Distinct time pattern of complement activation and cytotoxic T cell response in Guillain–Barré syndrome

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Summary
Humoral and cellular immune mechanisms are involved in the pathogenesis of Guillain–Barré syndrome (GBS). While activation of complement has been implicated in the initiation of myelin damage, we provide data here on the role of cellular cytotoxicity in GBS. Archival autopsy tissues including spinal roots, dorsal root ganglia and peripheral nerve were examined from 11 subjects who died 1 day to 8 weeks after onset of symptoms from GBS exhibiting a primary demyelinating pathology. In order to study the extent of humoral and cellular immune processes with regard to disease duration, a broad panel of antibodies to immunological and cellular markers was used to visualize the stage of demyelination, the deposition of complement components and expression of CD59, and to characterize cell infiltrates. Deposits of C9neo antigen on degenerating myelin sheaths were predominantly detected in acute cases. Expression of CD59 was upregulated on demyelinating fibres, but did not correlate with the presence of C9neo antigen or duration of disease. Quantitative analysis of endoneurial T cells showed a correlation between the density of CD3+ T cells per square unit and the degree of demyelination, but not with the duration of disease. The ratio of CD8+ to CD3+ T cells, however, was significantly increased in cases of GBS with a subacute course. Granzyme B positive lymphocytes and upregulation of MHC class I molecules on Schwann cells and myelin sheaths were detected in cases with more than 4 weeks disease duration. These findings implicate an important role of cytotoxic T cell responses for myelin damage in subacute stages of GBS.

Keywords: CD59; cytotoxic T-cell response; C9neo antigen; Granzyme B; Guillain–Barré syndrome

Abbreviations: GBS = Guillain–Barré syndrome; MAG = myelin-associated glycoprotein

Introduction
A cascade of both humoral (Saida et al., 1978; Hahn et al., 1980) and cellular (Lampert and Kies, 1967; Astrom et al., 1968; Hughes et al., 1981; Linnington et al., 1984; Hartung et al., 1990; Kiefer et al., 2001) immune responses against antigen epitopes on Schwann cells, myelin or axons causes the paralysis of Guillain–Barré syndrome (GBS). However, the extent of humoral and cellular immune responses leading to damage of the peripheral nervous system in GBS is incompletely defined. Evidence of complement activation has been found in serum (Koski et al., 1987) and CSF (Sanders et al., 1986; Hartung et al., 1987) of patients with GBS. In the presence of complement, sera from some patients with GBS exhibited demyelinating activity in vitro and in vivo (Saida et al., 1982; Harrison et al., 1984; Sawant-Mane et al., 1991, 1994). In addition, transient deposition of the terminal complement complex C5b-9 on Schwann cells and myelin sheaths has been shown in the early course of GBS (Hafer-Macko et al., 1996) and experimental allergic neuritis (EAN) (Stoll et al., 1991). The transient deposition of C5b-9 on Schwann cells precedes overt demyelination; therefore, a pathogenic role of complement in the initiation of immune-mediated myelin damage has been suggested. Formation and function of the complement system is regulated by several host proteins such as the membrane inhibitor of reactive lysis C9neo antigen (Vedeler et al., 1994; Koski et al., 1996) and hence protects Schwann cells from complement-dependent cytolysis (Sawant-Mane et al., 1996).

Apart from humoral immune responses, a decisive role of T cells in the pathogenic sequence of immune-mediated nerve...
damage was shown in EAN induced by P2 and P0 proteinspecific T cell lines (Linnington et al., 1984; Linington et al., 1992). T cell involvement in the pathogenesis of GBS is indicated by elevated levels of soluble interleukin-2 receptors in serum of GBS patients (Hartung et al., 1991) and increased frequencies of circulating activated T lymphocytes in these patients (Taylor and Hughes, 1989; Van den Berg et al., 1995). Post mortem and biopsy studies have established multifocal lymphocytic infiltration as the basis of histopathology of most cases of GBS (Asbury et al., 1969; Honavar et al., 1991; Schmidt et al., 1996), but insight into the role of T cells is still limited (Hartung et al., 2002). T cell activation may contribute in several ways to the pathogenesis of demyelinating neuropathies. Activated CD4+ T cells may help B cells to produce antibodies against peripheral nerve components, thereby inducing activation of either complement or antibody-dependent cytolytic cells, or may operate by recruiting macrophages to exert damage on peripheral nerve tissue. Activated CD8+ T cells or their soluble products can have a direct cytotoxic effect on myelin and Schwann cells or, alternatively, can play a role as suppressor cells in recovery from GBS (Hartung and Toyka, 1990). Activation of cytotoxic CD8+ T cells induces the expression of granule components, including perforin and granzymes, a series of serine proteases, which may trigger caspase-dependent and/or -independent pathways of cell death (Kagi et al., 1994; Santamaria, 2001).

Here we used archival autopsy material from 11 subjects who had acute or subacute GBS to study the deposition of complement components, the expression of the complement regulatory protein CD59, and the relative proportion and activation state of the CD8+ T cell subset with regard to disease duration and activity of the demyelinating process.

### Material and methods

#### Post mortem material

Autopsy material from 11 patients with GBS and four control patients without disease of the peripheral nervous system was investigated. Paraffin-embedded tissue blocks of spinal roots, dorsal root ganglia and peripheral nerve were retrieved from archival material collected between 1958 and 1999 at the Institute of Neurology, University of Vienna, and the Department of Pathology, University of Innsbruck, Austria. Spinal roots were examined in 10 GBS cases. In one GBS case only peripheral motor nerve was available. In five GBS cases dorsal root ganglia could also be studied. In all control patients, spinal roots and dorsal root ganglia were examined. The clinical characteristics of 10 patients (case nos 1–8, 10 and 11) have been reported previously (Maier et al., 1997), and fulfill standardized diagnostic criteria for GBS (Asbury et al., 1978). Patient 9 presented with a rapidly ascending tetraplegia and loss of tendon reflexes. Neurophysiological studies showed delayed distal motor latencies, absent F waves and severely reduced nerve conduction velocities consistent with a demyelinating neuropathy. CSF protein was elevated to 59 mg/dl and cell count was normal. Antiganglioside GM1 antibodies were detectable in serum and CSF. None of the cases had evidence of metabolic disturbance or toxin exposure. In all cases complete autopsy excluded systemic neoplastic disease or other systemic causes for the neuropathy (Maier et al., 1997). Demographic data, disease duration, treatment modalities (only four patients had been treated) and cause of death are summarized in Table 1. The disease course of GBS was defined as acute when patients survived less than 4 weeks after onset of the disease, and as subacute in patients with 4–8 weeks survival.

### Table 1 Patients' clinical data

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Duration (years)</th>
<th>Age</th>
<th>Sex</th>
<th>Steroid therapy</th>
<th>Plasmapheresis</th>
<th>Immunoglobulins</th>
<th>Antecedent event</th>
<th>Cause of death</th>
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<tr>
<td>GBS patients</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>1 day</td>
<td>78</td>
<td>F</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>2 days</td>
<td>67</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Tongue carcinoma</td>
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<tr>
<td>3</td>
<td>6 days</td>
<td>41</td>
<td>M</td>
<td>Yes</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Upper respiratory tract infection</td>
</tr>
<tr>
<td>4</td>
<td>9 days</td>
<td>78</td>
<td>M</td>
<td>Yes</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Influenza-like illness</td>
</tr>
<tr>
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<td>14 days</td>
<td>56</td>
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<td>–</td>
<td>Influenza-like illness</td>
</tr>
<tr>
<td>6</td>
<td>3 weeks</td>
<td>71</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Arterial bypass</td>
</tr>
<tr>
<td>7</td>
<td>4 weeks</td>
<td>79</td>
<td>M</td>
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<td>–</td>
<td>–</td>
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<td>Influenza-like illness</td>
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<tr>
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<td>5 weeks</td>
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<td>M</td>
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<td>69</td>
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<td>19</td>
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<td>–</td>
<td>–</td>
<td>Fever</td>
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<td>Control patients</td>
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<tr>
<td>1</td>
<td>62</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>2</td>
<td>75</td>
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<td>M</td>
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<td>–</td>
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</table>

M = male; F = female; NA = no antecedent event.
Histopathology and immunohistochemistry

Paraffin sections (4 μm thick) were routinely stained with haematoxylin/eosin, Kluever–Barrera stain for myelin and Bodian silver impregnation for axons. Immunocytochemistry was performed using an avidin–biotin or an alkaline phosphatase/anti-alkaline phosphatase technique as previously described in detail (Vass et al., 1986; Bien et al., 2002). The following primary antibodies were directed to CD45 (PD7/26+2B11, 1 : 10; Dako, Glostrup, Denmark), CD3 (polyAb, 1 : 50; Dako), CD8 (mAb, 1 : 100; Labvision, Fremont, CA, USA), CD20 (L26, 1 : 50; Dako), CD68 (KP1, 1 : 100; Dako), Ki-M1P (Ki-M1P, 1 : 10; Dr Wacker, Kiel Germany; Radzun et al., 1991), C3 (polyAb, 1 : 200; Department of Biochemistry, Cardiff, UK), C4 (polyAb, 1 : 300; Department of Biochemistry, Cardiff), C8 (polyAb, 1 : 200; Department of Biochemistry, Cardiff), C9 (MC47, 1 : 20; Department of Biochemistry, Cardiff), C9neo antigen (B7, 1 : 20; Department of Biochemistry, Cardiff), CD59 (MEM43, 1 : 1000; Monosan, Uden, The Netherlands), Fas (Apo-L, 1 : 50; Dako), FasL (sc-956, 1 : 100; Santa Cruz, Heidelberg, Germany), caspase-3 (CM-1, 1 : 3000; Srinivasan et al., 1998), Granzyme B (GZB01, 1 : 1000; NeoMarkers, Fremont, CA, USA), β2-microglobulin (polyAb, 1 : 200; Dako), myelin-associated glycoprotein (MAG) (B11F7, 1 : 4000; Dobersen et al., 1985) and P0 (1 : 100; Archelos et al., 1993). CD8 and Granzyme B staining was enhanced using biotinylated tyramine (King et al., 1997).

Lesional staging and quantitation of cellular infiltrates

Lesional activity was classified in all tissue blocks by the presence of myelin degradation products of different stages in macrophages (Brück et al., 1994). Immunoreactivity for the myelin proteins P0 and MAG visualized early myelin degradation products in actively demyelinating lesions. The amount of complement C9neo antigen deposition, expression of CD59 and β2-microglobulin, and the numbers of macrophages were assessed semiquantitatively. To quantify CD3+ and CD8+ T cells, 16 representative areas per section within the endoneurium were selected. Numbers of cells were counted with a 40× objective using an ocular morphometric grid, which covered an area of 0.0625 mm². The CD8+ to CD3+ ratios were calculated by determining the cell densities per mm².

Statistical analysis

For statistical evaluation, Spearman’s rank and Pearson’s correlation coefficients were used. P values <0.05 were considered as significant.

Results

Autopsy material from all 11 patients with GBS exhibited a primary demyelinating pathology characterized by inflammatory infiltrates (Fig. 1A), myelin disruption (Fig. 1B), mild to moderate axonal damage and endoneurial fibrosis. The degree of changes varied between the patients and lesions were multifocally distributed with rather ill-defined borders. The single exception was a case with 5 weeks duration, where large plaque-like areas of selective demyelination were sharply delineated. Early and late active demyelinating lesions were defined by the presence of myelin degradation products in macrophages immunoreactive for P0 (Fig. 1C) and/or MAG. In active demyelination macrophages were loaded with neutral lipids. Varying proportions of early and late active demyelination occurred in close proximity in severely affected cases of acute and subacute course. Extent of lesions and stages of demyelinating activity were not related to disease duration. The amount of inactive demyelination and associated axonal degeneration, however, was higher in cases with longer survival, as summarized in Table 2.

In controls, complement components C3, C4, C8 and C9 were diffusely present throughout the endoneurial space and labelled endothelial cells. In contrast, tissues from GBS cases showed greater amounts of complement deposition within the endoneurium, probably due to a disruption of the blood–nerve barrier. In addition, in GBS cases with <4 weeks duration complement deposition was accentuated along the surfaces of myelinated fibres, on mononuclear cells and at sites of active myelin breakdown (Fig. 1D). The C9neo antigen, a component of the terminal complement complex, was found on single plasmocytes scattered within the endoneurium in control tissues. In tissues of patients with GBS, distinct granular deposits of C9neo co-localized with degenerating myelin sheaths and macrophages (Fig. 1E) and were mainly detected in cases with an acute course. The membrane inhibitor of reactive lysis, CD59, was expressed on Schwann cell membranes, satellite cells in spinal ganglia and endothelial cells in GBS and control tissues. Notably, numbers of immunoreactive Schwann cells showed marked variability between fascicles. In some cases of GBS the expression of CD59 was increased at sites of florid myelin disruption (Fig. 1F and G), but this finding did not coincide with the presence of C9neo or the duration of disease.

In GBS tissues, inflammatory infiltrates, composed of macrophages and lymphocytes, were pronounced in dorsal root ganglia and spinal roots. In eight out of 11 cases monocytes/macrophages were the predominant cell type. All GBS cases exhibited varying densities of lymphocytic infiltrates in the endoneurial space, either clustered around blood vessels or scattered within fascicles. They consisted mainly of CD3+ T cells, whereas CD20+ B cells were either rare or absent. Quantification of CD3+ T cells and the CD8+ T cell subset revealed a high cell density in cases with extensive active demyelination. The degree of demyelination was significantly associated with the density of CD3+ T cells (r = 0.739, P < 0.01), but not with the density of CD8+ T cells (r = 0.326, not significant) or the duration of the disease (r = 0.063, not significant). However, duration of disease was...
correlated with the ratio of CD8+/CD3+ T cells ($r = 0.68, P < 0.05$) (Fig. 2). Only in subacute cases of GBS with extensive infiltrates did a portion of lymphocytes showed a vesicular staining pattern for Granzyme B. In a case with 5 weeks duration, Granzyme B positive cells were numerous, and lay in close apposition to neurons of the dorsal root ganglion (Fig. 3A). On corresponding sections these cells were identified as CD8+ T cells (Fig. 3B). Expression of β2-microglobulin, a constituent of the MHC class I molecule, paralleled increased CD8+/CD3+ T-cell ratios and was highly upregulated on endothelium, infiltrating cells and Schwann cells, and, notably, decorated myelin sheaths in subacute cases of GBS (Fig. 3C). FasL was expressed on a portion of macrophages and rarely on lymphocytes in GBS tissues, but

Fig. 1 (A) Mononuclear inflammatory infiltrates within the endoneurium of a spinal root (anti-LCA, 20× original magnification, case no. 9). (B) Severe myelin breakdown in a spinal root of a subacute GBS case (Klüver-Barrera, 20×, case no. 11). (C) Granular myelin degradation products immunoreactive for P0 (arrows) in an early active demyelinating lesion of a subacute GBS case (anti-P0, 40×, case no. 9); inset: myelin debris within macrophages (arrowhead, 100×). (D) Deposition of the complement component C9 on mononuclear cells and along myelin sheaths (arrowheads) in an acute GBS case (anti-C9, 40×, case no. 4). (E) Granular deposits of C9neo antigen on degenerating fibres in an acute GBS case (anti-C9neo antigen, 40×, case no. 4) and mononuclear cells immunoreactive for C9neo antigen (inset: high magnification of a degenerating fibre with C9 deposition, 100×). (F, G) Upregulation of CD59 on demyelinating fibres (anti-CD59, 40×, case nos 1, 10).
no Fas-positive cells were observed. In none of the cases did we find evidence for apoptosis of Schwann cells or lymphocytes by morphologic criteria or caspase-3 expression.

Discussion

There is consensus that both the humoral and the cellular immune system contribute to the pathogenesis of GBS. Most studies have addressed the role of humoral factors, because the beneficial effect of plasmapheresis (Osterman et al., 1984) and intravenous immunoglobulins (Van der Meche and Schmitz, 1992). The present study was performed on a series of 11 autopsy-confirmed GBS cases to investigate the pattern of humoural and cellular immune responses at acute and subacute stages of the disease. All cases of our series exhibited a primary demyelinating pathology. However, the extent of lesions and stages of demyelinating activity showed no correlation with disease duration. Importantly, active demyelination, as defined by the antigenic profile of myelin degradation products within macrophages (BruÈck et al., 1994), was still detectable at subacute stages. This observation indicates ongoing immune processes leading to sustained myelin damage in severely affected cases of GBS with a prolonged disease course. In our series, pronounced deposition of complement components along myelinated fibres and of C9neo antigen at sites of active myelin breakdown was mainly detected in acute GBS cases of less than 4 weeks duration. These findings are in line with previous studies that described a binding of C9neo antigen on peripheral nerve tissues in a single case (Koski et al., 1987) and a series of three very early cases of GBS emphasizing a role of complement activation in the initiation of immune-mediated myelin damage (Hulet et al., 1984).

In our series, pronounced deposition of complement components along myelinated fibres and of C9neo antigen at sites of active myelin breakdown was mainly detected in acute GBS cases of less than 4 weeks duration. These findings are in line with previous studies that described a binding of C9neo antigen on peripheral nerve tissues in a single case (Koski et al., 1987) and a series of three very early cases of GBS emphasizing a role of complement activation in the initiation of immune-mediated myelin damage (Hulet et al., 1984).

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The presence of CD59 on various cells of the human nervous system suggests a functional role of CD59 in neural host defense mechanisms (Vedeler et al., 1994; Koski et al., 1996). In accordance with experimental findings (Vedeler et al., 1999), we observed upregulation of CD59 at sites of active myelin disruption on the human material, which supports the protective role of CD59 in inflammatory diseases of the peripheral nervous system. However, we were unable to demonstrate upregulation of CD59 concurrent with deposition of C9neo antigen and no obvious relationship with disease duration was seen.

Evidence of sustained tissue damage in cases with a subacute course of GBS, as reflected by the finding of actively demyelinating lesions without, or with only minute, detectable amounts of C9neo antigen, emphasizes the importance of cellular immune mechanisms in the development and/or perpetuation of the inflammatory disease process. In contrast to the well-defined functions of macrophages during the effector and recovery phases of autoimmune neuropathies (Kiefer et al., 2001), the functional role of T cells that constitute a major component of the nerve lesion in GBS is largely unknown (Hartung et al., 2002). Controversy exists with respect to the degree and evolution of T cell infiltration at various stages of demyelinating neuropathies (Asbury et al., 1969; Mancardi et al., 1988; Cornblath et al., 1990; Honavar et al., 1991; Schmidt et al., 1996; Bosboom et al., 1999). Recently, γδ T cells, which are capable of recognizing non-protein antigens such as lipopolysaccharides, were isolated from a patient with GBS following infection with Campylobacter jejuni (Winer et al., 2002). The presence of T cells of a γδ T cell receptor phenotype in peripheral nerve tissues of GBS patients and expansion of human peripheral blood γδ T cells after exposure to C. jejuni in vitro (Van Rhijn et al., 2003) suggest that T cell reactivity against gangliosides may play a pathogenic role in GBS. Furthermore, a differential expression pattern of specific chemokines and chemokine receptors required for selective recruitment of inflammatory cells in inflamed peripheral nervous system tissues underlines possible cellular immune responses in human demyelinating neuropathies (Kieseier et al., 2002). Despite these accumulating data, clear evidence of Schwann cell lysis mediated by cytotoxic T cells is lacking (Hartung et al., 2002).

In our series of GBS patients, the density of endoneurial CD3+ T cells correlated with the extent and stage of demyelination. This finding is in contrast to previous reports,

**Fig. 3** (A) Dorsal root ganglion with abundant Granzyme B+ lymphocytes in close apposition to neurons (anti-Granzyme B, 40×, case no. 9); inset: high magnification of Granzyme B+ cells, 100×. (B) On corresponding sections, the lymphocytes are identified as CD8+ T cells (anti-CD8, 40×, case no. 9). (C) Upregulation of β2-microglobulin on Schwann cells, mononuclear cells and myelin sheaths (inset, 100×) in the same subacute GBS case (anti-β2-microglobulin, 40×, case no. 9). (D) Weak expression of β2-microglobulin on myelin sheaths (arrows) in an acute GBS case (anti-β2-microglobulin, 40×, case no. 1). (E) In an uninfammed spinal root of a control case, expression of β2-microglobulin is seen on blood vessel endothelia and endoneurial macrophages, but not on myelin sheaths (anti-β2-microglobulin, 40×).
demonstrated reactivity of Schwann cells for lymphocytes expressing Granzyme B, identified as CD8+ T cells, within the respective lesions suggest that they can contribute to tissue damage in GBS. Thus, the most salient observation of the current study was the detection of CD8+ T cells and tissue damage in the peripheral nervous system of patients with sepsis or critical illness neuropathy (unpublished data).

In conclusion, humoral immune responses appear to predominate at early stages of GBS. Our data suggest that at later stages additional cellular mechanisms mediated by cytotoxic T cells contribute to myelin damage via granzyme release. Thus, if standard treatments such as intravenous immunoglobulins or plasmapheresis (Hughes, 2002; Van Doorn and Garssen, 2002) are not sufficiently effective in severe GBS cases with a prolonged disease course and persistent symptoms, therapeutic approaches that target cellular immune responses (Rosen and Vastola, 1976; Korn et al., 2001) might be considered.

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References


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