the formulation of this hypothesis, research has narrowed down to the search for support for it, with the main goal being the identification of a pathogenic, crossreactive antibody and the antigen it recognizes.

The scope of the project "T lymphocytes in inflammatory neuropathies" is the possible involvement of T lymphocytes in the pathogenesis of inflammatory neuropathies. T lymphocytes are the central theme in this thesis and the focus has been shifted deliberately from the characterization of antibodies toward the role of T lymphocytes. In my opinion the most obvious gap in the knowledge about inflammatory neuropathies is the absence of data concerning the role of T cells during pathogenesis. In this thesis, the T cell response to myelin and to the microbial pathogen *C. jejuni*, and the status of the T cells during inflammatory neuropathy will be described. In the general discussion the newly obtained data will be put together into a hypothesis on the pathogenesis of GBS.

The rest of this general introduction will describe clinical aspects of inflammatory neuropathies, data on the status of the humoral and cellular immune system during inflammatory neuropathies, correlation with infectious diseases, and current hypotheses of autoimmune pathogenesis. Furthermore, the human γδ T lymphocyte will be introduced and placed in a context of basic immunology.

Inflammatory neuropathies

Damage to the axon or myelin sheath of the peripheral nerve leads to dysfunction called neuropathy. In addition to toxic substances, metabolic disturbances, degenerative processes, and physical damage, it is well recognized that components of the immune system can also be involved in pathogenesis. If antibodies, complement, T cells, or macrophages are likely to be involved in the pathogenesis of neuropathy, it is called an inflammatory or immune-mediated neuropathy. Though the main focus of this thesis is on GBS, Chapter 2 and 3 include chronic inflammatory demyelinating neuropathy (CIDP). A mayor clinical difference between CIDP and GBS is the chronic course of CIDP versus the acute character of GBS. Acute onset of disease means that the nadir is reached between a couple of days and 6 weeks, whereas chronic disease may relapse and remit or worsen continually for months or years. Another way to classify peripheral neuropathies is based on electrophysiological data. Damage to the myelin sheath can be distinguished from damage to the axon. This distinction is of clinical interest, since damage to myelin recovers better than axonal damage. A troubling factor is that primary axonal damage leads to secundary demyelination and vice versa. The two primary axonal variants of GBS that can be distinguished are Acute Motor Axonal Neuropathy (AMAN) and Acute Motor Sensory Axonal Neuropathy (AMSAN), and a primary demyelinating form is Acute Inflammatory Demyelinating Neuropathy (AIDP). In addition, there is a variant of GBS in which mainly the cranial nerves are affected, called Miller Fisher syndrome (MFS).
GBS patients benefit from therapy with intravenous immunoglobulins (IvIg). Though GBS is a selflimiting disease, trials with large amounts of patients have shown that the severity of the disease is less if the patient is given IvIg. The working mechanism of this therapy is not understood. Therapy given to CIDP patients consists of corticosteroids and/or immune suppressive drugs like cyclosporin, cyclophosphamide, or dexamethasone, and also IvIg.

The immune system during inflammatory neuropathies

In rather few cases, biopsies and postmortem material of peripheral nerve of GBS patients were studied. An infiltrate consisting of macrophages and lymphocytes has been described[1], and also the presence of complement activation products[2]. Material of CIDP patients is more easily accessible and therefore the histopathology of this disease is much better studied, including the presence of B cells and αβ and γδ T cells, and the Vβ chain expression of the infiltrating T cells[3]. All available data concerning inflammatory neuropathy point to aspecific infiltration, which

means that the T cell population present in peripheral nerve does not differ much from the population in blood.
The study of affected nerve material provides a local morphological and immuno-phenotypical description of a certain stage of a neuropathy. Additional data concerning the status of the immune system have been obtained from blood of patients suffering from inflammatory neuropathy. The circulating T lymphocyte population of CIDP patients has been shown to have a higher frequency of mutations in the HPRT gene, which is an indication of frequent activation[4]. Aberrant amounts of T lymphocyte-related cytokines in serum of GBS patients have been described, like IL-2[5], γIFN[6], TGFβ[7], and IL-18[8], and increased levels of soluble IL-2 receptor[9], suggesting that T cell activation takes place during inflammatory neuropathy. The presence of auto-antibodies has extensively been studied, especially antibodies recognizing the glycosylations of glycolipids or glycoproteins that are present in peripheral nerve. Antibodies against the gangliosides GM1 and GD1 occur frequently in GBS, and antibodies against GQ1 are found in most cases of MFS. In CIDP and GBS, antibodies against P0 and P2 have been implicated[10]. A primary pathogenic role for these antibodies has been tentatively proposed, but is hard to prove. Moreover, not all patients have these antibodies, and not all people with these antibodies suffer from neuropathy, so if they play a role in pathogenesis, it is not the only pathogenic factor.

Correlation between GBS and infectious disease

A remarkable feature of GBS which distinguishes it from other neuropathies is its close association with preceding infectious disease. An infection with the enteric pathogen *Campylobacter jejuni* has most frequently been reported. Depending on the geographical area where the studies were performed, 26 – 41% of GBS cases are preceded by a *C. jejuni* infection. Cytomegalovirus infection is the second most common (5 – 15%) GBS-preceding event. Other significantly associated infectious agents are Epstein-Barr virus, *Mycoplasma pneumoniae*, and *Hemophilus influenzae*.

Hypotheses of autoimmune pathogenesis of inflammatory neuropathies

Some of the characteristics of inflammatory neuropathies point to an autoimmune pathogenesis. It is thought that CIDP is a predominantly T cell mediated disease because high amounts of infiltrating T cells can be found in nerve biopsies, effective therapy consists of T cell suppressive drugs, soluble IL-2 receptor is found in serum, and high frequencies of T cells carrying a mutation in the HPRT gene are found. GBS on the contrary is often considered to be mainly antibody

mediated because of the anti-ganglioside antibodies that are present in the serum of GBS patients, and because the earliest abnormality found in GBS-affected nerve is complement deposition[2]. Traditionally, an autoimmune disease is regarded as T cell mediated or antibody or B cell mediated, but this distinction is in most cases questionable, and totally overlooks the innate immune system as possible pathogenic mediator. A strict attribution of T- versus B cell mediated pathogenesis to CIDP and GBS is not plausible since autoantibodies can be found in CIDP patients, and signs of T cell activation in GBS. In addition, for the production of antibodies of the IgG1 and IgG3 isotype, like the anti ganglioside antibodies found in GBS, T cell help is needed. Most likely, the innate immune system, the adaptive immune system including both B and T lymphocytes, and regulatory systems are all involved in the development of autoimmune disease.
The molecular mimicry hypothesis has been thought out in most detail for the anti-ganglioside antibodies in GBS patients. The concept is based on structural resemblance between a micro-organism and the host. Glycosylations of the outer core of the lipo-oligosaccharide (LOS) molecule of some *C. jejuni* strains resemble or are identical to glycosylations of the human gangliosides, and antibodies that were raised against these epitopes on *Campylobacter* cross-react with the corresponding eitopes on peripheral nerve gangliosides, thus leading to GBS. The concept lead to the question whether there are bacterial strains that are more likely to lead to GBS than others, the so called 'bad bugs'. This appears indeed to be the case. In Japan for example, serostrain O:19 is more frequently found in GBS patients than in patients with uncomplicated enteritis[11]. The same holds for serostrain O:41 in South Afrika[12]. However, other studies argue against simple epitope differences as the basis of the development of GBS. It has been demonstrated that although anti-ganglioside antibodies in *Campylobacter*-induced GBS are more frequently found than in uncomplicated *Campylobacter* enteritis, the bacterial strains that are isolated from both groups of patients are similar in their ganglioside-like epitopes on their LOS[13]. A recent discovery may clarify this controversy: The high molecular weight LPS containing the O-antigens-of *Campylobacter* is biochemically and genetically unrelated to LOS that in some strains contains epitopes that resemble human gangliosides[14;15]. Furthermore, seroconversion of *C. jejuni* strains has been reported [16]. But besides the ganglioside-mimicking issue, it is conceivable that other aspects of *Campylobacter* strains account for their GBS-inducing potential, and that these factors are coincidentally linked to their serotype.
Although there is a strong correlation between GBS and certain preceding infectious diseases, it is obvious that other factors play a role as well. Only two thirds of the GBS cases is preceded by an infectious disease (though undetected infections can not be excluded), and not all patients suffering from one of the infectious diseases that typically precedes GBS will develop GBS. It has been estimated that only one in a thousand clinically overt *Campylobacter* infections leads to

GBS. Genetic and non-genetic host factors have been put forward as risc factors for GBS. Examples of genetic factors that have successfully been studied and are correlated with the development of GBS are: Fc receptor polymorphisms[17] and TNFα polymorphisms[18], Of note, HLA linkage has been studied but is absent or very weak[19].

Immunological introduction

Immune responses against pathogens have extensively been described and can be classified according to the part of the immune system that is involved or to the type of antigen against which the reaction is directed. Both classifications are workable and will be used here.
An important division is between innate and adaptive immune systems. Adaptive immunity has been studied more extensively than innate immunity, and comprises mainly T and B cell mediated immunity that depends on rearrangment/recombination of antigen receptor gene segments in combination with diversification and clonal selection. The adaptive immune system is able to cope with rapidly changing pathogens. The innate immune system utilizes more preformed, germ-line encoded receptors. This enables the innate immune system to react quicker than the adaptive immune system, but only to conserved molecules of pathogens. Furthermore, the innate immune system is able to recognize and eliminate cells that express 'danger' or 'stress' signals. Examples of innate receptors are the LPS receptor CD14, Toll-like receptors, the mannose receptor, and NK receptors and NKT cell receptors.
Because the molecular mimicry hypothesis put forward the importance of identical glycosylations on pathogen and human ganglioside, the distinction between immunity against proteins and other molecules may be of particular interest to GBS. A classical, adaptive immune response against a protein of an extracellular pathogen comprises a MHC-restricted T cell response, T/B interaction, and antibody production by the B cell, mainly of the IgG1 and IgG3 isotype. There are however other scenarios possible. T-independent (TI) antigens that fulfill certain structural requirements may lead to the production of antibodies of the IgM or IgG2 isotype. TI-2 antigens are mostly molecules such as bacterial cell wall polysaccharides with highly repetitive structures[20;21]. There are also non-MHC restricted T cells like γδ T cells and CD1 restricted αβ T cells. Especially the recently described CD1b-restricted ganglioside-specific αβ T clones may play a role in the development of GBS[22], although the significance of this type of cells *in vivo* remains uncertain.

γδ T lymphocytes

Because the γδ T cell will play an important role in Chapter 4 and 5, a specific introduction will be given here. γδ T cells are generally considered as innate lymphocytes, and they make up a minor population in human blood. Their TCR variability is not as high as found in αβ T cells, but neither do they have a semi-invariant TCR like NKT cells. In blood, the expression of Vδ gene segments is limited to Vδ2, and to a lesser extent, Vδ1. Subpopulations of γδ T cells expressing a certain Vδ appear to have different distribution patterns and antigen specificity.
Circulating Vδ2 cells usually co-express the Vγ9 chain, and these Vδ2/Vγ9 cells expand in numerous infectious diseases. They appear to react quicker than αβ T cells, produce γIFN[23], and have strong cytotoxic potential. Vδ2 cells recognize antigens in unprocessed form, and no known antigen presenting molecule is needed. The antigens they recognize are phosphoantigens, which are intermediates in the bacterial deoxyxylulose pathway, and alkylamine antigens[24].

Aims of the study

The aims as described here reflect the questions that were raised before the project was started. They were partially based on certain expectations, like for example the discovery of a T cell stimulatory compound of myelin. As the project proceeded, unexpected results guided the refinement of the aims, but the original form of the aims is presented below.
1) Describe the conditions for local T cell activation in peripheral nerve
2) Describe the direct reactivity of *ex vivo* T cells of GBS and CIDP patients against PNS myelin
3) Study cross-reactivity of T cells between *Campylobacter* and myelin constituents
4) Describe the T cell response of healthy donors and GBS patients against *Campylobacter*

Chapter 2

Expression of accessory molecules for T cell activation in peripheral nerve of patients with CIDP and vasculitic neuropathy

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Abstract

Vasculitic neuropathy and chronic inflammatory demyelinating polyneuropathy (CIDP) are neuropathies characterized by a T lymphocyte infiltrate in the peripheral nerves. The microenvironment in which these T cells become activated and the molecules and cells that play a role in this process are incompletely understood. In immunohistochemical analysis, we studied the presence of adhesion-, costimulatory-, and antigen presenting molecules on different cell types as conditions for local T cell activation in human sural nerve biopsies of 7 CIDP and 3 vasculitic neuropathy patients and 3 healthy controls. In biopsies from CIDP and vasculitic neuropathy patients, but not healthy controls, Schwann cells expressed the adhesion/T cell stimulatory molecule CD58 (LFA-3). The CD58 molecule was also present on endothelial cells of all vasculitic neuropathy patients and 1 CIDP patient. In biopsies from normal controls and patients, CD54 (ICAM-1) expression was detectable on microvascular endothelial cells. In addition, expression of the co-stimulatory molecule CD86 was detected on vascular tissue in patients with vasculitic neuropathy. Although macrophages were always present in all subjects, expression of the MHC-like molecule CD1a by macrophages was restricted to biopsies from 2 CIDP patients and 1 vasculitic neuropathy patient. Unexpectedly, Schwann cells of a single vasculitis patient strongly expressed CD1b, a molecule involved in the presentation of self glycolipids to T cells. Schwann cells in biopsies from patients and normal controls expressed high levels of the invariant chain (CD74), a molecule involved in intracellular sorting of MHC class II molecules. There was no evidence for the presence of dendritic cells in sural nerve biopsies. These findings support a model in which T cell activation can be initiated and/or perpetuated locally in sural nerve biopsies of patients with CIDP and vasculitic neuropathy and predict an important role for Schwann cells and endothelial cells.

Introduction

Chronic inflammatory demyelinating polyneuropathy (CIDP) and vasculitic neuropathy are peripheral nerve disorders of presumed autoimmune etiology. A role for T lymphocytes has been suggested based on the observation that T cell infiltrates are present in biopsy specimens and postmortem material of peripheral nerves[1;25-28]. Further support for T cell involvement in the pathogenesis of CIDP comes from the notion that these patients have increased frequencies of circulating activated and chronically stimulated peripheral T lymphocytes and elevated levels of soluble interleukin-2 receptors[4;5].

Although T lymphocytes are accepted to play a role in the development and/or perpetuation of the inflammatory disease process, little is known about the processes and (auto)antigen(s) responsible for the initiation and chronic course of these neuropathies[29;30]. Generally, T cell activation and migration into peripheral tissues is a selective, multi-step process involving a myriad of molecules expressed or induced on different cell types. The initial events leading to T cell activation are: 1) adhesion and extravasation, 2) recognition of previously captured and processed antigen, presented on MHC class I or II by a professional antigen presenting cell (APC) and 3) recognition of costimulatory molecules like the classic examples CD80 (B7.1) and CD86 (B7.2) on the APC. Subsequently the T cell will proliferate and differentiate into an effector cell. Normally these events take place in the lymphnodes, but in autoimmune disease the immune response directed to specific autoantigens may be initiated locally. Unlike in Guillain-Barré syndrome (GBS), in CIDP or vasulitic neuropathy a link between preceding infections with micro-organisms and the occurrence of disease has not been established. Thus, although a role for exogenous antigens cannot be excluded, the initial immune response may also be induced by and directed against a nerve-specific or vascular autoantigen. In that case the presence of molecules for adhesion, antigen presentation and costimulation in peripheral nerve tissue is a prerequisite.
The anatomical site(s), cells and molecules that contribute to the autoimmune response resulting in the development of CIDP or vasculitic neuropathy have not been well-established. Here, we have performed an immunohistochemical analysis of cells and molecules involved in adhesion, antigen presentation and T cell costimulation in sural nerve biopsies of patients with CIDP, vasculitic neuropathy and, as a control, individuals without peripheral neuropathy. The results support the concept that in the sural nerve microenviroment in CIDP and especially vasculitic patients, presentation of autoantigen and local T cell stimulation may contribute to the initiation and/or perpetuation of disease.

Materials and Methods

Patients

Nerve biopsies from 7 patients with CIDP and 3 patients with vasculitic polyneuropathy without evidence for systemic vasculitis were included in the present study. The characteristics of the patients and nerve biopsies are listed in Table 1. The CIDP biopsies widely varied in the number of infiltrating T cells as we reported recently[28]. Patients 1-3 had increased numbers of T cells compared with normal controls[28]. Patient 2 had received treatment with intravenous immunoglobulin 2 months before the biopsy was taken. As normal controls, biopsy specimens

were obtained from individuals that did not suffer from polyneuropathies and succumbed from subarachnoidal bleeding or pulmonary embolism.

Table 1 *Clinical data of patients with CIDP and vasculitic neuropathy*

Pat	Age	Sex	Biopsy	M/S	Course	CSF	EMG	T cells	
	y		m			prot		dy	% CD8+
CIDP									
1	45	F	4	M>S	Progr	340	D	111	37
2	49	F	5	M=S	RR	350	D	87	59
3	29	F	5	M=S	Progr	330	D	57	61
4	31	M	2	M>S	RR	40	D	20	88
5	43	M	37	S>M	Progr	50	D	16	54
6	45	M	14	M>S	RR	80	D	14	15
7	29	M	168	M>S	Progr	80	D	12	84
Vasculitis									
1	64	M	5	M=S	Progr	60	A	183	42
2	78	F	2	M=S	Progr	30	A	161	30
3	58	F	8	M=S	Progr	nd	A	75	41

Immunohistochemical techniques

Serial transverse tissue sections of 6 µm were made on a cryostat and fixed in acetone. For staining with the S100 antibody, acetone treatment was preceded by fixation with 4% paraformaldehyde. The staining procedure for all antibodies (Table 2) was as follows: biopsies were incubated overnight with the primary antibody diluted in PBS/1% BSA, supplemented with 5% horse or goat serum. Biopsies were rinsed with PBS and incubated for one hour with the biotinylated secondary antibody (rabbit IgG/IgM or mouse IgG; Vector, Peterborough, England) diluted 1:200 in PBS/1% BSA, followed by an one hour incubation with ABC peroxidase (Vector). Color was developed with Nickel and Cobalt enhanced diaminobenzidine. The sections were counterstained with Nuclear fast red, which also stains the myelin sheaths, before dehydration and mounting. Black and white microphotographs were taken without filter resulting in a grey counterstaining and a black diaminobenzidine staining in the pictures.

Optimal dilution for primary antibodies were first determined using tissue sections of human tonsil and skin. These tissues also served as positive controls during each round of staining. In negative control stainings, the same procedure was followed except that the primary antibody was omitted.

In a first round of staining, sets of three serial sections were cut and the middle section was stained with the CD3 monoclonal antibody to confirm the presence of infiltrating T lymphocytes. The first and third section were used for staining with monoclonal antibodies specific for

adhesion (CD54, CD58), antigen presenting (HLA-DR, CD1a, CD1b, CD1c, CD1d), or costimulatory (CD40, CD80, CD86) molecules. When positive staining of adhesion-, costimulatory- or antigen presenting molecules was found, new sets of serial section were made and stained with the positive monoclonal antibody alternating with monoclonal antibodies specific for macrophages (CD68), Schwann cells (S100), endothelial cells (CD31) and B cells (CD20). All biopsy specimens were analyzed for the presence of CD83 positive cells, as a marker for the presence of dendritic cells. Table 2 lists the antibodies and dilutions used in the present study.

Table 2 *Antibodies used against different molecules*

Antigen		**Supplier**	**Batch/clone**	**Dilution**	**Isotype**
Adhesion					
CD31	(PECAM-1)	Monosan	EN-4	1:2500	IgG1
CD54	(ICAM-1)	Becton/Dickinson	LB2	1:200	IgG2b
CD58	(LFA-3)	PeliCluster	bric5	1:150	IgG2a
Antigen presenting					
HLA-DR		Becton/Dickinson	L1125	1:16000	IgG2a
CD1a		Gift*	Okt6	1:1000	IgG1
CD1b		Gift*	BCD1b3.1	1:1000	IgG1
CD1c		Gift*	F10/21A3	1:1000	IgG1
CD1d		Gift*	CD1d55	1:1000	IgG1
Co-stimulating					
CD40		Gift**	BE-1	1:1000	IgG1
CD80	(B7-1)	Pharmingen	BB1	1:2000	IgM
CD80	(B7-1)	Innogenetics	5B5	1:200	IgG3
CD80	(B7-1)	Innogenetics	B7.24	1:200	IgG2a
CD86	(B7-2)	Pharmingen	2331 (fun-1)	1:500	IgG1
Other					
CD3	(pan-T cells)	Dako	035 (202)	1:200	Rabbit polycl.
CD20	(B cells)	Dako	L26/91	1:600	IgG2a
CD68	(Macrophages)	Dako	KP/1 047(101)	1:1000	IgG1
CD83	(Dendritic cells)	Immunotech	HB15a/lot01	1:50	IgG2b
S100	(Schwann cells)	Sigma	SH-B1	1:60000	IgG1

*CD1 antibodies were kindly provided by Dr. S.A. Porcelli, Boston

**CD40 was kindly provided by Dr. T.W. LeBien, Minneapolis

Results

Immunohistochemical procedures and dilutions of antibodies were validated by staining tissue sections of human skin and tonsil. Using the dilutions listed in Table 2, all antibodies showed specific staining with anticipated distribution patterns of positive cells in human skin sections (anti CD1a) or human tonsil sections (all other antibodies)[31-33]. No detectable background staining

Table 2 *Antibodies used against different molecules*

Antigen		Supplier	Batch/clone	Dilution	Isotype
Adhesion					
CD31	(PECAM-1)	Monosan	EN-4	1:2500	IgG1
CD54	(ICAM-1)	Becton/Dickinson	LB2	1:200	IgG2b
CD58	(LFA-3)	PeliCluster	bric5	1:150	IgG2a
Antigen presenting					
HLA-DR		Becton/Dickinson	L1125	1:16000	IgG2a
CD1a		Gift*	Okt6	1:1000	IgG1
CD1b		Gift*	BCD1b3.1	1:1000	IgG1
CD1c		Gift*	F10/21A3	1:1000	IgG1
CD1d		Gift*	CD1d55	1:1000	IgG1
Co-stimulating					
CD40		Gift**	BE-1	1:1000	IgG1
CD80	(B7-1)	Pharmingen	BB1	1:2000	IgM
CD80	(B7-1)	Innogenetics	5B5	1:200	IgG3
CD80	(B7-1)	Innogenetics	B7.24	1:200	IgG2a
CD86	(B7-2)	Pharmingen	2331 (fun-1)	1:500	IgG1
Other					
CD3	(pan-T cells)	Dako	035 (202)	1:200	Rabbit polycl.
CD20	(B cells)	Dako	L26/91	1:600	IgG2a
CD68	(Macrophages)	Dako	KP/1 047(101)	1:1000	IgG1
CD83	(Dendritic cells)	Immunotech	HB15a/lot01	1:50	IgG2b
S100	(Schwann cells)	Sigma	SH-B1	1:60000	IgG1

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was observed using these conditions. Staining of skin sections with the CD1a-specific monoclonal antibody showed the presence of CD1a+ Langerhans cells. In tonsil sections, CD40 positive clusters of B lymphocytes, CD80 and CD1d positive germinal center B cells, and CD86**,** CD83, and CD1b positive interdigitating reticulum dendritic cells were clearly identified (not shown). Table 3 and Figures 1-6 show the results of the immunohistochemical staining of peripheral nerve biopsies.with antibodies specific for molecules involved in adhesion, antigen presentation or costimulation.

Table 3 *Results of the immunohistochemical analysis on sural nerve biopsies of patients and controls*

Pat	CD54	CD58	HLA-DR	CD1a	CD1b	CD1c	CD1d	CD40	CD80	CD83	CD86
CIDP											
1	+ (e)	+ (s)	+ (s, e, m, p)	-	-	-	-	-	-	-	-
2	+ (e)	+ (s, e)	+ (s, e, m)	-	-	+ (b)	-	-	-	-	-
3	+ (e)	-	+ (s, e, m)	-	-	+ (b)	-	-	-	-	-
4	+ (e)	+ (s)	+ (s, e, m)	-	-	+ (b)	-	-	-	-	-
5	+ (e)	-	+ (s, e, m)	+ (m)	-	-	-	-	-	-	-
6	+ (e)	+ (s)	+ (s, e, m, p)	+ (m, p)	-	-	-	-	-	-	-
7	+ (e)	+ (s)	+ (s, e, m, p)	-	-	+ (b)	-	-	-	-	-
Vasculitis											
1	+ (e)	+ (s, e)	+ (s, e, m)	-	-	+ (b)	-	-	-	-	-
2	+ (e)	+ (s, e)	+ (s, e, m)	+ (m, s)	+ (s)	+ (b)	-	-	-	-	+ (e)
3	+ (e)	+ (s, e)	+ (s, e, m, p)	-	-	-	-	-	-	-	+ (e)
Normal											
1	+ (e)	-	+ (s, e, m)	-	-	-	-	-	-	-	-
2	+ (e)	-	+ (s, e, m)	-	-	-	-	-	-	-	-
3	+ (e)	-	+ (e, m)	-	-	-	-	-	-	-	-

Pat = patients, **+** = positive staining, **-** = no staining. Localisation of positive staining: **e** = endothelial cells, **s** = Schwann cells, **m** = macrophages, **p** = perineurial cells, **b** = B cells

Expression of adhesion molecules in sural nerve biopsies

Serial sections of sural nerve biopsies were stained with the monoclonal antibodies specific for CD54 and CD31. CD31 (platelet/endothelial cell adhesion molecule 1, PECAM-1) is known to be expressed on all continuous endothelia; CD54 (intercellular adhesion molecule 1, ICAM-1) is typically expressed at high levels on activated endothelial cells. Representative results are shown in Figure 1. As expected, endothelial cells lining the epineurial and endoneurial blood vessels stained positive for the CD31 molecule in normal controls and in patients with CIDP and vasculitic polyneuropathy (Fig. 1a). In the sural nerve biopsies of all normal controls and patients with polyneuropathy analyzed, CD54 expression was found on the endothelial cells lining endoneurial vessels, whereas no staining of epineurial arterioles was observed (Fig. 1b). No other cell types in the biopsy specimens expressed the CD54 molecule.

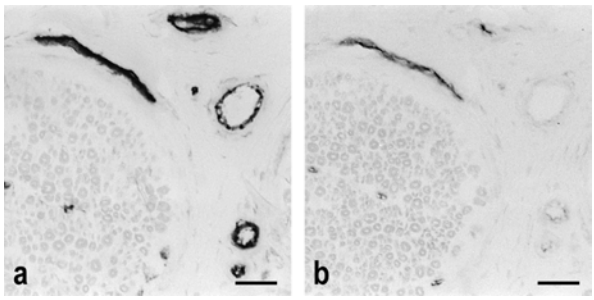

Figure 1
Serial sections of a nerve biopsy from CIDP patient C6. Endothelial cells, stained with the EN-4 antibody, (a) are positive for CD54 (b). Note that some epineurial arterioles do not stain whereas all endoneurial vessels are positive. Bars: 50μm.

The adhesion molecule CD58 (lymphocyte function-associated molecule 3, LFA-3), is known to be expressed on the surface of epithelial and endothelial cells and erythrocytes. We observed expression of CD58 by myelinating (Nuclear fast red-positive) and non-myelinating (Nuclear fast red-negative) Schwann cells in sural nerve biopsies of all 3 vasculitic neuropathy patients and 5 CIDP patients (Fig. 2a). In all 3 vasculitic neuropathy patients but only in 1 out of 5 CIDP patients, CD58 was also present on endothelial cells in both the epineurium and endoneurium (Fig. 2e). In contrast, no CD58 staining was observed in the sural nerve biopsies of normal controls (Fig. 2d).

Staining with an antibody specific for CD68, a widely used marker for cells of the macrophage/monocyte lineage and of dendritic cells, unveiled the presence of CD68+ cells in the endoneurium and epineurium. The CD68+ cells did not co-localise with the CD58+ Schwann cells (Fig. 2b).

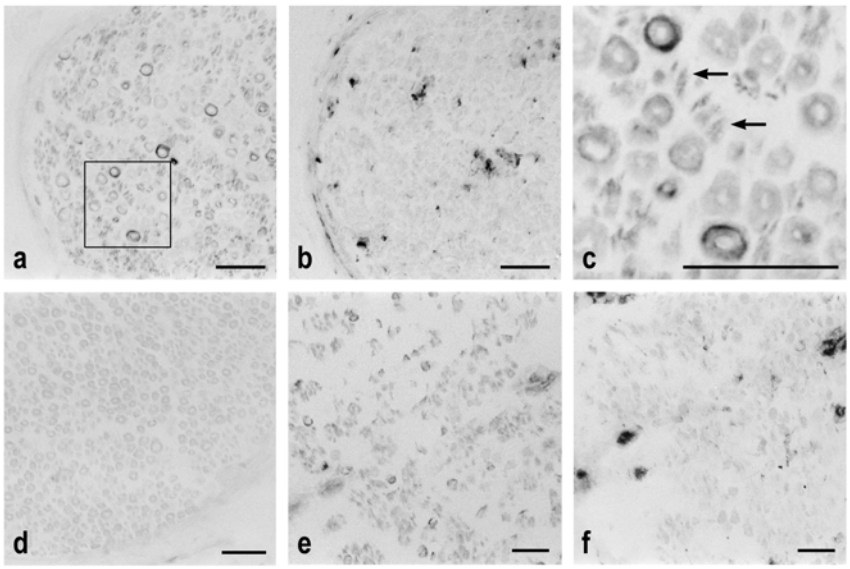

Figure 2

Figure a and b represent serial sections of a nerve biopsy from CIDP patient C6. The anti CD58 monoclonal antibody stains Schwann cells (a). Positivity is not associated with surrounding macrophages, as shown by the localization of CD68+ cells (b). The square in a is enlarged in c. CD58 positivity is associated with the rim of myelin sheats, probably Schwann cell plasmalemma, and non-myelinating Schwann cells (arrows). A section of a nerve biopsy from a normal control is negative for CD58 (d). Serial sections from biopsy specimens of vasculitic neuropathy patient V3 stained with anti CD58 (e) and anti CD31 (f) monoclonal antibodies shows that endoneurial vessels are CD58+. Bars: 50μm.

Expression of antigen presenting molecules

HLA-DR expression was observed on CD31+ endothelial cells and on CD68+ cells in both epineurium and endoneurium in biopsy specimens of all patients and normal controls. In most patients and normal controls, we observed HLA-DR expression by non-myelinating Schwann cells, identified by expression of the S100, an intracellular Ca-binding protein, and the absence of myelin (Nuclear fast red staining). Myelinating Schwann cells were always HLA-DR-negative. Some perineurial cells of unknown lineage were also HLA-DR+. Representative results are shown in Figure 3.

Antigen presenting capacity has recently been ascribed to members of the CD1 gene family[34]. In addition, CD1a, b, and c are markers for subsets of dendritic cells[35;36]. In one out of three patients with vasculitic neuropathy and two out of seven CIDP patients, weak CD1a expression was associated with macrophages (not shown). No CD1a staining was observed in sural nerve biopsies of normal controls. In a single vasculitic neuropathy patient, Schwann cells weakly expressed

CD1a (not shown) and strongly expressed CD1b (Fig 4a). In two vasculitic neuropathy patients and 4 CIDP patients, CD1c expression was observed in cells with a perivascular localization (Fig 4b). Staining of serial sections with a CD20 antibody confirmed that these CD1c+ cells belong to the B lymphocyte lineage (Fig. 4c) No CD1d expression was detected (not shown).
Finally, CD83 expression was not detectable in any of the nerve biopsy specimens studied.

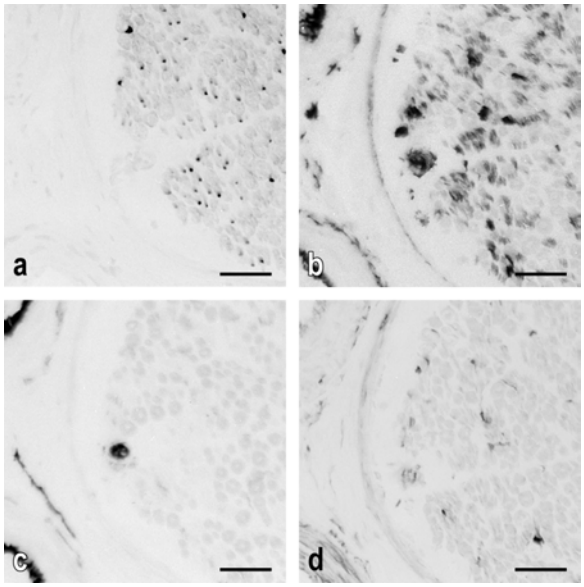

Figure 3
Serial sections of a nerve biopsy from CIDP patient C4 stained for the Schwann cell marker S100 (a), HLA-DR (b), CD31, a marker for endothelial cells (c) and macrophage marker CD68 (d). HLA-DR positivity (b) is not associated with the presence of macrophages (d) or vascular tissue (c), but likely associated with non-myelinating Schwann cells. Bars: 50μm.

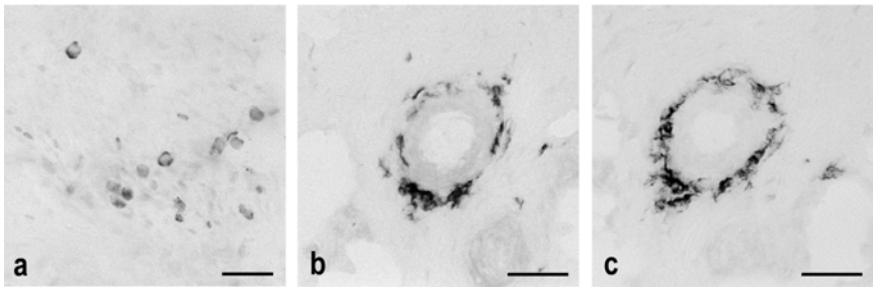

Figure 4
Section of a nerve biosy from vasculitis patient V2 demonstrate myelinating Schwann cells expressing high levels of CD1b (a). Perivascular localization and colocalization of CD1c (b) and the B cell marker CD20 (c) in serial sections of vasculitic neuropathy patient V1. Bars: 50μm.

Expression of costimulatory molecules

Expression of the costimulatory molecule CD40 was not detectable in biopsy specimens from patients or normal controls. CD80 (B7.1) and CD86 (B7.2) are costimulatory molecules expressed on activated B cells, macrophages and dendritic cells. The CD80-specific IgM monoclonal antibody BB1 from Pharmingen (San Diego, CA, USA) brightly stained both myelinating and non-myelinating Schwann cells in all biopsy specimens (Fig. 5a), whereas a complete absence of staining was observed with two IgG monoclonal antibodies specific for CD80 (Innogenetics, Ghent, Belgium), even in T cell rich areas (Fig. 5b and 5c). It was recently shown that the BB1 monoclonal antibody recognizes both CD80 and the cell surface form of the invariant chain (CD74)[37]. We conclude that the Schwann cells do not express CD80 yet express high levels of CD74, a molecule that associates with MHC class II molecules to prevent peptide binding. We were unable to confirm CD74 expression with anti-CD74 monoclonal antibodies because of the unacceptably high background in immunohistochemical analysis. Note that other MHC class II-positive cells like endothelial cells and perineurial cells did not stain with the Pharmingen anti CD80/CD74 antibody.

Weakly CD86-positive endothelial cells were detected in 2 vasculitic neuropathy patients but not in the other biopsy specimens, despite the presence of CD3+ T lymphocytes (Fig. 6a-c).

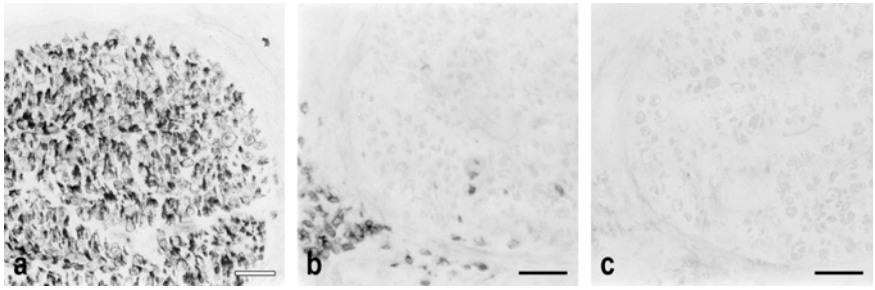

Figure 5

Nerve biopsy section of CIDP patient C5 stained with the Pharmingen anti CD80 antibody (a). Serial sections of the nerve biopsy of patient C3 demonstrate the presence of T cells using anti CD3 antibodies (Fig 6b) and the absence of CD80 using the anti CD80 (Innogenetics, 5B5) monoclonal antibodies. Bars: 50μm.

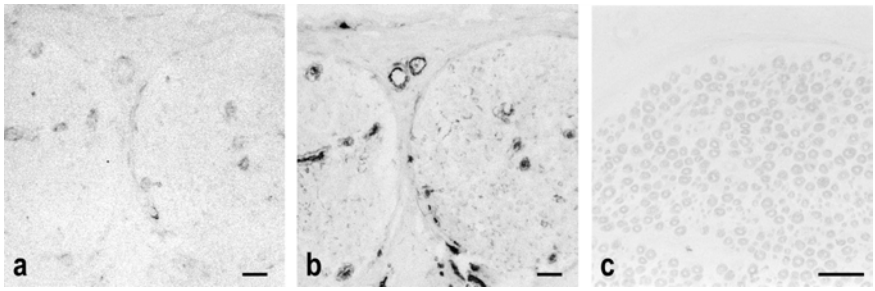

Figure 6

Vasculitis patient V2 (a) stained for CD86. Positivity is mainly localized on endoneurial vessels, as shown by the adjacent section stained with anti CD31 (b). Normal control N3 stained for CD86 (c) illustrates the absence of CD86 positivity as seen in most other specimens. Bars: 50μm.

Discussion

We have studied the expression of adhesion-, antigen presenting-, and costimulatory molecules by cells present in peripheral nerves of normal controls and patients with CIDP and vasculitic polyneuropathy. These molecules are involved in homing, priming and expansion of T lymphocytes and the analysis of their expression patterns in normal and disease-associated peripheral nerve tissue may contribute to the understanding of the pathogenesis of inflammatory peripheral neuropathies. Although in our study only the sural nerve was investigated and the disease process may well involve various other and more proximal nerves, the sural nerves in all vasculitic neuropathy patients and 3 of the 7 CIDP patients showed increased numbers of T cells compared with normal and non-inflammatory disease controls as was measured in a previous study[28].

One of the most salient findings of the current study is that the CD58 (LFA-3) molecule was found on myelinating and non-myelinating Schwann cells in sural nerve biopsies of all 3 vasculitic neuropathy patients and 5 CIDP patients. In striking contrast, no CD58 staining was observed in the sural nerve biopsies of normal controls. The CD58 molecule, a member of the Ig superfamily, is expressed on both hematopoietic and non-hematopoietic cell types. CD58 functions as an adhesion molecule and mediates signal transduction through its counter-receptor CD2 expressed on natural killer cells and T lymphocytes. Signaling via CD58/CD2 induces IL-12 responsiveness of activated T cells during their maturation into effector cells, resulting in T cell proliferation, development of cytotoxic potential and production of □-IFN[38]. In model systems, it has been shown that increased levels of CD58 expression correlates with increased production of the cytokines □-IFN, IL2 and TNF□ by effector T lymphocytes and may increase susceptibility

of CD58+ target cells to lysis[39]. Neonatal rat Schwann cells exert immune functions *in vitro* and, based on their ability to produce complement regulatory proteins and cytokines *in vivo,* human Schwann cells have been postulated to possess the capacity to present antigen to autoreactive T cells in inflammatory neuropathies[40;41]. CD58 expressed by Schwann cells in both CIDP and vasculitic material, but not in normal controls is in line with these findings and suggest that Schwann cells may function as an alternative accessory cell for T cell activation and/or constitute a target for lysis by activated cytotoxic T cells.

CD58 expression was also found on endothelial cells in sural nerve biopsy specimens in all three vasculitic neuropathy patients, one out of six patients with CIDP but not in sural nerve biopsies of normal controls. Endothelial cells have been shown to stimulate T lymphocytes *in vitro*, resulting in the induction of proliferation, cytokine production or development of cytotoxic potential[42-45]. A number of studies has shown that stimulation of T lymphocytes is dependent on expression of CD58 by endothelial cells and independent of the expression of CD80 or CD86 co-stimulatory molecules[42-44;46]. In the current study, the co-stimulatory molecule CD86 was found on endothelial cells in two patients with vasculitic neuropathy but not in CIDP patients or normal controls, whereas we did not observe expression of CD80. *In vitro* cultured and presumably activated microvascular endothelial cells express CD86 that acts as a co-stimulatory molecule for T cell activation[47;48]. Collectively, our experiments suggest that CD58+ and/or CD86+ sural nerve endothelial cells may play a role in the local activation of T lymphocytes in CIDP and vasculitic neuropathy. Of note, the observation that the endothelial cells of all three vasculitic neuropathy patients and only one of six CIDP patients expressed CD58 and that CD86 expression was entirely restricted to endothelial cells of patients with vasculitic neuropathy suggests that endothelial cells may play a more prominent role in local T cell activation in vasculitic neuropathy than in CIDP.

We observed that endoneurial but not epineurial blood vessels in normal controls and patients constitutively express the CD54 (ICAM-1) adhesion molecule. CD54 is constitutively expressed on endothelial cells in some tissues and contributes to T cell activation and migration across the endothelium[49]. A role for CD54 in immune-mediated disorders of the nervous system is suggested by its immunohistochemical localization in the lesions of experimental allergic encephalomyelitis and in multiple sclerosis plaques[50-52]. In the peripheral nervous system, upregulation of CD54 on endoneurial macrophages and endothelial cells in Lewis rat experimental autoimmune neuritis (EAN) was found and the administration of a monoclonal antibody to CD54 abrogated disease in EAN[53;54]. However, increased concentrations of the soluble form of this molecule were detected in serum or CSF from patients with multiple sclerosis but not in GBS[55;56]. In vasculitic neuropathy and CIDP, the role of the CD54 adhesion molecule remains uncertain as T cells are

located predominantly in the epineurium in these diseases[28], and ICAM-1 expression is predominantly in the endoneurium and does not seem to be upregulated in disease biopsies compared with normal controls. This is in contrast with the endothelial leukocyte adhesion molecule (ELAM-1) which was shown to be expressed in epineurial, not endoneurial, endothelial cells in sural nerve biopsies of 5 out of 10 CIDP patients and 1 out of 5 vasculitis patients, but not in the controls[57]. It is possible that the adhesion molecules which are involved in the extravasation of infiltrating cells are different in epineurial or endoneurial endothelial cells.

Dendritic cells are professional antigen presenting cells involved in the initiation of T cell-dependent immune responses. They have been implicated in a number of human autoimmune diseases as well as in animal autoimmune models[35;58-60]. Using MHC class II, CD1, CD40, CD54, CD68, CD80, CD86, and CD83-specific antibodies, we found no evidence for the presence of dendritic cells in sural nerve biopsies, which is in contrast to other chronic autoimmune conditions such as rheumatoid arthritis and psoriasis[61].

Our observation that Schwann cells strongly reacted with the BB-1 antibody but lacked reactivity with two other anti CD80 antibodies, in combination with the recent finding that BB-1 cross-reacts with an epitope on the CD74 molecule led us to conclude that Schwann cells express high levels of CD74. We could not directly confirm this observation by immunohistochemical analysis with an anti CD74 antibody because of an unacceptably high background staining in nerve tissue. BB-1 staining did not unveil obvious differences between myelinating or non-myelinating Schwann cells or between normal and diseased tissues. In contrast, non-myelinating but not myelinating Schwann cells expressed MHC class II molecules. CD74 functions to prevent peptide binding to MHC class II, facilitates MHC class II transport and enhances its localization to antigen-processing compartments[62]. CD74 is produced in molar excess of MHC class II and some is expressed at the cell surface independent of or in association with MHC class II molecules[63]. CD74 has been reported to act as a co-stimulatory molecule via binding of CD44 to chondroitin sulphate linked to CD74[64]. A putative role for CD74 in antigen presentation and/or co-stimulation by Schwann cells remains to be established. Previous studies on MHC class II expression by Schwann cells are not consistent. Initially, class II expression was reported on both myelinating and non-myelinating Schwann cells [65]. Others found no class II expression[66], or a distribution pattern similar to our results[67]. These differences may reflect immunohistochemical techniques or antibodies used.

CD1a and b have recently been reported to be expressed on macrophages and myelinated nerve fibres in sural biopsies of patients with inflammatory neuropathies[68]. Our results with regard to CD1a and CD1b were similar: Weak CD1a expression on macrophages was observed in 2 CIDP patients and one vasculitic neuropathy patient, and CD1a and CD1b expression on Schwann cells

was observed in one patient with vasculitic neuropathy. Stainings with anti CD1c and anti CD20 revealed that the expression of CD1c was restricted to B cells, and not found in normal controls. Our CD1d antibody clearly showed that there was no CD1d expression in diseased or normal peripheral nerve. We unexpectedly found weak CD1a and strong CD1b expression by Schwann cells in a single patient with vasculitic neuropathy. These MHC-like molecules are commonly expressed by professional antigen presenting cells and have hitherto not been associated with Schwann cells. In patients with multiple sclerosis, increased numbers of CD1b-restricted T cells that are specific for self-glycolipids are found in the peripheral blood[69]. Collectively, these findings suggest that CD1b+ Schwann cells may be capable of presenting self glycolipids to autoreactive T lymphocytes.

The results of the present study support a model in which T cell activation can be initiated and/or perpetuated locally in sural nerve biopsies of patients with CIDP and vasculitic neuropathy and predict an important role for Schwann cells and endothelial cells.

Chapter 3

Ex vivo T cell stimulations with peripheral nerve myelin

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Departments of [1]Neurology and [3]Immunology, University Medical Center Utrecht, [2]Division Immunological and Infectious Diseases, TNO Prevention and Health, Leiden, and [4]Crucell NV, Leiden, The Netherlands

Abstract

In the inflammatory peripheral neuropathies chronic inflammatory demyelinating polyneuropathy (CIDP) and Guillain-Barré syndrome (GBS), immune recognition of peripheral nerve myelin has been suspected as the basis for autoimmune destruction. To unveil the identity of T cell stimulatory antigens and to gain insight in the nature of the response, we prepared a panel of peripheral nervous system (PNS) myelin antigens suitable for T cell stimulations. Peripheral blood mononuclear cells (PBMC) of eight CIDP, three GBS patients, and eleven controls were stimulated directly, or with autologous dendritic cells (DC) as antigen presenting cells (APC), with different PNS myelin preparations. Also, T cell lines reactive to *Campylobacter jejuni*, a pathogen associated with GBS, were generated and tested for cross reactivity with myelin preparations. Although a strong memory T cell response against a standard recall antigen was always present, no response against any of the antigen preparations of PNS myelin could be demonstrated. In addition, we did not find evidence for cross-reactivity of *Campylobacter jejuni*-reactive T cells with PNS myelin antigens.

Introduction

Peripheral nervous system (PNS) myelin is most likely the target of immune attack in inflammatory neuropathies like chronic inflammatory demyelinating polyneuropathy (CIDP) and Guillain-Barré syndrome (GBS). Both diseases are characterized by weakness and sensory deficits, though GBS has an acute onset and monophasic course and CIDP is a chronic or relapsing-remitting disease. Most GBS cases are triggered by a preceding infectious disease, most often a *Campylobacter jejuni* enteritis[70-72]. Immune attack of myelin in CIDP and GBS is suggested by histological and electrophysiological evidence for demyelination and the presence of antibodies against constituents of myelin. Among these, antibodies against P0[73], P2[10], PMP22[74], and gangliosides GM1 and GQ1b have been identified[13].

The involvement of T cells in the pathogenesis of CIDP and GBS is suggested by several observations. Patients have a high titre of sIL2R in serum or CSF[9], a T cell infiltrate is present in affected peripheral nerves[1], and a high frequency of HPRT-mutated T cells can be found in patients[4]. In general, work on T cell reactivity is hampered by technical incoveniences like the preparation of antigen suitable for T cell culture, the inevitability of working with living cells, and the lack of standardized, satisfactory methods and protocols. In the field of T cell research, working with clones is common practice because of the advantages of high reproduceability and

the possibility to perform multiple experiments with the same cells. However, it is questionable whether clones still reflect the *in vivo* situation after a long period of *in vitro* culture and manipulation. Clones are usually produced on a priory selected antigens, and the mere fact that a clone reacting to a certain self-antigen exists does not mean that the antigen is relevant during disease, nor that the clone is potentially pathogenic. This is supported by the fact that clones or cell lines against self antigens can often be isolated from healthy donors as well as patients suffering from autoimmune disease. Because of these drawbacks of working with clones, we chose to perform our experiments with freshly isolated PBMC.

For practical reasons, antigens selected for T cell stimulations are often proteins that are major constituents of myelin. Minor protein constituents, as well as non-protein antigens, are easily overlooked as potential auto-antigens, but have been shown to be able to trigger responses, despite their low abundancy[75]. Recently, T cells have been described that react to a variety of non-protein antigens like self and non-self glycolipids[76;77], isoprenoid derivatives[78], and alkylamine antigens[24]. Lipids are a major part of myelin and may as well play a role in autoimmunity. Therefore we prepared lipid extracts in addition to a complete set of proteins with a broad range of hydrophobicity from normal PNS myelin. We also used a crude preparation of CIDP-affected nerve material. These different antigen preparations were used to stimulate *ex vivo* PBMC of GBS and CIDP patients and healthy controls. In addition, we generated *Campylobacter*-reactive polyclonal cell lines and studied the cross-reactivity of these cell lines with myelin preparations.

Material and methods

Patients and controls

For experiments with PBMC, eight CIDP patients with different levels of disease activity and three GBS patients in the acute phase of disease were used. An overview of the clinical status of the patients that were included in this study is given in Table 1. We intentionally included patients that were recovered or in remission because we anticipated differences in stimulatory capacities between acitvated T cells and resting memory T cells. PBMC from healthy blood bank donors were used as controls.

Normal control PNS myelin was obtained from obductions from individuals that did not suffer from polyneuropathies and succumbed from subarachnoidal bleeding or pulmonary embolism.

Table 1 Patient data

Patient	Sex	Age (years)	Duration disease (months)	Infection
CIDP				
C1	F	42	3	-
C2	F	49	8	-
C3	M	35	5	-
C4	M	45	4[a,b]	-
C5	M	48	4[a]	-
C6	M	60	14	-
C7	M	55	11	-
C8	M	32	9	-
GBS			(days)	
G9	M	64	10[a]	*C. jejuni*
G10	F	49	5	*C. jejuni*
G11	M	35	23	-

[a]PBMC of these patients were also tested 6 months later

[b]This patient received IVIg therapy between two experiments.

Antigen preparation

PNS myelin was fractionated as described for CNS myelin[79]. In short, cauda equina was homogenized in 0.29 M sucrose solution, layered over a 0.80 M sucrose solution and after centrifugation, the interface was washed in distilled water, pelleted and freeze dried. This preparation was called 'sucrose myelin'. For delipidation, myelin was dissolved in 2:1 chloroform methanol containing 1% TFA and precipitated in four volumes of ether which was repeated four times. After the last precipitation, delipidated myelin was dissolved in 2 chloroethanol containing 0.1% TFA, and mixed with three volumes of distilled water. This suspension was filtered and loaded on a C3 HPLC column. Fractions were eluted in solvent A (water with 0.1% TFA) with an increasing percentage of sovent B (80% THF, 20 % acetonitrile, 0.1% TFA), or with solvent B only. These preparations were called 'HPLC fractions' and 'HPLC myelin' respectively. Eluted material was freeze dried, dissolved in 2-chloroethanol, and stored at –20°C. Prior to use in a T cell stimulation, a small amount of the fractions was dialyzed in an eppendorf tube against water with a 3.5 kDa cut-off regenerated cellulose membrane (Spectrum Medical Industries, Laguna Hills, CA). The protein concentration of the dialyzed preparations was assessed by the DC Protein Assay (Bio-Rad, Hercules, CA).

To assess the resolution of the HPLC run, fractions were analyzed by SDS-PAGE on 12% polyacrylamide gels using the mini protean II system of Bio-Rad. One hour electroblotting on nitrocellulose membrane (Bio-Rad) was carried out using the mini transblot system (Bio-Rad). Fixed proportions of each fraction, 15 µg on average, was run per lane. Gels were stained with Coomassie Brilliant blue for protein detection Blots were incubated with anti-MBP clone B103.1 (Biogenensis, Poole, UK), anti-αB crystallin clone JAM07, or anti-P0 clone D4IE4 (kindly provided by Dr. B. Erne, Basel, Switzerland) in PBS/1%BSA, followed by biotinylated horse anti-mouse secondary antibody (Vector, Peterborough, England) diluted 1:200 in PBS/1% BSA,

followed by an one hour incubation with ABC peroxidase (Vector). Color was developed with Nickel and Cobalt enhanced diaminobenzidine.

Folch extraction of lipids was performed on 500 mg freeze-dried 'sucrose myelin'. The myelin was sonicated in 2.7 ml distilled water, and 9 ml 2:1 chloroform methanol was added. After vortexing and centrifugation, both the upper and lower phase were preserved. A small amount of each extract was dried and weighed to determine the contents of recovered material. Prior to use in T cell stimulations, a small amount of the fractions was dried under a stream of nitrogen, and sonicated into culture medium.

It has been suggested that abnormalities in the myelin itself like mutations in certain proteins, or infections, or aberrant expression of certain proteins, may lead to immune activation and autoimmunity[80;81]. In order to study whether CIDP-derived nerve material is more immunogenic than control material, we tested CIDP-derived material of two patients. Because we could obtain only small amounts of tussue, no purifications were performed on this material. Sural nerve biopsies are in some cases taken for diagnostic purposes from patients with CIDP. During the process of freezing this material for immunohistochemical analysis, the ends of the biopsy are usually discarded. We collected the ends of two CIDP biopsies (of patients C2 and C6), homogenized the tissue, followed by sonication.

Campylobacter jejuni strain D, an isolate of a GBS patient[82] with GM1-like epitopes was used. Bacteria were cultured at 37°C on 5% lysed horse blood-Columbia agar under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2).

Bacteria from overnight cultures were washed in PBS, suspended in H_2O, heated for 10 minutes at 100°C, and sonicated.

T cell cultures and proliferation assays

Heparinized blood from a CIDP or GBS patient and from a healthy control subject was collected on the same day and processed in parallel. Blood was diluted 1:1 in PBS and centrifuged over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) at 900 g during 20 minutes. After two washes in PBS, the cells were counted and resuspended at 10^6 cells/ml in culture medium. Culture medium consisted of RPMI 1640 (Life Technologies, Paisley, Scotland) with L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 5% heat inactivated human pooled serum. For direct proliferation assays, 100 µl aliquot of the PBMC and 100 µl culture medium with antigen or at double the final concentration were mixed in roundbottom 96-wells microtiter plates and cultured. 1 µCi of ^3H labeled thymidine was added to triplicate wells at day 5 of culture. After 18 hours cells were harvested onto filter papers and counted in a □-counter. Stimulation index was defined as (^3H thymidine incorporation with antigen) / (^3H thymidine incorporation without antigen).

In some experiments we used cultured autologous dendritic cells (DC) as antigen presenting cells (APC). These cells have been shown to possess strong antigen presenting and costimulatory capacities[83;84]. Crosspresentation of external protein antigens on MHC class I molecules has been reported[85;86], and antigen presentation on CD1 isoforms[87]. PBMC were allowed to adhere to tissue culture flasks during 2.5 hours. The non-adherent cells were frozen and the adherent cells were cultured for 3 to 5 days in medium containing 1000 U/ml IL-4 (Strathmann Biotech Hannover, Germany) and 50 ng/ml GMCSF (Schering-Plough, Kenilworth, NJ). In some cases these DC were cultured for an additional two days in medium containing 2.5 ng/ml TNF-α (Intergen, Oxford, UK), 0.5 μg/ml IL-1β (Srathmann Biotech), 1 μg/mlin PGE_2 (Sigma, St. Louis, MO), and 1000 U/ml IL-6 (Roche Diagnostics, Basel, Switzerland) in addition. DC were irradiated (2500 rad) and washed in culture medium before use. The non-adherent cells were thawed and used as responder cells in a ^{3}H thymidine incorporation assay.
Polyclonal *C. jejuni*-reactive T cell lines were generated by stimulation of PBMC of two different healthy donors with *Campylobacter* sonicate in culture medium without cytokines. After 17 days, a second round of stimulation was performed in the presence of 10 ng/ml IL-15 (PeproTech Rocky Hill, NJ), in the presence of autologous irradiated PBMC as APC. The reactivity of the cells was tested with APC and without cytokines at 17 days after restimulation.
Tetanus toxoid (TT) (SVM, Bilthoven, The Netherlands) was used at 10 μg/ml.
Toxicity of myelin preparations was tested by adding the highest concentration myelin to a TT stimulation. The amount of ^{3}H thymidine incorporation of the TT stimulation with or without myelin was compared.

Results

A chromatogram of a HPLC run is shown in Figure 1. In order to get an idea of the resolution of the fractionation, and of the contents of the fractions, we ran SDS-PAGE on the fractions, followed by western blotting. The presence of MBP, αB crystallin, and P0 in the fractions was monitored because they represent a hydrophilic, a rare, and a hydrophobic protein respectively. Figure 1 shows the distribution of these proteins, relative to the chromatogram.

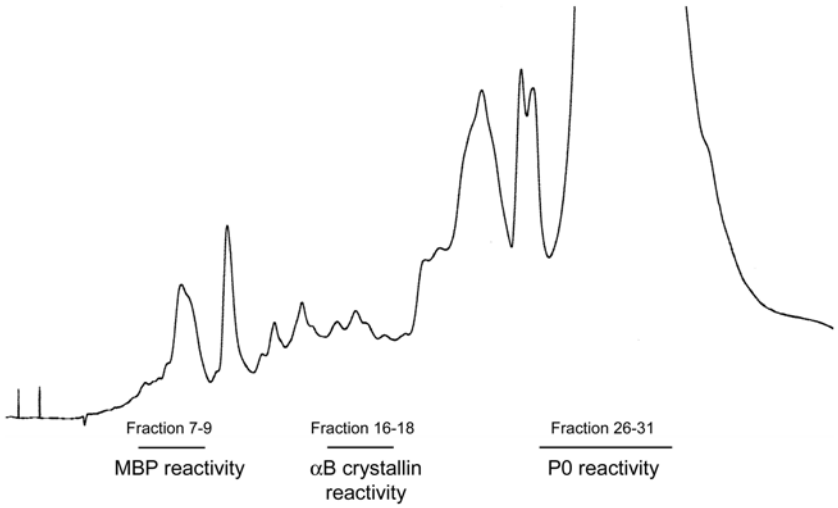

Figure 1

RP-HPLC chromatogram of sucrose gradient purified, delipidated PNS myelin. Myelin was applied to a C3 column and eluted with an increasing percentage of sovent B (80% THF, 20 % acetonitrile). The 36 fractions that were collected were analyzed by SDS-PAGE followed by immunoblotting. The bars show the projections of immunoreactivity on the chromatogram.

A total of 8 CIDP patients, 3 GBS patients, and 11 healthy blood donors was tested for anti-myelin T cell reactivity. Upon direct *in vitro* stimulation of PBMC, all subjects reacted to stimulation with TT with stimulation indices ranging from 13 - 25, but none of the subjectes showed a stimulation index higher than 3.8 to any of the myelin preparations. Eight subjects were also tested with autologous DC as APC. TT-specific proliferation indices were higher than without DC (up to 79), but no higher myelin-specific stimulation indices were obtained using DC. Many authors consider a stimulation index >2 as significant. Therefore, we looked at all myelin preparations that elicited a stimulation index >2 in our experiments. In the group of controls we observed 11 stimulation indices >2, spread over 9 different myelin preparations, and in the group of patients we observed 12 stimulation indices of >2, spread over 11 different myelin preparations.

An example of a direct PBMC stimulation and a stimulation with DC are given in Figure 2. Toxicity of 'HPLC myelin' was tested by adding the highest concentration tested to a TT stimulation. We never noticed a substantial decrease of the TT response.
Campylobacter-specific cell lines were sucessfully created from PBMC from three out of three different healthy blood bank donors. The reactivity to *Campylobacter*, sucrose myelin, and upper and lower phase of a Folch extraction of PNS myelin are shown in Figure 3. The *Campylobacter*-specific cell lines were reactive to *Campylobacter*, but not to the myelin preparations tested.

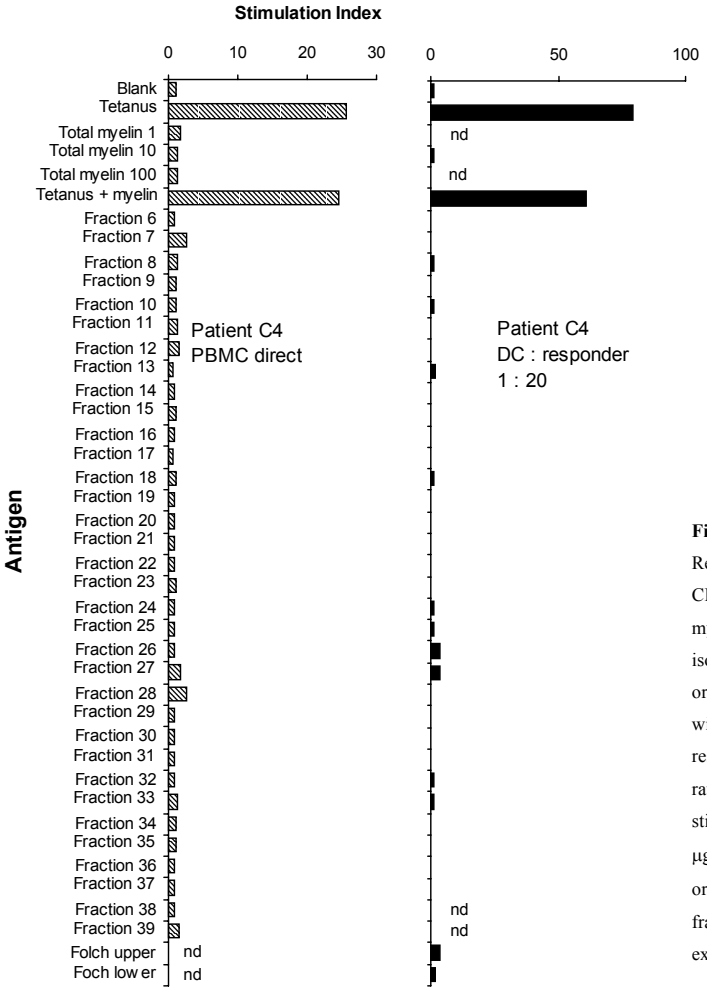

Figure 2
Reactivity of two different CIDP patients to PNS myelin antigens. Freshly isolated PBMC (left graph) or DC that were generated with GMCSF and IL-4 and responder cells in a 1:20 ratio (right graph) were stimulated with 1, 10, or 100 μg/ml total HPLC myelin, or 10 μg/ml of the HPLC fractions or the Folch extracts.

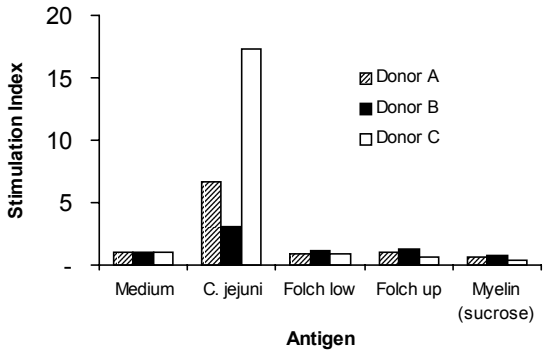

Figure 3
PBMC of three different healthy blood bank donors were used to generate *C. jejuni*-specific cell lines. After two stimulations with crude *C. jejuni* sonicate, the reactivity of the cell lines was tested with autologous irradiated PBMC as APC.

Discussion

In order to test T cell reactivity of patients with inflammatory neuropathy against suspected auto-antigens, PNS myelin lipids and proteins were isolated. Proteins were fractionated by HPLC, and since we successfully eluted MBP, αB crystallin, and P0 from the column, we expect that all proteins with intermediate hydrophobicity were also eluted. None of the antigenic preparations was toxic for T cells. Using these preparations for T cell stimulations we could only measure very low and inconsistent proliferative responses.

A conclusion that a certain phenomenon does not exist, should be supported by strong indications that the experimental set up was actually suitable to measure the phenomenon at all. In all our experiments, TT was taken along as a model recall antigen. Since most people are vaccinated against tetanus, this antigen is used by many researchers as a standard control for the presence of a functional memory response in the CD4+ T cell compartment. Comparing the observed vigorous responses to TT to the low responses to myelin, we conclude that a conventional CD4+ memory T cell response against peripheral myelin components does not exist in CIDP and GBS patients. We were also unable to detect low but consistent responses against any of the peripheral myelin components we isolated. Instead, we detected low responses against different myelin antigen preparations, both in patients and healthy controls. This means that there is no universal immunodominant antigen present in PNS myelin that was hitherto not recognized as such.

Addition of DC to cultures of PBMC increased the proliferative response to TT, but higher myelin-specific stimulation indices could not be measured. Proper controls for cross-presentation by DC in our experiments were not available. Therefore we can not conclude that MHC Class I antigen presented myelin antigens do not play a role in CIDP and GBS.

Polyclonal *Campylobacter*-reactive T cell lines that could be effectively restimulated *in vitro* with *Campylobacter*, did not react to any of the PNS myelin preparations. This indicates that T cell

stimulatory components of crude preparations of *Campylobacter* are not present in PNS myelin preparations. Although technical limitations of the assays used can not be excluded, these data suggest that a T cell stimulatory cross-reactive compound in PNS myelin and *Campylobacter* preparations does not exist. Of note, T cell clones against gangliosides have been described[69], but whether these clones cross-react with *Campylobacter* strains that carry ganglioside-like epitopes has not been reported.

Strong proliferative responses of primary T cells, in the order of magnitude of a TT response, to constituents of PNS or CNS myelin have never been shown. In some publications, low but reproducable and statistically significant responses have been reported. It is unclear which role these proliferative responses play in pathogenesis. One publication shows a significant response of PBMC from patients with peripheral neuropathy to a human PNS myelin protein. P0 elicited a stimulation index >2 in 6/19 GBS patients tested[10]. Because in our study one GBS patient, one CIDP patient, and one control reacted to a P0-containing fraction, we consider our data not in conflict with this publication. The same authors noted responses to P2 in patients with peripheral neuropathy and controls and conclude that the immune responses in peripheral neuropathy is heterogeneous, which is also suggested by our data.

The current techniques and ideas that are used to address this issue are clearly limited. New technical and theoretical approaches may include a closer look at T-independent B cell activation or the role of innate immunity in this disease. Also, research in the field of regulatory immune responses that may prevent tissue damage may prove useful. Some new insights are provided by recent developments in the field of cytokine expression. Vδ1-expressing γδ T cell clones from GBS patients appear to express Th2 cytokines stronger and at a higher frequency than Vδ1 clones from other sources[88]. γδ T cells, showing signs of recent activation in GBS patients, do not recognize peptides presented by MHC, indicating that other antigens may play a role in the development of GBS[89]. Additional input from, for example, work on T cell independent B cell activation, other innate immune responses, or a combination of these, may refine the view that anti-myelin autoimmunity is mainly caused by the wrong adaptive T cell response.

Chapter 4

Vigorous and oligoclonal expansion of human blood γδ T cells after *in vitro* stimulation with *Campylobacter jejuni*

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Abstract

Campylobacter jejuni is currently the prime cause of food-borne bacterial gastro-enteritis. An important complication of *C. jejuni* enteritis is Guillain-Barré syndrome (GBS), an immune-mediated disorder of peripheral nerve. It has been suggested that molecular mimicry plays a role in the pathogenesis of GBS, based on the observation that antibodies against *C. jejuni* lipopolysaccharide (LPS) crossreact with several components in peripheral nerve. Because little is known about T cell reactivity to *C. jejuni*, we have analyzed the *in vitro* immune response of normal individuals against five isolates of *C. jejuni* representing five different serotypes. We found a preferential expansion of peripheral blood γδ T cells after exposure to crude sonicates of all five *C. jejuni* serotypes. Expansion of γδ T cells was dependent on the presence of CD4+/□ □+ T cells in the cultures or addition of exogenous IL-2 or IL-15. *C. jejuni* stimulation was mediated via the T cell receptor and appeared to be induced by a non-proteinaceous bacterial antigen, most likely of phosphoantigenic origin.

Introduction

Campylobacter jejuni is the leading cause of bacterial food-borne diarrheal disease throughout the world[90]. Elevated serum IgG antibodies against *C. jejuni*, suggestive of previous exposure to *C. jejuni*, can be found in 90% of the healthy population[91]. Humans are usually infected by consumption of contaminated poultry, milk products or water, frequently resulting in inflammatory gastro-enteritis in naïve individuals. An important complication of *C. jejuni* enteritis is Guillain-Barré syndrome (GBS), an acute inflammatory demyelinating polyneuropathy which is the leading cause of acute flaccid paralysis in western countries. It has been postulated that the pathogenesis of GBS involves molecular mimicry because sera of patients with *C. jejuni* associated GBS have high titers of antibodies against peripheral nerve gangliosides[71;92;93], and the LPS from various *C. jejuni* serotypes contain a terminal tetrasaccharide identical to that of the GM1 or GQ1b ganglioside[94]. It has been suggested that these cross-reactive antibodies mediate the immune-related destruction of peripheral nerve tissue in GBS and that the diversity of clinical features may relate to the serotype of *C. jejuni*[12]. Serotype O:19[11;95] and O:41[12] are over-represented in GBS patients in Japan and South Africa respectively.

Although the humoral immune response to *C. jejuni* and the occurrence of crossreactive antibodies to peripheral nerve tissues has been addressed in many studies, relatively little is known about the role of T lymphocytes in GBS. Histological studies of peripheral nerve from

patients with GBS show evidence for T lymphocytes infiltrating the affected tissue[1;96;97] and evidence for the presence of activated T cells and T cell products in the blood and serum has been found[4;5;98]. Interestingly, a T cell line established from a nerve biopsy of a patient with GBS with preceding *C. jejuni* infection was shown to consist entirely of T cells expressing the γδ TCR[99]. *In vitro* stimulation of blood mononuclear cells from GBS patients, gastroenteritis patients and healthy volunteers with *C. jejuni* yielded T cell lines that were enriched for γδ T cells[100].
As a prelude to studies on the role of *C. jejuni*-reactive T cells in patients with GBS, we have analyzed the *in vitro* immune response of healthy individuals against five isolates of *C. jejuni* representing five serotypes and obtained from both GBS patients and uncomplicated enteritis patients.

Materials and Methods

Bacterial strains and antigen preparation

A *C. jejuni* strain was isolated from the stool of a GBS patient and was serotyped as O:2 according to the system described by Penner[101]. *C. jejuni* strains with serotypes O:1 (#43429), O:18 (#43446), and O:19 (#43445) were obtained from the American Tissue Culture Collection (Manassas, VA). A strain with serotype O:4 (#10938) was from the Culture Collection University of Göteborg (Göteborg, Sweden). *C. jejuni* strains were cultured under micro aerobic conditions, at 37ºC, on Blood Free Campylobacter Selective Agar Base (Oxoid**,** Basingstoke, Hampshire, England) supplemented with Cefoperazone (32mg/litre) and Amphotericine B (5mg/litre). Bacteria were scraped off the plates, washed twice in PBS, and heated at 100º C for 40 minutes, followed by probe sonication for two minutes. The protein concentration of the preparation was assessed by the DC Protein Assay (Bio-Rad, Hercules, CA).
LPS was extracted from *C. jejuni* by the phenol-water method as described by Westphal[102]. In short, crude antigen preparations were extracted at 100° C in a mixture of water and phenol. The aqueous phase was dialyzed against water, centrifuged at 300.000 g for 2 hours, and freeze-dried. The pellet was weighed and dissolved in water at a concentration of 1mg/ml. The LPS content was analyzed by SDS-PAGE. Preparations that contained no detectable protein were used for T cell stimulation studies.
Isopentenyl pyrophosphate (Sigma, St. Louis, MO) was dried under a stream of nitrogen, dissolved in medium by water bath sonication, and used at 15 μg/ml.

Partial characterization of *C. jejuni* preparations

Aliquots of delipidated preparations of *C. jejuni* containing 150-200 μg of protein were treated

with 1 μg proteinase K (Boehringer-Mannheim, Almere, The Netherlands) for 30 minutes at 37º C and for 5 minutes at 70º C. This procedure was repeated twice. Subsequently 1 μg pronase E (Boehringer-Ingelheim, Heidelberg, Germany) was added and incubated at room temperature for 1 hour. The enzymes were finally inactivated at 100º C for 5 minutes.

SDS-PAGE

SDS-PAGE was performed on 14% polyacrylamide gels using the mini protean II system of Bio-Rad. One hour electroblotting on nitrocellulose membrane (Bio-Rad) was carried out using the mini transblot system (Bio-Rad). Antigen preparation containing 15 μg of protein, the equivalent amount of protease-digested antigen preparation, or 1 μg LPS was run per lane. Blots were stained with Coomassie Brilliant blue for protein detection or with a silver stain kit (Novex, San Diego, CA), modified after Tsai[103] for detection of LPS.

Determination of anti-*C. jejuni* antibodies in serum

Serum from healthy donors was used in serial dilutions in an ELISA to determine the presence of IgM, IgG and IgA anti-*C. jejuni* antibodies[91]. Ratios were obtained by comparing with negative reference serum. IgM or IgA ratios >1 were considered to be indicative of recent *C. jejuni* infection, and IgG ratios of >2 in the absence of IgM or IgA antibodies indicative of previous *C. jejuni* infections in a more distant past[91].

T cell cultures and proliferation assays

For proliferation assays, peripheral blood mononuclear cells (PBMC) from healthy donors lacking IgM or IgA anti-*C. jejuni* antibodies but with IgG ratios >2 were used. PBMC were isolated from heparinized blood by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density centrifugation and resuspended in culture medium consisting of RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 5% heat inactivated pooled human AB serum. For proliferation assays, serial 7-fold dilutions of antigen were added to triplicate wells in roundbottom 96-wells microtiter plates containing 10^5 cells. At day 5 (PBMC) or day 3 (clones) of culture, 1 μCi of [^{3}H]thymidine was added. After 18 hours cells were harvested onto filters and counted in a β-counter. PBMC were cultured for 12 days prior to flow cytometric analysis. The final antigen concentration in these T cell cultures corresponded with 3 μg protein/ml. For inhibition assays, 20 μg/ml monoclonal antibody against the γδ TCR, IL-2 or IL-15 was added to the cultures at days 1, 3, and 6. Human IL-2 was obtained from Strathmann Biotech (Hannover, Germany) and used at 10 U/ml, and IL-15 was from PeproTech (Rocky Hill, NJ) and used at 10 ng/ml.

Cloning and restimulation was performed at 17 days after the last stimulation, using irradiated

allogeneic PBMC as feeder cells, 10 ng/ml IL-15, and antigen at 3 μg protein/ml. Note that PHA was not used. Proliferation assays were performed without cytokines.

Cell depletion and sorting

For depletion experiments, PBMC from individuals with γδ T cells that were >90% CD4-/CD8- were used. CD4+ or CD8+ cells were removed from PBMC by incubation with anti-CD4 or anti-CD8 antibody and magnetic M450 Dynabeads coated with goat anti-mouse IgG (Dynal, Oslo, Norway), according to the manufacturers protocol. CD4+, CD14+, CD16+, CD19+, CD33+, Vα24-, and αβ TCR+ populations were depleted from PBMC by cell sorting on a FACStar^{plus} cell sorter (Becton Dickinson, San Jose, CA). PBMC populations depleted for a population of cells by magnetic beads or cell sorting contained less than 1% of the depleted population as determined by flow cytometry.

Monoclonal antibodies and flow cytometry

Anti-human IL-2 (clone B-G5) and IL-15 (clone 34593.11) monoclonal antibodies for use in cell culture were purchased from Diaclone (Besancon Cedex, France), and from R&D systems (Minneapolis, MN) respectively. The anti-γδ TCR monoclonal antibody producing hybridoma 5a6.e9 was obtained from the ATCC (Manassas, VA). Hybridoma culture supernatant was purified on a protein A column (Bio-rad, Hercules, CA), according to the manufacturers protocol. The protein concentration of the preparation was assessed by the DC Protein Assay (Bio-Rad, Hercules, CA). Isotype control Ig was obtained from ICN Biomedicals (Eschwege, Germany). For flow cytometry, anti-CD3 monoclonal antibody conjugated to PECy5 and unconjugated anti-Vα24 were obtained from Immunotech (Marseille, France), unconjugated anti-Vδ1 (clone A13) was a gift of Dr L. Moretta, (Genova), unconjugated anti-Vδ2 (clone 4G6), anti-Vγ4 (clone 4A11) and anti-Vγ9 (clone B3) were gifts of Dr G. De Libero (Basel), unconjugated anti-CD4 was from Dako (Glostrup, Denmark), goat anti-mouse-PE was from Southern Biotechnology (Birmingham, Alabama) and all other antibodies were from Becton Dickinson (San Jose, CA). 10^5 freshly isolated or cultured cells from 24 pooled wells were used for each staining. All monoclonal antibody incubations were carried out on ice. Washing of cells and dilutions of antibodies were carried out with PBS containing 1% BSA. The unconjugated antibodies were used undiluted and removed by washing before incubation with goat anti mouse-FITC (1:50) or goat anti mouse-PE (1:500). Cells were washed again and incubated with normal mouse serum (1:1000) before anti CD3-PECy5 was added. Flow cytometric analysis was performed on a FACScan (Becton Dickinson).

Calculation of the T cell expansion index

The expansion of γδ T cells was monitored by flow cytometric analysis as described[104] with minor modifications. Stimulated cells were double-stained with anti-γδ TCR or anti-αβ TCR antibodies in combination with an anti-CD3 antibody, and the frequency of γδ or αβ cells in the CD3 and life gate was determined. The absolute number of γδ or αβ T cells was calculated by multiplying the total number of viable cells in a culture with the frequency of γδ or αβ T cells. The expansion index was calculated by dividing the absolute number of γδ or αβ T cells at day 12 of culture by the absolute number of γδ or αβ T cells seeded at the onset of culture.

PCR and nucleotide sequence analysis of TCR Vδ regions

Cultures with expanded populations of TCR Vδ2+ T cells after stimulation with the *C. jejuni* O:1 antigen were harvested, processed for nucleotide sequence analysis of expressed Vδ2 genes, and compared to nucleotide sequences expressed in TCR Vδ2+ T cells at the onset of the culture. RNA extraction was performed with Dynabeads Oligo$(dT)_{25}$ (Dynal, Oslo, Norway) according to the manufacturers protocol, followed by two washes in 1* AMV-RT buffer (Boehringer Mannheim). After the last wash 1 µl RNAguard, 8.5 µl water, 5 µl DTT (100 mM), 5 µl dNTP (10 mM), 5µl 5* AMV-RT buffer and 0.5 µl AMV-RT were added and incubated at 42º C for 90min for cDNA synthesis.

PCR conditions and primers were identical to those described previously[105;106]. PCR products were ligated in the pGEM-T Vector (Promega, Madison, WI) and transformed into *E. coli*. Plasmid DNA was isolated from randomly-picked bacterial colonies and used for nucleotide sequence analysis. The T7 promoter primer was used for sequencing with the Terminator Ready Reaction DyeDeoxy Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Samples were analyzed by automated fluorescence detection.

Clones that were not recognized by the anti-Vδ2 antibody 4G6, nor by the anti-vδ1 antibody A13, were analyzed by Vδ2 PCR as described. Amounts of cDNA were standardized in an β-actine PCR.

Results

Crude preparations of *C. jejuni* induce vigorous T cell proliferation

PBMC from four different healthy donors, with no serological evidence of a recent *C. jejuni* infection, were stimulated *in vitro* with serial dilutions of crude antigen preparations of *C. jejuni* isolates of five different serotypes. Representative dose-response curves of a single donor

Figure 1

stimulated with the five *C. jejuni* serotypes and of four donors responding to a single *C. jejuni* serotype are shown in Fig. 1. In all experiments, significant proliferation (> 3 times the background of non-stimulated cells) was observed at protein concentrations as low as 8.7 ng/ml or less. Maximum proliferation was observed at 3 or 21 μg/ml. Using [^{3}H]thymidine incorporation as a read-out, only minor differences in the dose-response curves upon stimulation with different *C. jejuni* serotypes were observed (Fig. 1). In 40 individuals analyzed to date, we have found only a single individual that completely and repeatedly failed to respond to all five *C. jejuni* isolates (results not shown).

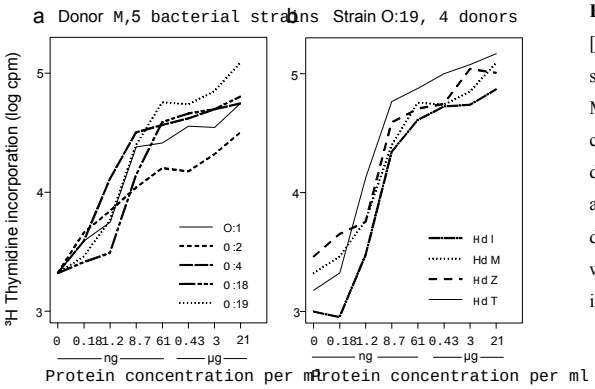

Figure 1
[^{3}H]thymidine incorporation after stimulation of PBMC from donor M with different concentrations of crude *C. jejuni* preparations of five different Penner serotypes (panel a). Variability in the responses of donor T, Z, M, and I to stimulation with the O:19 strain is illustrated in panel b.

Crude preparations of *C. jejuni* induce selective outgrowth of γδ T cells

For identification of the proliferating cells in the cultures stimulated with *C. jejuni* crude antigen preparations, PBMC at the onset and after 12 days of culture were double-stained with γδ or □□ TCR-specific antibodies in combination with an anti-CD3 monoclonal antibody and analyzed by flow cytometry. The expansion index was calculated by dividing the absolute numbers of γδ T cells before and after stimulation and provides a direct measure of T cell expansion during culture. In all donors tested, the frequency of circulating γδ T cells was in the normal range, varying between 0.5 and 10% of the total CD3+ T cell population (not shown). Selective outgrowth of γδ cells, and minimal or no outgrowth of αβ T cells was induced upon stimulation of PBMC of 4 healthy donors with crude preparations of all five isolates of *C. jejuni*. A representative flow cytometric analysis of T cells before and after cell culture is shown in Fig. 2a. The results of these experiments, expressed as γδ or αβ T cell specific expansion indices, are summarized in Table 1. The γδ T cell-specific expansion index ranged from 2 to 151 (median 11.5), whereas the expansion index of αβ T cells in the cultures was significantly lower, ranging from 0.8 to 2.9 (median 1.6).

A

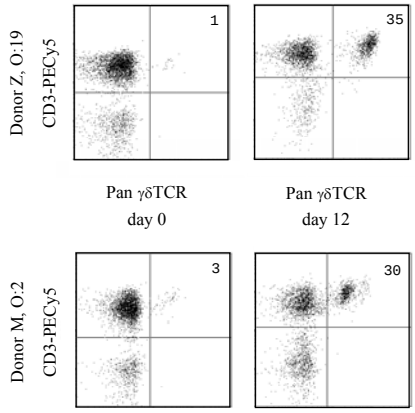

Figure 2

Flowcytometric analysis of PBMC of donor Z and M at the onset and after 12 days of culture with *C. jejuni*. Cells from triplicate or more wells were pooled and double-stained with the CD3-PECy5 and pan γδ TCR-F monoclonal antibodies (panel a), or with the CD3-PECy5 in combination with the Vδ1-F, Vδ2-F, Vγ4-F, or Vγ9-F antibodies (panel b). Percentages of γδ T cells are expressed as percentages within the live, CD3+ population. Note that the sum of the two Vγ families does not match the total amount of γδ T cells of donor M at day 12, as determined by the pan γδ TCR antibody.

B

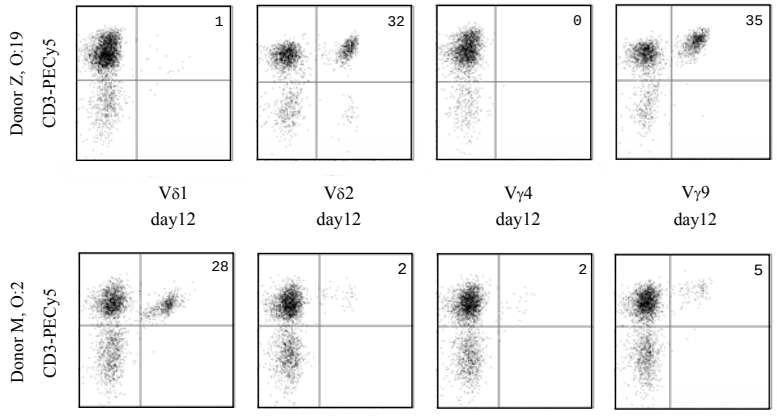

Table 1 αβ and γδ T cell expansion

Donor	**Donor Z**		**Donor T**		**Donor R**		**Donor M**	
T cell population	□□	□□	□□	□□	□□	□□	□□	□□
Bacterial strain								
O:1	4.9	2.9	4.1	1.3	2.0	1.4	20.6	1.6
O:2	29.3	2.0	9.0	0.8	5.9	1.0	15.3	1.0
O:4	13.4	2.2	6.8	1.4	5.5	1.7	11.6	2.2
O:18	27.0	2.2	10.7	1.5	4.9	2.0	31.6	2.2
O:19	151.3	2.1	7.4	1.0	12.5	1.3	32.7	1.2

Expansion indices of αβ and γδ T cells after 12 day culture of PBMC of four healthy donors with crude preparations of five different *C. jejuni* isolates.

Vγ and Vδ chain utilization in γδ T cells stimulated with *C. jejuni*

TCR Vγ and Vδ utilization among γδ T cells before and after stimulation with crude preparations of *C. jejuni* was analyzed by flow cytometry using monoclonal antibodies specific for Vγ4, Vγ9, Vδ1, and Vδ2, and a pan-γδ T cell receptor monoclonal antibody. In three of the four donors analyzed (T, R and Z), the number of (Vδ1+ plus Vδ2+) and (Vγ4+ plus Vγ9+) cells matched the number of γδ T cells detected with the pan-γδ TCR antibody. The results obtained with donor Z are shown in Fig. 2, upper panels. In donor M, the number of γδ T cells detected with the pan-γδ TCR antibody exceeded the sum of the individual Vγ and Vδ families, suggesting the expansion of γδ T expressing V regions not detected with the available monoclonal antibodies (Fig. 2 lower panels).

In donor T, Z, and I, prior to culture 95%, 56%, and 53% of the peripheral blood γδ T cells respectively expressed a TCR encoded by the Vδ2 and Vγ9 gene segments. After culture with all five serotypes of *C. jejuni*, the Vδ2/Vγ9 combination was expressed by virtually all γδ T cells. A representative experiment is shown in Fig. 3. In donor M, a different pattern was observed. In this donor we observed outgrowth of T cells expressing Vδ1 in combination with a Vγ gene segment not identified with our monoclonal antibodies after stimulation with four of the serotypes, and outgrowth of an undetermined Vδ in combination with Vγ9 after stimulation with the fifth serotype. γδ T cells in the non-stimulated PBMC of this donor expressed TCR encoded by Vδ2 (24%) and Vδ1 (76%) showing that T cells expressing the undetermined Vδ comprised only a minor population at the onset of culture. PCR analysis of RNA extracted from γδ T cells clones that were not recognized by the anti-Vδ1 and the anti-Vδ2 antibodies showed that they all express a Vδ2 chain (not shown).

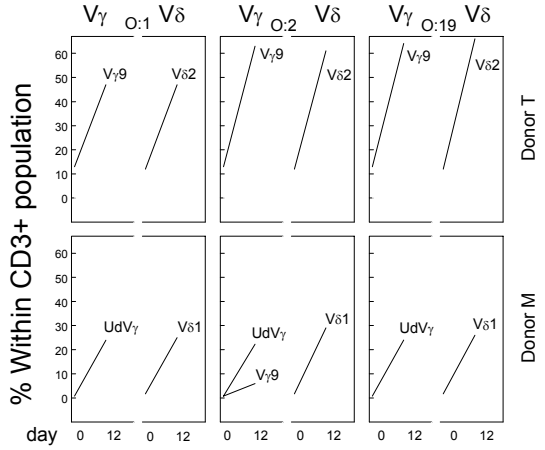

Figure 3

Vγ and Vδ gene utilization in γδ T cells before and after *in vitro* stimulation with *C. jejuni*. V gene families showing less than 5% increase after the cultures are not shown. Representative data of the PBMC of two donors, stimulated with three different crude *C.jejuni* serotypes are shown.

Expanded γδ T cells of all donors, stimulated with all bacterial strains were mostly CD4-/CD8- with a variable percentage (range 0-35%) CD4-/CD8+ γδ T cells (not shown).

Nucleotide sequence analysis of TCR Vδ regions

We assessed the clonal diversity of γδ T cells proliferating in response to *in vitro* stimulation with *C. jejuni*. Freshly-isolated PBMC from donor R and Z were were compared to cultures that had been stimulated for 12 days with the crude antigen preparation of *C. jejuni* strain O:1. The cells were used for RNA extraction and first strand cDNA synthesis and amplified with a Vδ2 specific primer pair. PCR fragments were cloned and the nucleotide sequence of 80 independent Vδ2 inserts was obtained comprising 40 inserts from each donor, equally divided between PBMC before and after culture. The Vδ-Dδ-Jδ junctional sequences are shown in Table 2. All Vδ2 sequences analyzed were in frame. In the collections of 20 sequences from freshly-isolated PBMC, all sequences from donor Z and 19/20 sequences from donor R were unique. After culture, the collections of Vδ2 sequences of the two donors (R and Z) showed clear evidence of oligoclonal expansion as evidenced by the recurrence of identical Vδ-Dδ-Jδ junctional sequences in independently picked bacterial clones.

The *C. jejuni* antigen with strong γδ T cell-stimulatory capacity is a protease resistant compound

Human γδ T cells have been reported to respond to a wide variety of antigens. To determine the nature of the antigen with strong γδ T cell-stimulatory capacity, crude *C. jejuni* preparations were subjected to a number of treatments and separation procedures.

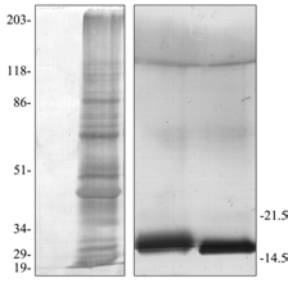

Figure 4
Protease-treated (left panel, left lane) and untreated (left panel, right lane) *C. jejuni* O:19 antigen preparation on a Coomassie stained blot, and phenol-water extracted LPS of O:1 and O:19 on a silver stained gel (right panel).

LPS, known to stimulate murine γδ T cells[107], was isolated from *C. jejuni* isolates O:1 and O:2 by phenol-water extraction and identified on silver stained SDS-PAGE gels (Fig. 4, right panel). In dose-response curves with concentrations ranging from 1.2 ng/ml to 3 μg/ml, LPS induced a just above background proliferative response in 12 day cultures of PBMC. At day 12 of culture, no γδ T cells were detectable by flow cytometry (results not shown), indicating that LPS is not the compound responsible for the γδ T cell response to *C. jejuni* antigen preparation.

The crude antigen preparations of serotypes O:8 and O:19 were delipidated, treated with pronase E and proteinase K and analyzed by SDS-PAGE to monitor the efficiency of protein degradation.

Table 2 VDJ sequences derived from donor R and Z, before and after *in vitro* stimulation with *C. jejuni*.

Freq.	DV	N	DD1/N/DD2	N	DD3	N	DJ1/DJ2/DJ3		CDR3
Donor R, unstimulated									
2\20	cctgtgac	cgg	atatcctac		actgggg	cca	acaccgataaactcat	DJ1	13
1\20	cctgtgac		tcta	t	actgggggа		cgataaactcat	DJ1	9
1\20	cctgtgacacc		atagcatcct	cttt	actgggggata	ggaggag	caccgataaactcat	DJ1	17
1\20	cctgtgacacc		ctac		tggg	aa	acaccgataaactcat	DJ1	10
1\20	cctgtgacacc			g	tgggg	actaag	accgataaactcat	DJ1	10
1\20	cctgtgacacc	gtgg	gaagt		actgggg	aacg	caccgataaactcat	DJ1	13
1\20	cctgtgac	ccccgagc	ccttccta	aa	actgggggatac	acgaaggg	cgataaactcat	DJ1	17
1\20	cctgtgac		tcct	t	actgggggatacg	cgggggt	acaccgataaactcat	DJ1	14
1\20	cctgtgacacc			ggaggttatt	ctgggg		cctgggacacccga	DJ3	14
1\20	cctgtgacacc		acaggctac	g	ctgggg	cttcgg	ccgataaactcat	DJ1	13
1\20	cctgtgacacc	gtaaaga	ctac	gtcgcct	actgggggatacg	aggggt	acaccgataaactcat	DJ1	19
1\20	cctgtgac	ccactggggcgagga	ccta	gg	actgggg	aaaaaac	caccgataaactcat	DJ1	17
1\20	cctgtgacac			tt	gggga	ctccgggaggg	taaactcat	DJ1	10
1\20	cctgtgacacc	ttaggtgtc			ggggatacg	gaaatta	acaccgataaactcat	DJ1	15
1\20	cctgtgaca			attt	actggggg	cca	acaccgataaactcat	DJ1	11
1\20	cctgtgac			ggga	tgggggatac	tctct	ctttgacagcacaact	DJ2	13
1\20	cctgtgacac			g	tgggggа		acaccgataaactcat	DJ1	9
1\20	cctgtgacacc		gaa		gggga	cagggggggg	cgataaactcat	DJ1	11
1\20	cctgtgacacc				gggggatac	aag	accgataaactcat	DJ1	10
Donor R, after stimulation with O:1									
5\20	cctgtgacacc	gtggg	tag	gta	tgggg	cgaccgaaaacggg	aaactcat	DJ1	14
3\20	cctgtgac	tcac	tcc	ga	ggggatacg	cgaattcgcgggac	caccgataaactcat	DJ1	16
3\20	cct		cct	cacaccg	gggggа	gggtggggatctc	aaactcat	DJ1	11
1\20	cctgtgac			tcactccga	ggggatacg	cgaattcgcgggac	caccgataaactcat	DJ1	16
1\20	cctgtgac	ccagt	gaaaattccta	tatcggt	actgggggatac	cagggggg	accgataaactcat	DJ1	19
1\20	cctgtga		tcc	cgt	actgggggat	ggaaagt	acaccgataaactcat	DJ1	13
1\20	cctgtgacacc	g	tagttcc	c	gggga	aaagggac	ccgataaactcat	DJ1	13
1\20	cctgtgacac	t	ctac	c	ggggatac	taagag	ctcctgggacacccga	DJ3	16
1\20	cctgtgacac			actcgg	tgg		caccgataaactcat	DJ1	9
1\20	cctgtgacacc				ctgggggatac	caccca	caccgataaactcat	DJ1	12
1\20	cctgtgac			ccgcccgt	actgggggatacg		tcctgggacacccga	DJ3	15
1\20	cctgtgaca		tagttac		tggg	cccaccaatt	acaccgataaactcat	DJ1	13
Donor Z, unstimulated									
1\20	cctgtgacacc	gttgg	tagt	cga	gggat	ttttaag	aaactcat	DJ1	
1\20	cctgtgac	gttc	tag	gaa	tgggggat	cct	gataaactcat	DJ1	11
1\20	cctgtga		atcctt	aaggacgtccg	gggggatac	agttcggg	accgataaactcat	DJ1	16
1\20	cctgtgac			tcc	ctggg	acta	accgataaactcat	DJ1	9
1\20	cctgtgacac		tctt	attat	tgggggа	ac	caccgataaactcat	DJ1	12
1\20	cctgtgac			ct	actgggggatac	gggtgggg	ataaactcat	DJ1	11
1\20	cctgtgacacc		cttcttcctac	cgtg	actgg	acctgt	caccgataaactcat	DJ1	15
1\20	cctgtgacacc	gttat	tag		tgggggatac	tccgaactacacg	ccgataaactcat	DJ1	16
1\20	cctgtgacacc	g	tagttcc	c	gggga	aaagggac	ccgataaactcat	DJ1	13
1\20	cctgtgacacc	g	tag	g	actgggggatacg	cgggatactggggcgac	actcat	DJ1	15
1\20	cctgtgac			cccgccggt	actgggg	tttgt	acaccgataaactcat	DJ1	13
1\20	cctgtgacac	actagaca	gaaa	c	gggga	actttccgaat	acaccgataaactcat	DJ1	16
1\20	cctgtgac		gacatcc	gga	ggg	ccaagt	acaccgataaactcat	DJ1	12
1\20	cctgtgac			tcggc	ggggatacg	ctct	gataaactcat	DJ1	10
1\20	cctgtgaca			gtattaccg	tgggggat	ggcct	taaactcat	DJ1	11
1\20	cctgtga		tcc	ggtggag	gggggа	ctggagg	ccgataaactcat	DJ1	12
1\20	cctgtgacacc			ctcggg	actgggg	acctaagaaa	cgataaactcat	DJ1	13
1\20	cctgtga			tgggtt	actgggggata	taggtatca	ataaactcat	DJ1	12
1\20	cctgtgacacc	gtaggg	gaa	g	ctgggggg	caaa	accgataaactcat	DJ1	13
1\20	cctgtgac			cat	ctggg	tgatacaa	acaccgataaactcat	DJ1	11
Donor Z, after stimulation with O:1									
5\20	cctgtgacacc			gtc	ggga	ccc	acaccgataaactcat	DJ1	10
3\20	cctgtgacac			gt	tggggg		acaccgataaactcat	DJ1	9
3\20	cctgtgac			ccat	tggggg	ccct	cgataaactcat	DJ1	9
2\20	cctgtgacacc				tgggg	accg	accgataaactcat	DJ1	9
1\20	cctgt			att	ctgggggа	catgt	acaccgataaactcat	DJ1	10
1\20	cctgtgacacc			g	tgggg	ccccgggg	ctcctgggacacccga	DJ3	14
1\20	cctgtgacacc			gtgggt	actgggggatacg	cgttg	gataaactcat	DJ1	13
1\20	cctgtgac		ctacttcttcc	cgcgg	tgggggа	acct	acaccgataaactcat	DJ1	15
1\20	cctgtgac			gc	ctgggggg	ccgtat	accgataaactcat	DJ1	10
1\20	cctgtgac		ccgatcc	caat	ggga	gagggtcgtt	acaccgataaactcat	DJ1	14
1\20	cctgtga			tccc	atac	tgacc	gacacccga	DJ3	10

After blotting and Coomassie Brilliant blue staining, no protein could be detected (Fig. 4, left panel, left lane). PBMC from a healthy donor were stimulated with mock-treated or protease-treated antigen preparations and a [^{3}H]thymidine incorporation assay and flow cytometric analysis were performed. No differences in the proliferation-inducing capacity between the mock-treated and the delipidated/protease-treated *C. jejuni* preparations was observed (Fig. 5 upper panel). Flow cytometric analysis showed that stimulation of PBMC with either preparation resulted in the selective and extensive expansion of γδ T cells (Fig. 5 lower panel).

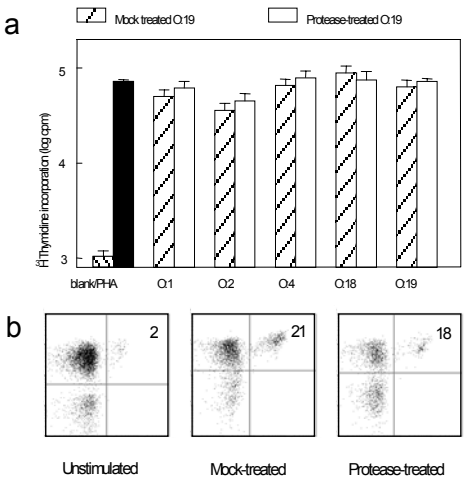

Figure 5
[^{3}H]thymidine incorporation (a) and FACS analysis (b) of non-stimulated PBMC of donor R (left panel) and PBMC stimulated with mock-treated (middle panel) or protease-treated/delipidated serotype O:19 *C. jejuni* preparations (right panel). Both mock and protease-treated/delipidated preparations induced selective outgrowth of γδ T cells, as shown by flow cytometric analysis of CD3-PECy5/pan γδ TCR-F stained cells (b).

Reactivity of γδ T cell clones and *C. jejuni* stimulated cell lines

We generated polyclonal cell lines from PBMC of three different donors by two cycles of stimulation with *C. jejuni* antigen. Clones were obtained from these cell lines, and polyclonal lines and clones were tested for reactivity with *C. jejuni* or isopentenlyl pyrophosphate (IPP). All cell lines proliferated in response to stimulation with *C. jejuni* and IPP. These results were confirmed by the proliferative response of three independent clones from two of the donors (Figure 6).

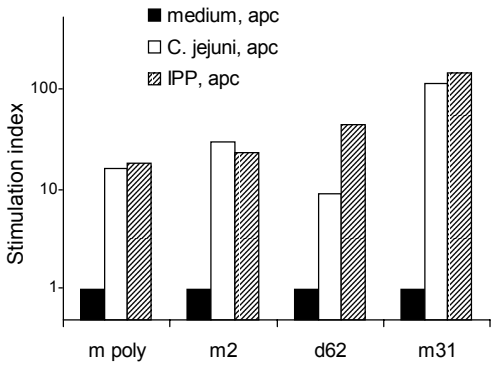

Figure 6
Proliferation of a polyclonal cell line and three independent clones to *C. jejuni* and IPP stimulation. Clones m2 and d62 are derived from different donors and are Vδ2 positive as they stain with the 4G6 antibody, and clone m31 gives a positive Vδ2 PCR signal although it does not stain with the available antibodies. The stimulation index is defined as the cpm obtained from wells with responder cells, irradiated feeder cells, and antigen, divided by the cpm from the wells with responder cell and irradiated feeder cells without antigen.

The presence of CD4+/αβ T cells is a prerequisite for the γδ T cell response to *C. jejuni*

To determine whether signaling via the γδ T cell TCR was essential for the observed stimulation of γδ T cells by *C. jejuni*, we administered a blocking anti-γδ TCR antibody[108] to the cultures. As shown in Fig. 7, control antibody does not influence the response of γδ T cells to *C. jejuni*, whereas the anti-γδ TCR 5a6.e9 monoclonal antibody completely abolishes γδ T cell expansion.

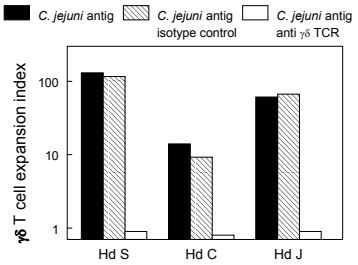

Figure 7

The γδ T cell response to stimulation with *C. jejuni* serotype O:18 is inhibited by addition of 20 μg/ml anti-γδ TCR monoclonal antibody but not by isotype-matched control monoclonal antibody. The γδ T cell expansion index of different donors in response to *C. jejuni* O:18 stimulation is depicted.

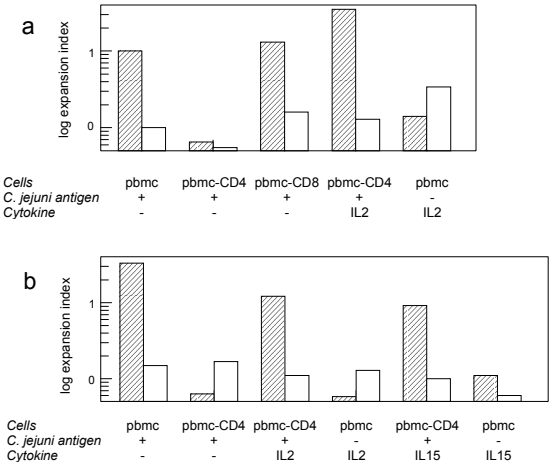

Figure 8

Immunomagnetic depletion of CD4+ cells, but not of CD8+ cells from PBMC of two different donors abrogated the response to *C. jejuni* O:19 antigen (a) and O:2 antigen (b). The response could be restored by the addition of 10 U/ml IL-2 (a and b) or 10 ng/ml IL-15 (b). Dashed bars represent the γδ T cell specific expansion index, and open bars the αβ T cell specific expansion index. The results are representative of experiments with PBMC from eight different donors.

It has been shown that γδ T cell expansion of PBMC upon stimulation with Daudi cells[109] or *Mycobacterium tuberculosis*[110;111] depends on the presence of CD4+ cells. To determine the contribution of CD4+ cells to the response to *C. jejuni*, PBMC were depleted with CD4 or CD8 monoclonal antibody-coated magnetic beads and subsequently stimulated with *C. jejuni*. Depletion of CD8+ cells did not affect the proliferative response to *C. jejuni*, whereas depletion of CD4+ cells completely abrogated γδ T cell expansion (Fig. 8). These findings were extended by depleting PBMC of a number of cell subpopulations by cell sorting. Thus, depleting PBMC of TCR□□+ T cells or CD4+ cells completely abrogated the proliferative response, whereas depletion of CD14+/CD16+, CD33+, CD19+, or V□24+ cells had no effect (Fig. 9).

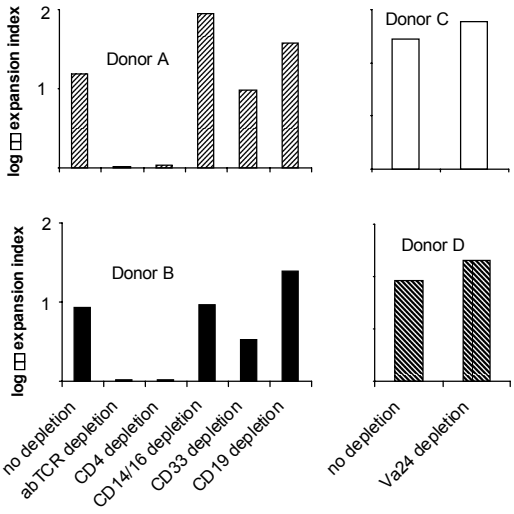

Figure 9

The γδ T cell expansion indices of *C. jejuni* strain O:1-stimulated PBMC from donor A and B, depleted for αβ TCR+, CD4+, CD14+/CD16+, CD33+, and CD19+ cells by cell sorting. As a control for the sorting procedure, unstained PBMC were run through the sorter and put in culture. In separate experiments with PBMC from donor C and D, the NKT cell population was depleted from the PBMC with an anti-Vα24 antibody (right panels).

The γδ T cell response to *C. jejuni* can be restored by adding IL-2 or IL-15 to CD4-depleted cultures of PBMC

The role of the T cell growth factors IL-2 and IL-15 was assessed by adding these cytokines to CD4-depleted cultures of PBMC stimulated with crude *C. jejuni* preparations. Low doses of both IL-2 and IL-15 were capable of restoring the γδ T cell response in CD4-depleted populations (Fig. 8). As noted previously by others[112;113], γδ T cells from some donors proliferated in response to stimulation with IL-2 alone, in the absence of *C. jejuni* antigen (results not shown). We did not include these donors in these experiments.

Monoclonal antibodies to IL-2 and IL-15 unveil a dichotomy in cytokine production in cultures of *C. jejuni* stimulated PBMC

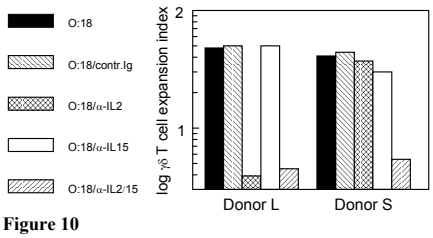

Figure 10

Inhibition of the γδ T cell response by anti-IL-2 monoclonal antibody (donor L), or a combination of anti-IL-2 and anti-IL-15 monoclonal antibodies (donor S). The γδ T cell expansion index of different donors in response to *C. jejuni* O:18 stimulation is depicted. Results are representative of experiments with PBMC from six donors.

The observation that low doses of IL-2 and IL-15 restored the γδ T cell response to *C. jejuni* in CD4-depleted PBMC populations were further supported by the finding that anti-IL-2 monoclonal antibody and in some donors the combination of anti-IL-2 and anti-IL-15 antibodies blocked the γδ T cell response to *C. jejuni* when total PBMC were stimulated (Fig. 10). Anti-IL-15 antibodies alone did not affect the γδ T cell response in 6 donors tested.

Discussion

C. jejuni has become recognized as the most frequent antecedent pathogen associated with the development of GBS. Although the humoral immune response to *C. jejuni* and the occurrence of cross-reactive antibodies to peripheral nerve tissues has been addressed in many studies, relatively little is known about the role of T lymphocytes in GBS. We therefore analyzed the human T cell response to crude preparations of five different serotypes of *C. jejuni*.

The results presented here show that all five different serotypes of *C. jejuni* induce *in vitro* proliferation of γδ T cells present in PBMC from healthy individuals. In 40 individuals analyzed to date, we found only a single individual that completely and repeatedly failed to respond to all five *C. jejuni* serotypes.

In three out of four donors extensively analyzed here, stimulation with all five *C. jejuni* serotypes resulted in the expansion of T cells expressing the Vγ9/Vδ2 TCR. In the fourth donor, proliferation of T cells expressing Vδ1 and an undetermined Vγ was observed. The Vγ9/Vδ2 T cell receptor pair is utilized in 50-75% of circulating γδ T cells, whereas the second major population of blood γδ T cells expresses Vδ1 in combination with one of several Vγ gene segments[114]. Expansion of circulating Vδ1 expressing T cells has only occasionally been reported in the context of HIV[115;116] and CMV after renal transplant[117]. Vγ9/Vδ2 T cell expansions are associated with many human infectious diseases including EBV-mononucleosis[118], CMV disease[119], tuberculosis[120], tularemia[121], listeriosis[122], toxoplasmosis[123], salmonellosis[124], and infection with *Coxiella burnetii*[125]. EBV and CMV are two pathogens that are also frequently found in association with GBS.

Previous reports have shown that the Vγ9/Vδ2 T cells expanding *in vitro* in response to crude preparations of *Mycobacterium tuberculosis* show extensive Vδ-Dδ-Jδ junctional diversity, reflecting a polyclonal T cell response[126;127]. In contrast, nucleotide sequence analysis of 80 Vδ2 regions of Vγ9/Vδ2 T cells in the two donors analyzed here before and after stimulation with *C. jejuni* provided evidence for an oligoclonal T cell outgrowth.

It has been previously shown that CD4+ T cells provide helper functions for the expansion of γδ T cells induced by mycobacterial extracts[110;111;128]. Similarly, the proliferation of *C. jejuni*-reactive γδ T cells could be completely abrogated by depleting CD4+ T or □□ TCR+ T cells from PBMC. Depletion of other cell types including B cells, NK cells, dendritic cells, monocytes, and NKT cells did not impair the ability of γδ T cells to respond to *C. jejuni* stimulation. The γδ T cell proliferation in CD4- or □□ TCR-depleted PBMC could be completely restored by addition of IL-2 to *C. jejuni* stimulated cultures, confirming previous findings that, at least *in vitro*, IL-2 is

a key cytokine for γδ T cell expansion provided by CD4+ T lymphocytes[110;129]. Addition of IL-15, a T cell growth factor that shares some activities with IL-2 *in vitro*[130], also completely restored γδ T cell proliferation in CD4-depleted PBMC. This is in line with previous reports that show that IL-15 contributes to the activation γδ T cells[131;132]. Of note, recent data show that IL-15 may have a prominent role in stimulating and maintaining memory T cells *in vivo* in mice and humans[130;133-135].

Blocking studies with monoclonal antibodies specific for IL-2 and IL-15 in cultures of unseparated PBMC stimulated with *C. jejuni* unveiled a dichotomy in cytokine production during culture of PBMC with crude lysates of *C. jejuni*. In three out of six donors tested, addition of anti-IL-2 monoclonal antibodies to the cultures completely inhibited γδ T cell proliferation, whereas addition of anti-IL-15 antibodies had no effect. In the cultures of PBMC of the remaining 3 donors analyzed, addition of anti-IL-2 or anti-IL-15 antibodies had no effect whereas the simultaneous addition of both anti-IL-2 and anti-IL-15 antibodies completely inhibited the outgrowth of *C. jejuni*-reactive γδ T cells. Apparently, in all donors IL-2 is produced during culture of PBMC with *C. jejuni*, requiring at least the addition of anti-IL-2 antibodies to block γδ T cell proliferation. In cultures of PBMC of a subset of donors, IL-15 appears to be produced in addition to IL-2, requiring the presence of both anti-IL-2 and anti-IL-15 antibodies for inhibition. IL-15 may be produced by a variety of cell types including monocytes, dendritic cells and T cells (reviewed by Fehniger et al.[130]).

The depletion studies suggest that CD4+ T cells are the source of IL-2 and IL-15 in *C. jejuni* stimulated cultures of PBMC. A possible mechanism of CD4+ T cell activation leading to cytokine production, but not proliferation, in the absence of antigen presenting cells and protein antigens is activation via immune receptors recognizing more conserved molecules, like the Toll-like receptor family.

C. jejuni-specific stimulation was mediated via the γδ TCR. We demonstrated that protease treatment of crude preparations of *C. jejuni* did not diminish the γδ T cell response further suggesting that conventional presentation of peptides by antigen presenting cells was not required. Indeed, a number of non-proteinaceous compounds have been identified that stimulate γδ T cells[24;136-140]. Although the stimulatory component for the γδ T cells in the crude preparations of *C. jejuni* was not purified in the current studies, the fact that *Campylobacter*-reactive clones and cell lines recognize isopentenyl pyrophosphate suggests that the active compound in *Campylobacter* antigen preparation is of phosphoantigenic origin.

Chapter 5

Long-lasting γδ T cell non-responsiveness in patients with *Campylobacter jejuni*-associated Guillain-Barré syndrome

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Departments of [1]Neurology and [3]Immunology University Medical Center Utrecht, [2]Departments of Neurology and Immunology, Erasmus University Rotterdam, and [4]Crucell, Leiden, The Netherlands

Abstract

Guillain-Barré syndrome (GBS) is an acute, autoimmune neuropathy. Two thirds of the GBS patients report an acute antecedent infectious disease, most frequently *Campylobacter jejuni* enteritis. Serum antibodies against *C. jejuni* lipopolysaccharides cross-react with peripheral nerve components, suggesting that molecular mimicry plays a role in the induction of GBS. Here, we aimed to seek further evidence for this hypothesis by studying *C. jejuni*-reactive T lymphocytes in GBS patients. PBMC of GBS patients and controls were stimulated *in vitro* with crude sonicates of *C. jejuni.* In healthy individuals, T lymphocytes expressing γδ but not αβ T cell receptors vigorously proliferated after stimulation with *C. jejuni*. In contrast, γδ T cells of acute GBS patients with antecedent *C. jejuni* infections completely failed to respond. GBS patients without evidence for antecedent *C. jejuni* infections and individuals with *C. jejuni* enteritis without GBS responded like healthy individuals. In some patients, the γδ T cell non-responsiveness could last for years after recovery from GBS. Supplementing cell cultures with the cytokines IL-2 or IL-15 resulted in restoration of the γδ T cell proliferative response. γδ T cell non-responsiveness in GBS patients reflects a lack of production of cytokines required to activate γδ T cells. T cell non-responsiveness and ensuing defective autoimmune regulation may be a more general mechanism leading to autoimmune disease.

Introduction

Guillain-Barré syndrome (GBS) is the leading cause of acute flaccid paralysis in most countries. GBS occurs worldwide and affects patients of all ages. In approximately two-thirds of patients, the neuropathy is preceded by an infectious illness, most commonly a respiratory-tract infection or gastroenteritis. *Campylobacter jejuni*, a gram-negative rod that is a major cause of bacterial gastroenteritis worldwide, is the most frequent antecedent pathogen[70-72].

An autoimmune etiology of GBS has generally been accepted[141]. Antibodies to nerve components have been detected in serum of GBS patients, complement deposits and lymphocytic infiltrates have been observed in sural nerve biopsies and postmortem peripheral nervous system material[1;96;97] and patients have increased levels of circulating IL-2, soluble IL-2 receptors and activated T lymphocytes[4;9;98] Based on the observation that anti *C. jejuni* lipopolysaccharide antibodies crossreact with gangliosides in peripheral nerve, it has been suggested that 'molecular mimicry' plays a role in the pathogenesis of GBS[94]. In this scenario, the immune response to the

infectious organism would generate antibodies and perhaps T lymphocytes that cross-react with components of peripheral nerve.

To gain further insight in the role of T lymphocytes in the pathogenesis of GBS, we analyzed the *in vitro* response of peripheral blood mononuclear cells from GBS patients with antecedent acute *C. jejuni* infections and from GBS patients without evidence of recent exposure to this micro-organism. As controls, we evaluated the *in vitro* response of healthy individuals and patients with a history of *C. jejuni*-induced enteritis.

Methods

Study subjects

An overview of the clinical status of the GBS patients whose blood was collected for this study is presented in Table 1. GBS was diagnosed according to established criteria[142]. Acute phase GBS patients were included in the study within one week after the diagnosis of disease. None of the patients received medication at the time blood samples were collected. Experiments with material from a GBS patient and from a healthy control subject were conducted on the same day. None of the healthy control subjects recorded recent symptoms of enteritis. Patients with a history of *C. jejuni* enteritis, as established by a positive *C. jejuni* culture from their stool during the acute phase of gastro-enteritis, were also included in the study. The Medical Ethical Committee of the University Medical Center Utrecht approved the study protocol.

Serologic studies and criteria for *C. jejuni* positivity

C. jejuni seropositivity was determined in an ELISA as described[91]. Acute phase serum of GBS patients was tested for the presence of anti-*C. jejuni* IgG, IgA, and IgM. Ratios were determined by dividing the OD of the serum by the OD of a negative reference serum representing the cut-off titer.

Patients with positive *C. jejuni* stool culture, patients suffering from diarrheal disease before development of GBS and anti-*C. jejuni* IgG ratios > 8, and patients with anti-*C. jejuni* IgM or IgA ratios > 1 were considered as *C. jejuni* positive. Patients with IgM and IgA ratios < 1 and no history of diarrheal disease were considered to be *C. jejuni* negative, irrespective of their IgG titer[71].

Table 1 *Study subjects*

Subject	Time after onset	Severity score	*C. jejuni*	CMV	EBV	Myco-plasm
acute GBS						
1/ac	acute	5	+	-	-	-
2	acute	4	+	-	-	-
3	acute	3	+	-	-	-
4	acute	3	+	-	-	-
5	acute	4	+	-	-	-
6	acute	4	-	-	-	-
7	acute	3	-	-	-	-
8	acute	2	-	-	-	-
9/ac	acute	3	-	-	-	-
10	acute	4	-	-	-	-
recovered from GBS						
1/ex	2 months	5	+	-	-	-
11	7 years	5	+	-	-	-
12	3 years	5	+	-	-	-
13	3 years	3	+	-	-	-
14	3 years	2	+	-	-	-
15	3 months	3	+	-	-	-
16	3 years	2	+	+	-	-
17	7 years	5	+	-	-	-
18	7 years	4	-	-	-	-
19	1 month	3	-	-	-	+
20	4 years	4	-	-	-	-
9/ex	7 months	3	-	-	-	-
recovered from *C. jejuni* enteritis						
21	4 years		+			
22	2 years		+			
23	2 years		+			
24	1 year		+			
25	3 years		+			

Clinical status of the GBS and *C. jejuni* enteritis patients included in this study. The period between onset of GBS or *C. jejuni* enteritis and inclusion in our experiments is depicted as 'Time after onset disease'. Severity of GBS was scored at the peak of their illness on a functional disability scale on which 0 denotes healthy; 1, minor symptoms; 2, able to walk > 10 m without assistance; 3, able to walk > 10 m with support; 4, bedridden or chairbound; 5, requiring assisted ventilation for at least part of the day; 6, dead. Antibodies against CMV, EBV, and mycoplasm were not determined in *C. jejuni* enteritis patients.

T cell cultures and proliferation assays

Twenty ml heparinized blood from a GBS patient and from a healthy control subject was collected on the same day and processed in parallel. Blood was diluted 1:1 in PBS and centrifuged over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) at 900 g during 20 minutes. After two washes in PBS, the cells were counted and resuspended at 10^6 cells/ml in culture medium. Culture medium consisted of RPMI 1640 (Life Technologies, Paisley, Scotland) with L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 5% heat inactivated human pooled serum. An 100 µl aliquot of the PBMC and 100 µl culture medium with antigen or mitogen at double the final concentration were mixed in roundbottom 96-wells microtiter plates and cultured during 12 days prior to cell counting and flow cytometric analysis. For proliferation assays, 1 µCi of ^{3}H labeled thymidine was added to triplicate wells at day 5 of culture. After 18 hours cells were harvested

onto filter papers and counted in a □-counter. Levels of ^{3}H thymidine incorporation in *C. jejuni*-stimulated cultures were calculated by subtracting background ^{3}H thymidine incorporation from cell cultures containing medium without the crude *C. jejuni* preparations.
The expansion index was calculated by dividing the absolute number of αβ or γδ T cells at day 12 after stimulation by the absolute number at day 0. IL-2 was obtained from Strathmann Biotech (Hannover, Germany) and used at 10 U/ml, and IL-15 was from Pepro Tech (Rocky Hill NJ) and used at 10 ng/ml.

Bacterial strains and antigen preparation

For stimulation of cells, eight *C. jejuni* strains were used. Four *C. jejuni* strains, differing in their serotype as described by Penner[101]. were obtained from the ATCC: ATCC 43429: serotype O:1; ATCC 43446: serotype O:19; ATCC 43445: serotype O:18; and CCUG 10938: serotype O:4. In addition, we used four *C. jejuni* strains isolated from the stool of GBS patients. The serotype of one of these strains could not be determined and the other strains had serotypes O:1, O:2, and O:19. *C. jejuni* strains were cultured under micro aerobic conditions at 37ºC, on Blood Free Campylobacter Selective Agar Base (Oxoid, Basingstoke, Hampshire, England) supplemented with Cefoperazone (32 mg/litre) and Amphotericine B (5 mg/litre). When growth was sufficient, the bacteria were scraped off the plates and washed twice in PBS. The pellet of approximately 0.5 ml was heated at 100ºC for 40 minutes, followed by sonication for two minutes. The protein concentration of the preparation was assessed by the DC Protein Assay (Bio-Rad, Hercules, CA). The crude *C. jejuni* antigen preparations were stored at -20ºC until use. *Mycobacterium tuberculosis* strain H37a was obtained from Difco (Detroit, MI). A vial containing 100 mg of dry material was dissolved in 2 ml water, sonicated, and used at a 1:1000 dilution in T cell cultures.

Antibodies and staining procedures

Anti-CD3-PECy5, goat anti-mouse-PE, unconjugated anti-FasL, unconjugated anti-TCR ζ chain, unconjugated anti-FasL, and anti-Fas-Fitc were obtained from Immunotech (Fullerton, CA). Unconjugated anti-TCR Vδ1 (clone A13) was a kind gift of Dr L. Moretta, Genova, unconjugated anti-TCR Vδ2 (clone 4G6), anti-Vγ4 (clone 4A11) and anti-Vγ9 (clone B3) were generous gifts of Dr G. De Libero, Basel, anti-CD94 was a kind gift of Dr M Lopez Botet, Madrid, and all other antibodies were from Becton Dickinson. 10^{5} freshly isolated or cultured PBMC were used per staining. All incubations were on ice. Washes and dilutions of antibodies were carried out with PBS containing 1% BSA. The unconjugated antibodies were used undiluted and removed by washing cells before incubation with goat anti-mouse-FITC (1:50) or goat anti-mouse-PE (1:500). Cells were washed again and incubated with normal mouse serum (1:1000) before other

antibodies were added. Flow cytometric analysis was performed on a FACSscan (Becton Dickinson, Franklin Lakes, NJ) and for each analysis 5000 ungated events were acquired. In the Figures, the percentage of CD3+ cells is expressed as the percentage of cells within a gate around the living cells. The γδ TCR+ cells and the Vγ and Vδ families are expressed as percentages of the living, CD3+ population. In case the number of γδ T cells detected with the pan-γδ antibody exceeded the sum of the individual Vγ and Vδ families, the presence of γδ T cells expressing a V region not detected with the available monoclonal antibodies was assumed and termed 'undetermined Vγ or Vδ'. Fas, FasL, CD8, HLA-DR, FcRIII, and CD94 positivity is expressed as a percentage of the living, CD3+, γδ TCR+ population.

Statistical analyses

The mean of triplicate measurements of ^{3}H thymidine counts and expansion indices was log-transformed prior to statistical evaluation. Values obtained from culture medium control stimulations were subtracted from experimental values. We used a two-tailed Mann-Whithney rank sum test to analyze the differences between healthy control subjects and different patient groups.

Results

Freshly isolated PBMC were stimulated with crude antigen preparation of eight different *C. jejuni* strains at a concentration of 3μg protein/ml that, in preliminary dose-response experiments, was determined to be the optimal stimulatory dose for all strains. The eight different *C. jejuni* strains did not differ in their capacity to stimulate the proliferation of T cells in either patient or healthy control groups (results not shown). Therefore, the experimental values obtained from stimulations with different strains were averaged and are referred to as '*C. jejuni* antigenic stimulation'. ^{3}H-labeled thymidine incorporation was measured at day 5 of the culture, whereas flow cytometric analysis was performed at day 12.

PBMC of healthy donors (n = 30) vigorously proliferated upon stimulation with crude *C. jejuni* preparations (median $30*10^3$ cpm, range 7.6 - $101*10^3$ cpm; Figure 1a). In contrast, PBMC of acute GBS patients with a preceding *C. jejuni* infection (n = 5) did not proliferate upon stimulation with *C. jejuni* antigen (median $1.2*10^3$ cpm, range 0.12 – $3.2*10^3$ cpm; Figure 1b). The group of acute GBS patients with a preceding *C. jejuni* infection differed significantly from the group of healthy donors in their proliferative response to *C. jejuni* antigenic stimulation ($P < 0.001$). PBMC of GBS patients without evidence for antecedent *C. jejuni* infections (n = 5), proliferated vigorously in response to *C. jejuni* stimulation. The magnitude of the response did not

differ significantly from that of healthy individuals (median $48*10^3$ cpm, range $4.6 – 139*10^3$ cpm, Figure 1b).

To assess whether the observed non-responsiveness was transient, we collected blood of patient 1 two months after the first experiment. In addition, we analyzed seven patients who had a recorded history of *C. jejuni* related GBS but were free of symptoms for prolonged periods of time, up till 7 years. Varying responses were observed (n = 8, median $3.9*10^3$ cpm, range 0.2 - $47*10^3$cpm) with two of the eight recovered patients responding similar to the majority of healthy individuals, three patients showing a very low response, and three patients showing absence of a γδ T cell response (Figure 1b). Patients 11 and 17, which were among these low and non-responders, were both free of clinical signs of GBS as long as 7 years prior to these experiments.

Patients with a history of gastro-enteritis caused by *C. jejuni* showed high responses (n = 5, median $52*10^3$ cpm, range 32 - $68*10^3$cpm, Figure 1b) and differed significantly ($P < 0.01$) from this group of recovered, *C. jejuni* positive GBS patients. The uncomplicated gastro-enteritis patients had recovered from their disease between one and three years at the time of inclusion in the experiments.

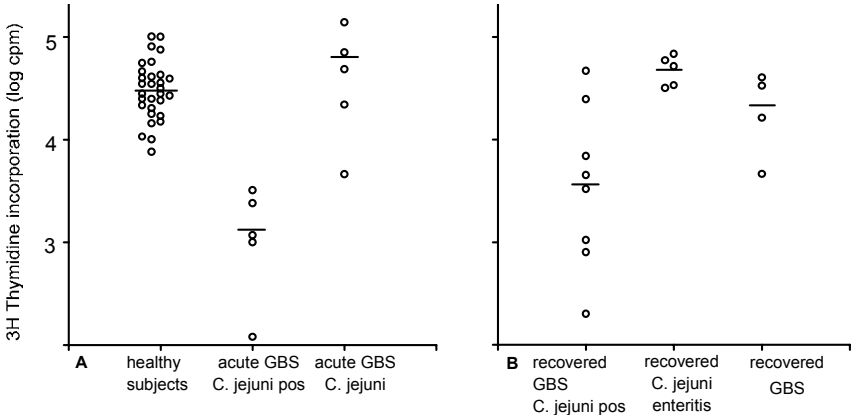


Figure 1

Proliferative responses of PBMC to *C. jejuni* antigen

Proliferative response to *C. jejuni* antigens of healthy donors, acute *C. jejuni*-induced GBS cases, ad acute GBS without preceding *C. jejuni* infection (a), and patients with a history of *Campylobacter* enteritis-induced GBS, uncomplicated *Campylobacter* enteritis, or *Campylobacter*-unrelated GBS (b). Incorporated ^{3}H thymidine counts are means of triplicate wells of stimulations with different bacterial strains.

The phenotype of expanded T cells was determined in subsequent experiments by double-immunofluorescent analysis with anti-CD3, anti-TCR αβ, and anti-TCR γδ monoclonal antibodies, and compared to the phenotype of T cells at the onset of cell culture. Flow cytometric analysis demonstrated extensive expansion of CD3+/TCRγδ+ T cells in the cultures of PBMC of all healthy individuals tested. A representative example is shown in Figure 2. The eight *C. jejuni* strains showed no differences in stimulatory capacity of γδ T cells.

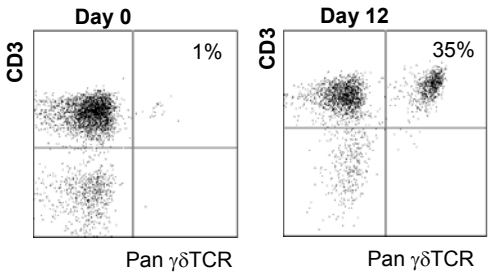


Figure 2

γδ T cell expansion after stimulation with *C. jejuni* antigen

Percentages of γδ T cells of a healthy donor at day 0 and day 12 after *in vitro* stimulation with *C. jejuni* antigen. Cells are stained with anti-CD3-Cy5 and anti-γδ TCR-FITC monoclonal antibodies and prior to flow cytometric analysis. The percentages given are in the living, CD3+ population.

In autoimmune and infectious diseases, an aberrant distribution pattern among TCR Vγ/Vδ families and significant expansion or reduction of the entire γδ T cell population has been reported. We therefore analyzed the frequency and Vγ/Vδ utilization of blood γδ T cells of GBS patients and compared the results to those obtained from healthy individuals. In addition, we performed a phenotypic analysis of blood γδ T cells with monoclonal antibodies against molecules that provide information on their activation status, like HLA-DR, anatomical origin, like CD8[143], TCR-mediated signal transduction capacity, like TCRζ chain, CD3, and FcRIII[144], apoptosis related molecules (Fas/FasL)[145], or killer cell inhibitory receptors (CD94)[146]. The results show no statistically significant difference between the GBS and healthy control group (not shown).

We next determined whether γδ T cells from *C. jejuni* non-responsive GBS patients were capable of proliferating after stimulation with other antigens. To that end, PBMC of non-responder GBS patients and healthy donors were incubated with a crude preparation of the γδ T cell antigen *M. tuberculosis*[147]. Healthy donors as well as *C. jejuni* non-responsive GBS patients showed varying γδ T cell responses to *M. tuberculosis*. The *C. jejuni* non-responder GBS patient group showed a significantly diminished response ($P = 0.044$), albeit that some individual patients demonstrated substantial γδ T cell proliferation after stimulation with *M. tuberculosis* (Figure 3). These experiments suggest that lack of proliferative capacity is not an intrinsic property of the γδ T cells in *C. jejuni*-related GBS patients. The diminished response in these patients to *M. tuberculosis* may be explained by shared γδ T cell-stimulatory antigens.

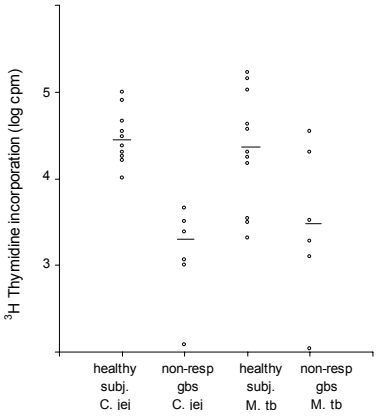


Figure 3

γδ T cell expansion after stimulation with *M. tuberculosis* and *C. jejuni*

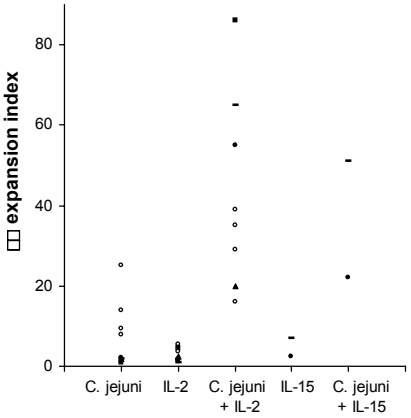
Responsiveness of healthy subjects and *C. jejuni* non-responsive GBS patients *to M. tuberculosis*. The γδ T cell expansion index is plotted on the y-axis.

The basis for γδ T cell non-responsiveness was further investigated by adding cytokines to the cell cultures. γδ T cells have been shown to constitutively express the high affinity IL-2 receptor and expand after stimulation with IL-2[112;113] or a combination of antigen and IL-2 or IL-15[131;148;149] PBMC of four non-responding GBS patients were stimulated with *C. jejuni*, IL-2, IL-15, or with *C. jejuni* antigen in combination with these cytokines. After 12 days of culture, expansion indices of γδ T cells were determined. Figure 4 shows that γδ T cells from *C. jejuni*-associated GBS patients do not respond to stimulation with any of the single compounds, but that stimulation with *C. jejuni* antigen in combination with IL-2 or with IL-15 results in a strong proliferation, comparable to that observed with PBMC from normal donors.

Figure 4

Restoration of the γδ T cell proliferative response by cytokines

Response of healthy donors (open circles) and *Campylobacter* non-responsive GBS patients (each patient represented by a different black symbol) to *Campylobacter*, IL-2 or IL-15 alone, and *Campylobacter* antigen in combination with IL-2 or IL-15. The γδ T cell expansion index is plotted on the y-axis.



It has been previously shown that *in vitro* stimulation of PBMC from *M. tuberculosis* infected patients with *M. tuberculosis* antigen induces apoptosis of γδ T lymphocytes within 48 hours[150]. A similar mechanism does not appear to be operative in cultures of cells derived from GBS patients stimulated with *C. jejuni* antigen. Flow cytometric analysis of *C. jejuni*-stimulated cells at 24 hour

intervals unveiled that the percentage of γδ T cells within the CD3+ population remained unaltered during the first 7 days in culture (results not shown).

Discussion

The most salient finding of this study is that γδ T cells in GBS patients with an antecedent acute infection with *C. jejuni* fail to proliferate after *in vitro* stimulation with a crude antigen preparation of this micro-organism. This is in striking contrast to the vigorous *C. jejuni*-induced proliferative response of γδ T cells obtained from healthy individuals, individuals with *C. jejuni*-related acute enteritis and GBS patients without evidence for an acute antecedent *C. jejuni* infection. We observed that in some patients, γδ T cell non-responsiveness lasted for prolonged periods of time after clinical recovery. In the most extreme case, the peripheral blood γδ T cells of two patients that had recovered from *C. jejuni*-related GBS 7 years prior to the time of the experiment, still completely failed to respond.

We did not detect quantitative or phenotypic differences in the blood γδ T cell population in GBS patients and healthy controls that could explain the non-responsiveness. PBMC from GBS patients contained normal numbers of circulating γδ T cells that, like γδ T cells in healthy individuals, predominantly expressed TCR encoded by Vγ9/Vδ2 variable genes. Moreover, expression levels of molecules related to γδ T cell activation status and TCR-mediated signal transduction capacity (MHC class II, TCR ζ chain, γδ TCR, CD3, and FcγRIII), apoptosis related molecules (Fas/FasL) or killer cell inhibitory receptors (CD94) were similar among γδ T cells of GBS patients and healthy controls, confirming recently published data[89]. Although the γδ T cells did not expand as a result of stimulation with *C. jejuni* antigen, we observed that they remained present during the culture. The combined data distinguish GBS-related γδ T cell non-responsiveness from *M. tuberculosis* related non-responsiveness where stimulation with *M. tuberculosis* induces Fas/FasL-mediated γδ T cell death and disappearance of γδ T cells from cell cultures[150].

Several observations point to a possible mechanism of non-responsiveness. Four out of six *C. jejuni* non-responder GBS patients also failed to respond to stimulation with preparations of *M. tuberculosis*, a well-studied antigen inducing γδ T cell proliferation. Thus, non-responsiveness does not appear to be restricted to a *C. jejuni* specific antigen but extends to a single or a category of γδ T cell stimulatory antigens at least partially shared by *M. tuberculosis* and *C. jejuni* bacteria. This hypothesis is further supported by the recent finding that γδ T cells display a diminished

responsiveness in a non-selected (no discrimination between *C. jejuni*-related and non-related) group of GBS patients to phosphoantigens, bacterial antigens that potently activate γδ T cells[89]. Furthermore, T cells of GBS patients were found to produce little IL-2 upon polyclonal T cell activation and showed impaired responses to IL-2[151]. We noted that γδ T cell responsiveness could be completely restored by simultaneous stimulation of patients PBMC with *C. jejuni* and the cytokines IL-2 or IL-15. Based on the combined data, we propose that the observed non-responsiveness in GBS patients is not an inherent property of γδ T cells but rather reflect a deficient cytokine production in the cell cultures, required to drive γδ T cell proliferation. Several reports have shown that γδ T cells can alter antibody responses (reviewed by Born et al.[152]). Transgenic mice lacking γδ T cells have an exacerbated autoimmune disease phenotype in murine lupus[153], and higher antibody titers against T cell-independent antigens of *Borrelia burgdorferi*[154] as compared to their littermates with functional γδ T cells. These results suggest that γδ T cells may regulate potentially auto-aggressive immune responses. In GBS, antibodies against *C. jejuni* have been shown to cross-react with peripheral nerve tissues. The concomitant lack of cytokine production upon *C. jejuni* stimulation and ensuing γδ non-responsiveness may result in deregulation of autoantibody production and disease. It is tempting to speculate that a similar scenario, based on non-responsiveness of T cells after infection by micro-organisms, unfolds during other autoimmune responses.

Chapter 6

Campylobacter DNA is present in circulating myelomonocytic cells of healthy individuals and patients with Guillain-Barré syndrome

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Abstract

Campylobacter jejuni is the prime cause of food-borne bacterial gastro-enteritis. An important complication of *C. jejuni* enteritis is Guillain-Barré syndrome (GBS), an immune-mediated disorder of the peripheral nerve. The presence of *C. jejuni* DNA in peripheral blood mononuclear cells (PBMC) from GBS patients, *C. jejuni* enteritis patients, and healthy subjects was studied. Two target genes, the flagellin and the *ceuE* gene, were used for PCR identification of *Campylobacter* species in DNA extracted from PBMC. Approximately 30% of the healthy individuals and 50% of the patients had PBMC containing *C. jejuni* DNA as verified by Southern blot analysis or sequencing of the PCR products. Cell sorting revealed that *Campylobacter* DNA was present in the CD14+ and CD33+ populations, indicating that cells from the myelomonocytic lineage are the *Campylobacter* DNA carrying cells. These findings show that *Campylobacter* DNA is present in blood cells from healthy human subjects, although viable bacteria could not be demonstrated.

Introduction

Campylobacter jejuni is the leading cause of bacterial food-borne diarrheal disease throughout the world. *Campylobacter* can penetrate and damage the intestinal mucosa leading to blood and inflammatory cells in the stool[155]. However, also patients with mild diarrhea and asymptomatic cases have been reported. Ongoing research focuses on possible bacterial virulence factors with as milestone the completion of the whole genome sequence of *Campylobacter*.

An important complication of *C. jejuni* enteritis is Guillain-Barré syndrome (GBS), an acute autoimmune inflammatory demyelinating polyneuropathy. Infection with *C. jejuni* has become recognized as the most frequent antecedent pathogen for GBS[95].

Several studies have shown that human monocytes derived from PBMC and monocytic cell lines can phagocytose *Campylobacter* in vitro[156]. The presence of intracellular *Campylobacter* in humans in vivo however, has not been reported. To investigate this possibility, we searched for the presence of *Campylobacter* in PBMC of GBS patients, patients who had suffered from uncomplicated *C. jejuni* enteritis, and healthy subjects.

Material and Methods

Patients and control subjects

PBMC from 65 healthy blood bank donors, 10 patients with a history of uncomplicated culture-proven *Campylobacter* enteritis, and 23 GBS patients, among which 11 patients with serological evidence of a *C. jejuni* infection that preceded the development of GBS, were used in this study. Acute phase serum of GBS patients was tested for the presence of anti-*C. jejuni* IgG, IgA, and IgM in an ELISA as described[91] and evaluated according to established criteria[71]. GBS was diagnosed according to the criteria formulated by Asbury[142].

Bacterial strains and culture

The *C. jejuni* strain 81116 and *C. coli* strain UA417 were grown (37°C, 22°C) on 5% lysed horse blood-Columbia agar under microaerophilic conditions. For revitalization of *Campylobacter*, one ml of PBS containing $5*10^6$ of PCR-positive PBMC, or a series of 10^3 to 10^{-3} *C. jejuni* strain 81116, were injected into the yolk sac of seven-day-old embryonated eggs from specific-pathogen-free (SPF) chickens as described[157]. After 48 h of incubation at 37°C, the vitellus fluid was harvested and plated.

DNA isolation

PBMC were isolated from heparinized blood by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density centrifugation. 10^6 cells were suspended QX1 buffer of a Qiaex II kit for DNA extraction (Qiagen, Hilden, Germany), and sonicated in a bath sonicator for 3 minutes. DNA was isolated according to the Qiaex protocol. To test the sensitivity of the PCR reactions, heat-killed bacteria (10 min, 100°C) were titrated in a PCR-negative PBMC sample prior to DNA isolation.

PCR conditions

For the amplification of the flagellin A and B intergenic sequences, semi-nested primersets were used as described[158]. For the PCR of the *ceuE* gene different semi-nested primersets were used, specific for the *C. jejuni* or the *C. coli ceuE* gene. In addition to the primers described by Gonzalez[159], we designed two 5'-located primers to obtain two semi-nested primersets (COL0: 5'-CCC TGT AAG TCA GAT TGA TGC CC-3', JEJ0: 5'-CGT AAG TCA AAT TAA TGG TAA-3'). Cycling conditions were as described, with minor modifications.

DNA sequencing

PCR products were cloned into the pGEM-T Vector (Promega, Madison, WI) and transformed into *E. coli*. Randomly-picked bacterial colonies were used for plasmid isolations and nucleotide sequence analysis. The T7 promoter primer was used for sequencing with the Terminator Ready

Reaction DyeDeoxy Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Samples were analyzed by automated fluorescence detection.

Southern blotting

Second round *ceuE* PCR products were blotted onto Nitrocellulose membrane (Bio-Rad, Hercules, CA) by overnight capillary blotting. Sequenced *C. coli* or *C. jejuni ceuE* PCR products were ^{32}P-labeled (RadPrime DNA labeling system, Life Technologies, Breda, Netherlands) and used as a probe. The hybridization procedure was performed at 42°C and was followed by three washes in 0.1% SDS/0.2*SSC, at 58ºC. Hybridization patterns were visualized by conventional autoradiography on films.

Cell sorting

All antibodies were obtained from Becton Dickinson (San Jose, CA). $CD14^+$, $CD19^+$, $CD33^+$, and $CD3^+$ populations were sorted from PBMC on a FACStar plus cell sorter. Purity of the cell populations after sorting was always 98% or higher. 3000 Cells were sorted and DNA was isolated as described.

Statistical analysis

SPSS 8.0 for Windows was used for statistical analysis. Chi-square tests with continuity correction (Yates' corrected chi-square) was used. If numbers smaller than 5 occurred, Fisher's Exact test was used.

Results

Evaluation of methods

Two target genes (*fla* and *ceuE*) were used for PCR identification of *Campylobacter*. A PCR on the intergenic sequence of the flagellin A and B genes was chosen for its reported high sensitivity[158]. This PCR amplifies flagellin fragments from *C. jejuni* and *C. coli*, but not from other *Campylobacter* species or other bacterial genera. Amplification of this region yielded a single band of the expected size of approximately 200 bp (not shown). Sequencing confirmed that the obtained PCR product consisted of *Campylobacter* flagellin intergenic sequence. Because considerable variability in the flagellin intergenic region has been reported[158], we considered Southern blotting unsuitable to increase the sensitivity of the detection.

The PCR on the *ceuE* gene, encoding a siderophore-binding protein involved in iron acquisition, does discriminate between *C. jejuni* and *C. coli*[159]. *CeuE* was detected in the samples by a

combination of PCR and Southern blotting. Using sequenced *ceuE* fragments as probes, we confirmed that there was no cross-reactivity between the two *Campylobacter* species (Figure 1A). For both the flagellin PCR and the *ceuE* PCR/Southern blotting procedure the sensitivity of detection was determined at about 10 *C. jejuni* or 10 *C. coli* in 25,000 PBMC. Controls without template DNA that were used in each PCR analysis were always negative. In order to exclude contamination from the environment as a source of positive PCR results, we performed several experiments in a lab where experiments with *Campylobacter* or *Campylobacter*-derived DNA had never been performed. Of the 27 blood samples of healthy donors that were processed under these circumstances, 6 were flagellin positive, demonstrating that contamination is unlikely to account for the observed results.

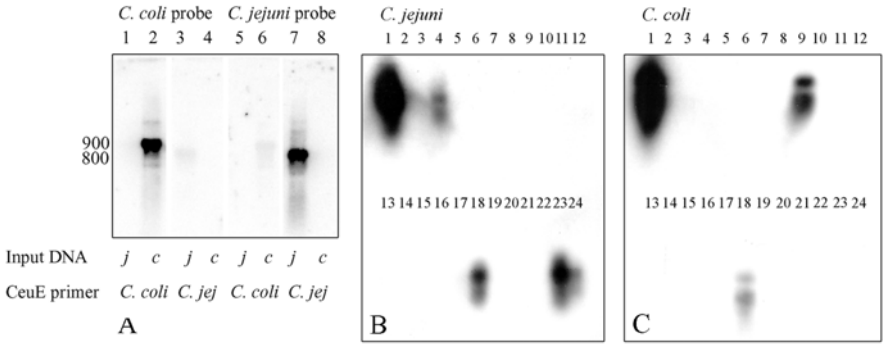

Figure 1

CeuE Southern blotting

A To demonstrate the specificity of the PCR/Southern blot procedure, we performed a PCR with primers specific for the *C. jejuni* or the *C. coli ceuE* gene on DNA isolated from a *C. jejuni* and a *C. coli* strain. This material was blotted and detected with sequenced *C. jejuni* and *C. coli ceuE* probes. Only when the input DNA, PCR primerset, and probe were from the same species, a strong signal of the correct size was detected (lane 2 and 7). Weak cross-reactivity was observed when *C. jejuni* DNA was amplified with *C. jejuni* specific primers and detected with the *C. coli* probe and vice versa (lane 3 and 6), a procedure not relevant for the experimental samples. *C. jejuni* DNA did not produce any signal when amplified with *C. coli ceuE* primers and detected with *C. coli* probe and vice versa (lane 1 and 8), demonstrating that our experimental set up led to highly specific results.

C. jejuni (**B**) and *C. coli* (**C**) specific PCR/Southern blot procedures performed on a positive control sample with DNA containing the equivalent of 25,000 PBMC and 10 *C. jejuni* or 10 *C. coli* (lanes 1) and on experimental samples containing the equivalent of 25,000 PBMC of 23 different healthy donors (lanes 2 – 24).

Screening of human PBMC for the presence of *Campylobacter* DNA

The distribution of *ceuE* and flagellin positive samples in the different groups of donors is summarized in Table 1. The *ceuE* PCR/Southern blotting procedure revealed that a considerable number of the samples was positive for *C. jejuni*, while a few samples tested positive for *C. coli* (Figure 1B). Three subjects were positive for both species. Sequencing of 32 cloned flagellin PCR

products yielded sequences which all scored as *Campylobacter*-derived in a BLAST search of the database available at www.ncbi.nlm.nih.gov/BLAST/.

In an attempt to characterize the *Campylobacter* DNA carrying cells, we sorted 3000 $CD14^+$, $CD19^+$, $CD33^+$, and $CD3^+$ cells from PCR-positive PBMC and performed flagellin PCR on the samples. Only the $CD14^+$ and $CD33^+$ positive populations were flagellin positive. This indicates that the *Campylobacter* DNA was present in monocytes and, possibly, myeloid dendritic cells.

Table 1 *Numbers of fla and ceuE positive samples*

	Flagellin		***C. jej* CeuE**		***C. coli* CeuE**	
	pos.	neg.	pos.	neg.	pos.	neg.
GBS patients						
Preceding *C. jejuni* infection (n = 11)	6	5	6	5	0	11
No preceding *C. jejuni* infection (n = 12)	5	7	6	6	0	12
C. jejuni enteritis (n = 10)	4	6	6	4	1*	9
Healthy donors (n = 65)	22	43	21	44	4**	61

* This patient was also positive for *C. jej* CeuE
** Two of these donors were also positive for *C. jej* CeuE

Table 1

Numbers of *fla* and *ceuE* positive samples

The number of positive and negative samples in the different groups of subjects is shown. The results of the *fla* PCR and the *C. jejuni* or *C. coli* specific *ceuE* PCR/Southern blot are shown separately. None of the patient groups differed significantly ($P < 0.05$, Fisher's Exact test) from the group of healthy donors in the *fla*- or the *ceuE* results. Only if the group of *C. jejuni* enteritis patients and the group of Guillain-Barré syndrome (GBS) patients with preceding *C. jejuni* infection were combined, this led to significant differences ($P < 0.05$, Chi-square tests) in the frequency of *C. jejuni* positivity as determined with the *C. jejuni*-specific *ceuE* PCR/Southern blot.

Persistence of *Campylobacter*

From five study subjects (three *fla* positive and two negative individuals) we were able to obtain a second blood sample one year or more after the first sample. PCR on the follow-up samples yielded identical results as found for the initial samples. The probability that this is accidentally is 0.013, suggesting that the presence or absence of *Campylobacter* DNA in the blood of a certain person is a stable characteristic on a 1-2 year time scale.

As the *fla* intergenic region is variable among *C. jejuni* strains[158], identical DNA sequence at different time points may indicate that certain *Campylobacter* DNA persists in the PBMC. The *fla* sequences obtained from the samples could be classified into three groups with only minimal variations within each group. Comparison of *fla* sequences obtained at different time points showed that initial and follow-up sequences always contained sequences of the same group, with only minimal differences between the first and second sample. In one case, 4 single base deviations from the group consensus sequence were again found in the follow-up sample, suggesting that the same *Campylobacter* DNA was being amplified.

Culturability of *Campylobacter* from PBMC

Direct plating of $3*10^6$ PCR positive PBMC before or after lysis in 1% saponin did not yield detectable growth of bacterial colonies. In vitro studies have shown that *C. jejuni* ingested by human macrophages convert to a coccal form[156]. These coccoid bacteria may be viable but not culturable by direct plating. Culturable *C. jejuni* has been recovered successfully from this state by inoculation in the yolk sacs of embryonated chicken eggs[157]. We applied this method to human PCR-positive PBMC derived from three different subjects. No *Campylobacter* were recovered, although the viability of a series of 10^3 to 10^{-3} log phase-grown *C. jejuni* was increased by 2 logs by this procedure.

Discussion

This study shows that circulating human PBMC contain *Campylobacter* DNA. Within the limits of the sensitivity of our assay, approximately 30% of all healthy individuals is positive, based on the detection of two independent *Campylobacter* genes by PCR, and confirmed by sequencing and Southern blotting. To our knowledge there are no previous reports of intracellular *Campylobacter* DNA in freshly isolated human cells. For GBS and enteritis patients, higher percentages of positive samples were found than for healthy blood donors, but the differences were smaller than might have been expected, and were not statistically significant. The high percentage of *Campylobacter* DNA positive blood donors implies that a large part of the population has a history of, perhaps non-symptomatic, *Campylobacter* infection or still carries the bacteria in an unidentified niche. The latter may also explain the presence of anti-*Campylobacter* IgG in the serum of a high percentage of the healthy population[91].

We have several possible explanations for the frequent presence of *C. jejuni* DNA in human myelomonocytic cells. One is that there may be a continuous supply of *C. jejuni* from the food or from an intestinal reservoir to these cells, which may efficiently engulf and destroy the bacteria. In this concept, an ongoing, subclinical gastro-intestinal infection with bacterial translocation of the intestinal mucosa and influx of *Campylobacter* into the bloodstream has to be assumed, which is unlikely.

An alternative scenario is that the ingested *Campylobacter* are killed, but that their DNA is resistant to degradation and persists within the cells. This phenomenon has been described for *Chlamydia pneumoniae*[160] and for several bacterial species that are found in the joints of patients with arthritis[161]. The inability to recover *Campylobacter* from PBMC is consistent with this hypothesis. However, in this scenario the apparent persistence of the DNA far beyond the normal life-time of the host cells remains unexplained.

A third possible explanation for our results is that during a *Campylobacter* infection, bacteria are ingested by the myelomonocytic cells and persist in the cells in a viable but not culturable form and without causing clinical symptoms. *Campylobacter* has several features that point towards a facultative intracellular life-style including a catalase and a superoxide dismutase gene that protect against oxidative killing[162;163]. A natural intracellular niche has not yet been described, but our data suggest human myelomonocytic cells as a likely candidate. But again, the observed long term persistence of the DNA seems incompatible with the limited life span of the cells, unless it is assumed that the viable bacteria are able to expand the life span of the host cells. This phenomenon has been described for *Mycobacterium bovis* (BCG) and *Brucella suis*. These bacteria increase the half-life of host monocytes by suppressing apoptosis probably via an active process[164;165]. Despite extensive effort, we were unable to recover viable *Campylobacter* from PBMC. However, the presence of a viable, non-culturable *Campylobacter* that are capable to influence the life span of the host cells, cannot be excluded.

Chapter 7

General discussion

Why a general discussion?

A general discussion is meant to add something to the discussions at the end of each Chapter The value of the thesis in its entirety has to be discussed, whether the goals have been properly addressed and attained, and what it adds to the field of interest. A disadvantage of the current practice to present experimental Chapters as independent papers is that certain issues that may have value for the project as a whole are not or only superficially mentioned in the Chapters because they did not fit in the narrow scope of a paper. Consequently, relevant aspects that were underexposed in the experimental Chapters are emphasized here.
The general discussion is not submitted for publication in a peer-reviewed journal, while the Chapters are. Therefore, critical review and speculations in the general discussion may reach a little further than in the Chapters.

Experimental approach

The goal of this thesis was to unravel how activation of the immune system causes inflammatory neuropathy, with particular focus on the role of T lymphocytes. In general, it is a difficult to prove theories concerning pathogenesis of human disease. In principle, the experimental approach entails the collection of supportive data from *in vitro* experiments with human material and the design and execution of experiments in mouse models of the disease under consideration. Also, prospective longitudinal studies are suitable for the evaluation of factors involved in pathogenensis if the studied disease has a sufficiently high incidence, which is not the case for inflammatory neuropathies. This thesis was however not meant to study risk factors for inflammatory neuropathies. My intention was to mechanistically describe the sequence of immunological events that lead to damage of the peripheral nervous system. This approach is most valuable if pathological and normal immune reactions are studied, and clear differences are found that support or reject the working hypothesis.
The search for a plausible theory on how T cells contribute to the development of inflammatory neuropathy was approached in different ways. Nerve tissue from patients suffering from peripheral neuropathy was studied to define the status of antigen presentation and T cell stimulatory molecules *in situ* using immunohistochemistry. Blood from patients was tested for T cell reactivity against peripheral nerve constituents. Alternative approaches were based on the observation that GBS is often preceded by an infection with *C. jejuni.* The *in vitro* T cell response against this pathogen was studied in experiments with PBMC and *C. jejuni* antigen. Molecular

biological techniques like PCR, Southern blotting, and sequencing, were used to detect *Campylobacter* DNA in blood cells from healthy donors and patients.

Observations and ensuing hypothesis

Chapter 2

In nerve biopsy material of inflammatory neuropathy patients, cell surface molecules were present that could play a role in activating primed T cells[166], but no support was found for the hypothesis that naive autoreactive T cells are locally activated to cause tissue damage. In a study from Bosboom *et al.*[3], the phenotype of the T cells that infiltrate the nervous tissue was described, and compared with that of blood. Many different T cell receptor (TCR) Vβ chains were expressed, and αβ TCR+, γδ TCR+, CD4+, and CD8+ T cells were all present. Collectively, these data argue against the presence in nerve tissue of an oligoclonal T cell population, or a T cell population that expresses another marker that points to a common basis of antigen recognition, like a mainly CD4 or CD8-positive T cell population, that accounts for the studied peripheral neuropathies. GBS-derived material was not used for these studies because biopsies for diagnostic purposes are not taken nowadays, and no cryopreserved old material was available. In publications that do describe GBS-derived material[1], the same overall picture was provided of a T cell population infiltrating affected nerve that does not differ from an average circulating T cell population.
These observations are therefore profoundly different from those found in some other organ-specific autoimmune diseases. For example, in polymyositis the phenotype of the infiltrating T cell population in affected muscle, the skewed or even uniform Vβ expression, and antigen presentation, collectively suggest that local damage is caused by Class I presentation to CD8+ T cells expressing a single or restricted set of Vβgenes[167-169]. Class I expression in muscle cells has been shown to be a hallmark in the development of myositis[170].
A possible scenario for the origin and perpetuation of peripheral neuropathies is that local nerve tissue damage is not primarily or directly caused by T cells, but by cytokines, antibodies and/or activated macrophages. T cells may play a role in the stimulation of antibody production and in the production of cytokines, but the localization of these processes is most likely not in the peripheral nerves, but for example in the lymphoid organs.

Chapter 3

In an attempt to gain insight into the reactivity of circulating T cells to peripheral nerve constituents, lipid extractions and protein fractions of normal PNS myelin, and crude homogenate of CIDP-affected peripheral nerve were used to stimulate *ex vivo* PBMC of GBS and CIDP

patients, and healthy controls. Although a strong memory T cell response against tetanus toxoid (TT) could be measured in all patients, no response against any of the antigen preparations of PNS myelin could be demonstrated. Because TT is widely accepted as the standard protein recall antigen that stimulates memory CD4+ cells, I conclude in this Chapter that there is no similar memory response against myelin proteins in patients or healthy controls.

Although it may undermine the conlusions of Chapter 3, some comments on the use of TT as a recal antigen are warranted. TT is a formaldehyde-treated form of tetanus toxin, which has inherent immunostimulatory qualities that point to innate stimulatory activities[171;172] in addition to the adaptive memory T cell response that it elicits. This general immunostimulatory property or adjuvant-like activity may be circumvented by using the synthetic TT peptide p2, containg amino acids 830 – 844 of TT, that has been shown to universally bind HLA-DR, and towards which the immunodominant memory T cell response is directed. However, even the *in vitro* T cell response to this peptide is probably not comparable to the response against other recall protein antigens because 1) it can associate with all known HLA-DR alleles[173], leading to a high percentage of DR molecules occupied by the peptide, 2) synthetic peptides can bind directly to class II MHC molecules, circumventing the natural processing route in the antigen presenting cells[174], which renders the peptide usuitable as control for the antigen presenting capacities of the *in vitro* system for which is serves as a control, 3) peptide interaction with class II MHC generates epitopes dissimilar to those resulting from intracellular processing[175], and 4) because the half-life of the peptide in human serum is approximately 10 minutes[176;177], which is much lower than what is found for most proteins, it is expected that the kinetics of both types of differs considerably. Optimally, in experiments concerning T cell memory, a negative responder population like adults that have not been vaccinated against tetanus should be used in order to exclude T cell responses that are not due to a real recall response.

It is even harder to conceive a proper positive control antigen for a MHC class I-restricted recall response *in vitro* using the experimental set up we used with PBMC as responder population. Taken together, this difficulty in finding proper positive and negative controls is a reason to reconsider the feasibility of primary, *in vitro* T cell stimulations.

Chapter 4

Because many patients with GBS have suffered from a preceding *C. jejuni* infection, the *in vitro* T cell response against this micro-organism was studied. The results of these studies were described in Chapters 4 – 6. The *in vitro* T cell response of healthy donors against *C. jejuni* was investigated in Chapter 4 and it was found that γδ T cells specifically expand upon such a stimulation. A phosphoantigen is the active compound, and the activation of the γδ T cells

depends on the presence of CD4+ αβ T cells or the addition of IL2 or IL15 during culture. Expansion of γδ T cells has been observed by others when T cells were stimulated *in vitro* with certain bacteria or bacterial antigens, suggesting an important role for these cells in anti-bacterial immunity. However, human γδ T cells have also been shown to be able to kill uninfected human target cells, and they have also been implicated in anti-viral immunity. This multifunctional character of γδ T cells makes it difficult to speculate about their role in the induction of autoimmunity. It is important to note that other T cell populations expressing αβ TCR may still be involved in the response against *C. jejuni*, but that they go undetected because of the vigorous γδ T cell proliferation.

Chapter 5

Experiments with T cells of GBS patients pointed to aberrant reactivity towards *Campylobacter* as compared to healthy individuals. The γδ T cells of acute GBS patients with antecedent *C. jejuni* infections completely failed to respond to in vitro *Campylobacter* stimulation. Insufficient cytokine production by CD4+, αβ T cells was proposed as an explanation for the observed lack of responsiveness, because these cells appeared to be indispensable for a normal the vigorous T cell response in healthy donors. The addition of low amounts of IL2 or IL15 to cultures of non-responding γδ T cells cultures rendered them responsive. Further support for an impaired cytokine production in GBS patients comes from the finding that polyclonally-acivated T cells from patients *in vitro* produce low quantaties of IL2[151].

In apparent contrast, GBS patients have been shown to harbor high titers of circulating IL2 and soluble IL2-receptors compared to healthy controls. Therefore the possibility of a higher cytokine-response threshold of the γδ T cells of non-responsive GBS patients instead of a lower cytokine production by the αβ T cells should be taken into account. Alternatively, it is conceivable that an IL2-ELISA does not distinguish between IL2 bound to the soluble IL2R or free IL2[178], and since sIL2R is present in serum of GBS patients, it is conceivable that there is no free IL2 in GBS serum.

γδ T cell non-responsiveness was not found in *Campylobacter* enteritis patients that did not develop GBS, showing that that this phenomenon may be involved in the pathogenesis of GBS. However, since γδ T cells are innate lymphocytes, involved in the rapid 'defense' against micro-organisms, an alternative hypothesis is that defective γδ T cell reactivity leads to more severe infection with stronger activation of the adaptive B and T cell mediated immunity and thus to GBS. This hypothesis is based on the assumption that γδ T cell non-responsiveness exists before infection with *C. jejuni* or develops at the onset and as a consequence of infection. The fact that

all acute *Campylobacter*-induced GBS patients are non-responders, and only part of the recovered *Campylobacter*-induced GBS patients are non-responders, and some are low-responders suggests that non-responsiveness develops at the time of GBS, and slowly subsides. This argues against the idea that GBS patients had a pre-existing slow and inefficient innate defense against *Campylobacter* which lead to a more severe infection and stronger adaptive B and T cell response. But it still leaves open whether γδ T cell non-responsiveness is caused by the *Campylobacter* infection and causes GBS, or whether it is a side effect of GBS. An attractive hypothesis is that T cell non-responsiveness leads to defective immune regulation and autoimmune disease.

Chapter 6

The idea that the severity, duration, or localization of the *C. jejuni* infection may influence whether or not a *C. jejuni* enteritis leads to T cell non-responsiveness, and is followed by GBS led to the experiments described in Chapter 6. The only aspect of prior *Campylobacter* exposure that was studied was whether or not *Campylobacter* DNA was still present in PBMC of the studied subjects. This led to the remarkable observation that 30% of the healthy donors carry *Campylobacter* DNA in their monocytes. Of the GBS patients and *Campylobacter* enteritis patients that were studied, 50% was positive. Though the work is by no means a comprehensive description of the history of the *Campylobacter* infection that an individual has experienced, these experiments suggest that the persistent presence of *Campylobacter* DNA and presumably antigen in antigen presenting cells of a large proportion of the human population does not render the T cells of these individuals unresponsive to *in vitro Campylobacter* stimulation.

Integration in existing hypothesis on pathogenesis of peripheral neuropathies

Usually, a hypothesis on the pathogenesis of a disease is based on a combination of environmental and host factors. Even descriptions of the molecular mimicry hypothesis of GBS and searches for the 'bad bug', both emphasizing environmental factors in the development of GBS, take host factors into account. Host factors that have been studied are restricted to straightforward genetic factors like cytokine and cytokine receptor genes and HLA- and FcR polymorphisms. The work described in this thesis may not be sufficiently worked out to add a fully-developed hypothesis to this field, but some novel ideas with potential importance, mainly concerning the status of the immune system of the host are presented.
A hypothesis on the sequence of events leading to GBS, combining established ideas with the findings presented in this thesis may be formulated as follows. An infection with a ganglioside-

mimicking pathogen is the first, initiating event in the development of GBS. This may be a *Campylobacter* infection, or other GBS-associated pathogens that have been shown to elicit anti-ganglioside antibodies[179]. It is also conceivable that other cross-reactive epitopes play a role. Initially, a normal immune response will develop which consists of a bactericidal or cytotoxic innate lymphocyte response, followed by an adaptive T cell response, and antibody production. For as yet unknown reasons a subset of T cells becomes hyperactivated followed by anergy[180] or a certain T cell population was already non-responsive to stimulation with antigen before the actual infection took place. If these nonresponsive T cells are the ones that should normally support regulatory T cells, or should perform a regulatory function themselves, a physiological immune response now turns into a pathogenic one, characterized by a long duration and heightened intensity. As a consequence, pathogenic autoantibodies and immune-stimulatory molecules are produced in high amounts and gain access to the peripheral nerve where complement and macrophage activation damage the nerve tissues. In this scenario, molecular mimicry alone is not sufficient to cause autoimmunity, but it is a condition for a pathogenic response. Only the combination of defective downregulation and a peripheral nerve-mimicking epitope on a pathogen may lead to GBS.

The question that arises immediately is why T cells become non-responsive. Potential answers to this question are: 1) the infection is very severe due to an inherent property of the pathogen, or 2) due to an inadequate early defense of the host, both leading to hyperactivation and anergy, or 3) genetic host factors, or 4) other immunological host factors contribute to the observed T cell non-responsiveness. It may for example be crucial how the host immune system was primed before the GBS-inducing infection was established. Most of the suggestions for additional studies that will follow below address these issues.

Because this thesis was meant to encompass not only GBS, but also CIDP as a model autoimmune peripheral neuropathy, I will make some comments about CIDP as well. The results from Chapter 2, suggesting that T cells are probably not locally causing primary damage, and from Chapter 3, that a classical *in vitro* CD4 memory T cell response against myelin is not found in CIDP patients, both point to the limitations of current techniques and ideas that are used to address this issue. New technical and theoretical approaches may include a closer look at T-independent B cell activation or the role of innate immunity in this disease. Also research in the field of regulatory immune responses that may prevent tissue damage may prove useful.

Suggestions for additional studies

In order to develop the aforementioned hypothesis concerning immune regulation in the pathogenesis of GBS, new experimental data should be collected.

The role of γδ T cells in the pathogenesis of GBS may be studied in γδ T cell knock out mice. Although the antigen reactivity of human and mouse γδ T cells widely differ, there may be a functional overlap. γδ T cell knock out mice may be infected with with *Campylobacter* and monitored for the development of GBS-associated features like clinical signs of weakness, demyelination, and higher antibody titers against *Campylobacter* than wild type mice.

The question arises whether the idea of hyperactivation of T cells followed by non-responsiveness is also applicable to other infections that are associated with GBS. *In vitro* T cell stimulation of healthy donors and GBS patients for with GBS-associated pathogens should shed light on this issue.

A final question that should be addressed in this thesis concerns the potential benefits that patients may ever have from this work. If the ideas concerning defective immune regulation hold, the question rises how this could contribute to the cure or prevention of GBS. Prevention is only a realistic option if a disease has a high incidence and mortality. Interventions in an ongoing immune response are notoriously difficult. However, the design of therapeutic strategies greatly benefits form a thorough understanding of the pathogenesis of the disease. In addition, an animal model that mimicks both the clinical characteristics and pathogenesis of the disease is urgently needed for the development of novel therapies.

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Summary

This thesis deals with three subjects: inflammatory neuropathies, the (human) immune system, and microbial pathogens. The work is mainly focussed on Guillain-Barré syndrome (GBS), an acute inflammatory neuropathy that is often induced by a bacterium called *Campylobacter jejuni*, and chronic inflammatory demyelinating polyneuropathy (CIDP). In the broadest sense, the goal is to reveil how activation of the immune system causes inflammatory neuropathy. Much less is known about the T cell response than about the antibody response in inflammatory neuropathy and therefore the thesis is entirely focussed on T cells. The T cell response to myelin and to the microbial pathogen *C. jejuni*, and the status of the T cells during inflammatory neuropathy are described. The newly obtained data will be put together into a hypothesis on the pathogenesis of GBS.

The presence of adhesion-, costimulatory-, and antigen presenting molecules on different cell types as conditions for local T cell activation in human sural nerve biopsies of CIDP and vasculitic neuropathy patients and healthy controls is described in **Chapter 2**. In biopsies from CIDP and vasculitic neuropathy patients, but not healthy controls, Schwann cells expressed the adhesion/T cell stimulatory molecule CD58 (LFA-3). Expression of the co-stimulatory molecule CD86 was detected on vascular tissue in patients with vasculitic neuropathy. Schwann cells of a single vasculitis patient strongly expressed CD1b, a molecule involved in the presentation of self glycolipids to T cells. There was no evidence for the presence of dendritic cells in sural nerve biopsies. These findings suggest that T cell activation can be perpetuated locally nerves of patients with CIDP and vasculitic neuropathy, but no support was found for the hypothesis that naive autoreactive T cells are locally activated to cause tissue damage.

In an attempt to gain insight into the reactivity of circulating T cells to peripheral nerve constituents, lipid extractions and protein fractions of normal peripheral nervous system myelin, and crude homogenate of CIDP-affected peripheral nerve were used to stimulate *ex vivo* T cells of GBS and CIDP patients and healthy controls. As described in **Chapter 3**, a strong memory T cell response against tetanus toxoid could be measured in all patients, but no response against any of the preparations of myelin could be demonstrated.

Because many patients with GBS have suffered from a preceding *C. jejuni* infection, the *in vitro* T cell response of healthy donors against this micro-organism was described in **Chapter 4**. We found a preferential expansion of peripheral blood γδ T cells after exposure to crude sonicates of *C. jejuni*. Expansion of γδ T cells was dependent on the presence of CD4+/□ □+ T cells in the cultures or addition of exogenous IL-2 or IL-15. *C. jejuni* stimulation was mediated via the T cell

receptor and appeared to be induced by a non-proteinaceous bacterial antigen, most likely of phosphoantigenic origin.

In contrast, γδ T cells of acute GBS patients with antecedent *C. jejuni* infections completely failed to respond, as described in **Chapter 5**. GBS patients without evidence for antecedent *C. jejuni* infections and individuals with *C. jejuni* enteritis without GBS responded like healthy individuals. In some patients, the γδ T cell non-responsiveness could last for years after recovery from GBS. Supplementing cell cultures with the cytokines IL-2 or IL-15 resulted in restoration of the γδ T cell proliferative response, suggesting that γδ T cell non-responsiveness in GBS patients reflects a lack of production of cytokines required to activate γδ T cells. T cell non-responsiveness and ensuing defective autoimmune regulation may be a more general mechanism leading to autoimmune disease.

In **Chapter 6** the presence of *C. jejuni* DNA in blood mononuclear cells from GBS patients, *C. jejuni* enteritis patients, and healthy subjects was studied. Because persistent antigen can cause T cell non-responsiveness, the presence of *C. jejuni* in GBS patients may provide an explanation for the observed T cell non-responsiveness in these patients. Two target genes, the flagellin and the *ceuE* gene, were used for PCR identification of *Campylobacter* species. DNA extracted from blood mononuclear cells of approximately 30% of the healthy individuals and 50% of the patients contained *C. jejuni* DNA as verified by Southern blot analysis or sequencing of the PCR products. Cell sorting revealed that *Campylobacter* DNA was present in the CD14+ and CD33+ populations, indicating that cells from the myelomonocytic lineage are the *Campylobacter* DNA carrying cells. These data did not provide us with an explanation for the T cell non-responsiveness in GBS patients as observed in **Chapter 5**.

A hypothesis on the sequence of events leading to GBS, combining established ideas with the findings presented in this thesis may be formulated as follows. An infection with a ganglioside-mimicking pathogen is the first event in the development of GBS. Initially, a normal immune response will develop which consists of a bactericidal or cytotoxic innate lymphocyte response, followed by an adaptive T cell response, and antibody production. For as yet unknown reasons a subset of T cells becomes hyperactivated followed by anergy or a certain T cell population was already non-responsive to stimulation with antigen before the actual infection took place. If these nonresponsive T cells are the ones that should normally support regulatory T cells, or should perform a regulatory function themselves, a physiological immune response now turns into a pathogenic one, characterized by a long duration and heightened intensity. As a consequence, pathogenic autoantibodies and immune-stimulatory molecules are produced in high amounts and gain access to the peripheral nerve where complement and macrophage activation damage the

nerve tissues. In this scenario, molecular mimicry alone is not sufficient to cause autoimmunity, but it is a condition for a pathogenic response. Only the combination of defective downregulation and a peripheral nerve-mimicking epitope on a pathogen may lead to GBS.

The question that arises immediately is why T cells become non-responsive. Potential answers to this question are: 1) the infection is very severe due to an inherent property of the pathogen, or 2) due to an inadequate early defense of the host, both leading to hyperactivation and anergy, or 3) genetic host factors, or 4) other immunological host factors contribute to the observed T cell non-responsiveness. It may for example be crucial how the host immune system was primed before the GBS-inducing infection was established.

Samenvatting

In dit proefschrift worden drie onderwerpen behandeld: inflammatoire neuropathieën, het menselijke immuunsysteem, en ziekteverwekkende bacteriën. Het werk gaat voornamelijk over Guillain-Barré syndroom (GBS), een acute inflammatoire neuropathie die vaak door de bacterie *Campylobacter jejuni* veroorzaakt wordt, en chronische inflammatoire demyeliniserende polyneuropathie (CIDP). Het doel in brede zin is erachter te komen hoe activatie van het immuunsysteem een inflammatoire neuropathie veroorzaakt. Aangezien er veel minder bekend is over de T cel respons dan over de antilichaamrespons in inflammatoire neuropathieën is dit proefschrift geheel gericht op de T cel respons. De T cel respons tegen myeline en *C. jejuni* en de status van de T cellen gedurende inflammatoire neuropathie worden beschreven. De nieuwe gegevens zullen in een hypothese over de pathogenese van GBS worden verwerkt.

De aanwezigheid van adhesie-, costimulatie-, en antigeenpresenterende moleculen die plaatselijk in nervus suralis biopten van patiënten met CIDP en vasculitisneuropathie en gezonde controles T cellen zouden kunnen activeren is beschreven in **Hoofdstuk 2**.

In de biopten van CIDP en vasculitisneuropathie patiënten, maar niet in die van gezonde controles, brachten de Schwanncellen het adhesie-/T cel stimulatiemolecuul CD58 (LFA-3) tot expressie. Het co-stimulatiemolecuul CD86 kwam tot expressie in vaatweefsel van vasculitisneuropathie patiënten. De Schwanncellen van een vasculitisneuropathie patiënt brachten CD1b, een molecuul dat bij de presentatie van glycolipiden afkomstig uit het eigen lichaam betrokken is, sterk tot expressie. Er waren geen aanwijzingen voor de aanwezigheid van dendritische cellen in nervus suralis biopten. Deze bevindingen suggereren dat T cel activatie in stand gehouden kan worden in zenuwen van CIDP en vasculitisneuropathie patiënten, maar er waren geen aanwijzingen dat naïeve, autoreactieve T cellen plaatselijk geactiveerd kunnen worden en weefselschade kunnen aanrichten.

Om inzicht te krijgen in de reactiviteit van circulerende T cellen tegen componenten van perifere zenuw werden vetextracten en eiwit fracties van normaal myeline uit de perifere zenuw, en ruw homogenaat van door CIDP aangedane zenuw, gebruikt om *ex vivo* T cellen van CIDP en GBS patiënten en gezonde controles te stimuleren. Zoals in **Hoofdstuk 3** beschreven is kon in alle patiënten een sterke secundaire respons door T cellen tegen tetanus toxoïde gemeten worden, maar tegen geen enkel myeline preparaat.

Omdat veel GBS patiënten aan een voorafgaande infectie met *C. jejuni* hebben geleden is de *in vitro* T cel respons van gezonde donoren tegen deze bacterie beschreven in **Hoofdstuk 4**. Er werd een expansie van uitsluitend γδ T cellen gevonden na blootstelling aan een ruw sonicaat van *C.*

jejuni. De expansie was afhankelijk van de aanwezigheid van CD4+/□□+ T cellen in de kweek of toevoeging van exogeen IL-2 of IL-15. Stimulatie door *C. jejuni* vond plaats via de T cel receptor en bleek geïnduceerd te zijn door een bacterieel antigeen dat geen eiwit is maar waarschijnlijk een fosfoantigeen.

γδ T cellen van acute GBS patiënten met voorafgaande *C. jejuni* infectie reageerden helemaal niet, wat beschreven is in **Hoofdstuk 5**. GBS patiënten zonder voorafgaande *C. jejuni* infectie en patiënten met een *C. jejuni* infectie zonder GBS reageerden als gezonde donoren. In sommige patiënten duurde de γδ T cel inactivatie jaren nadat ze hersteld waren van GBS. Als de cytokines IL-2 of IL-15 werden toegevoegd aan de kweken resulteerde dit in herstel van de proliferatieve respons van γδ T cellen, wat suggereert dat de inactivatie in GBS patiënten te wijten is aan een tekort aan cytokineproduktie die nodig is om de γδ T cellen te activeren. T cel inactivatie en daaruit voortvloeiende defecte immuunregulatie zou een algemeen principe kunnen zijn dat tot auto-immuniteit kan leiden.

In **Hoofdstuk 6** wordt de aanwezigheid van *C. jejuni* DNA in witte bloedcellen van GBS patiënten en gezonde personen bestudeerd. Omdat continu aanwezig antigeen tot T cel inactivatie kan leiden zou de aanwezigheid van *C. jejuni* in GBS patiënten een verklaring kunnen geven voor de T cel inactivatie in GBS patiënten. Twee genen, het flagelline gen en het *CeuE* gen, werden gebruikt voor PCR identificatie van *Campylobacter* soorten. Het DNA geïsoleerd uit witte bloedcellen van ongeveer 30% van de gezonde donoren en 50% van de patiënten bevatte *C. jejuni* DNA, en dat was geverifieerd met Southern blot analyse of sequentiebepaling van het PCR product. Het bleek dat cellen die gesorteerd waren op CD14 of CD33 positiviteit *Campylobacter* DNA bevatten, wat betekende dat myeloïde cellen of monocyten de *Campylobacter* DNA bevattende cellen zijn. Deze resultaten gaven echter geen verklaring voor de in **Hoofdstuk 5** gevonden T cel inactivatie bij GBS patiënten.

Een hypothese voor de opeenvolging van gebeurtenissen die tot GBS leidt en die gevestigde ideeën combineert met nieuwe ontdekkingen zal nu volgen. Een infectie met een pathogeen dat ganglioside-achtige epitopen draagt is de eerste gebeurtenis in de ontwikkeling van GBS. Er zal eerst een normale immuunrespons volgen die bestaat uit een bacteriedodende of cytotoxische aangeboren immuunrespons, gevolgd door een verworven immuunrespons bestaande uit antilichaamproductie en T cel reactiviteit.

Om een tot nu toe onbekende reden wordt een subpopulatie van T cellen overgeactiveerd, gevolgd door inactivatie, of een bepaalde T cel populatie was al inactief voordat de infectie tot stand kwam. Als deze inactieve T cellen degene zijn die normaal gesproken regulerende T cellen zouden moeten ondersteunen, of als zij zelf regulerende functies zouden moeten vervullen

verandert de normale immuunrespons een pathogene die gekenmerkt wordt door een lange duur en verhoogde intensiteit. Hierdoor worden pathogene antilichamen en immuunstimulerende moleculen in grote hoeveelheden geproduceerd en komen zij in de perifere zenuw terecht waar complement- en macrofaagactivatie tot zenuwschade leidt.
In dit scenario is moleculaire mimicry alleen niet voldoende om auto-immuniteit te veroorzaken, maar het is wel een voorwaarde voor een pathogene respons. Slechts de combinatie van falende immuunregulatie en een epitoop op een pathogeen dat op een epitoop op perifere zenuw lijkt leidt tot GBS.
De vraag die meteen naar voren komt is naar de reden van inactivatie van T cellen. Mogelijke antwoorden zijn: 1) de infectie is zeer ernstig wat komt door een eigenschap van het pathogeen zelf, of 2) door onvoldoende afweer van de gastheer, wat beide leidt tot overactivatie en inactivatie, of 3) genetische gastheerfactoren of 4) overige immunologische factoren dragen bij aan T cel inactivatie. Het zou bijvoorbeeld van belang kunnen zijn welke immuunresponsen het immuunsysteem van de gastheer al voortgebracht heeft voordat de GBS veroorzakende infectie tot stand kwam.

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