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SYMPOSIUM: Peripheral Neuropathies

Mechanisms of Immune Regulation in the Peripheral Nervous System

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The peripheral nervous system (PNS) is a target for heterogenous immune attacks mediated by different components of the systemic immune compartment. T cells, B cells, and macrophages can interact with endogenous, partially immune-competent glial cells and contribute to local inflammation. Cellular and humoral immune functions of Schwann cells have been well characterized *in vitro*. In addition, the interaction of the humoral and cellular immune system with the cellular and extracellular components in the PNS may determine the extent of tissue inflammation and repair processes such as remyelination and neuronal outgrowth. The animal model experimental autoimmune neuritis (EAN) allows direct monitoring of these immune responses *in vivo*. In EAN contributions to regulate autoimmunity in the PNS are made by adhesion molecules and by cytokines that orchestrate cellular interactions. The PNS has a significant potential to eliminate T cell inflammation via apoptosis, which is almost lacking in other tissues such as muscle and skin. *In vitro* experiments suggest different scenarios how specific cellular and humoral elements in the PNS may sensitize autoreactive T cells for apoptosis *in vivo*. Interestingly several conventional and novel immunotherapeutic approaches like glucocorticosteroids and high-dose antigen therapy induce T cell apoptosis *in situ* in EAN. A better understanding of immune regulation and its failure in the PNS may help to develop improved, more specific immunotherapies.

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Introduction

The peripheral nervous system (PNS) was counted among the special immuno-privileged tissues like retina, cornea, anterior chamber of the eye, testis, and liver (105). This concept of immune privilege, originally formulated by Medawar (79) defined the protection of tissue grafted to certain sites. Later it was extended to describe seclusion of particular areas of the body from the systemic immune compartment. Some ten years ago it was shown that indeed immune surveillance is operative in such tissues (113). Even vulnerable privileged sites like the nervous system are constantly patrolled by activated T lymphocytes. Hence specialized anatomic barriers like the blood-brain or blood-nerve barrier (31) or the absence of lymphatic drainage do not necessarily guarantee the integrity of these sites. Although the concept of immune privilege then had to be abandoned, it is beyond doubt that these tissues possess specialized mechanisms of immune protection, which ensure rapid and gentle elimination of inflammation once this has encroached on there. Here we summarize pathways of inflammation which are operative in the PNS and describe intrinsic counterregulatory mechanisms that contribute to termination of the assault. Both *in vitro* data obtained on glial cell cultures and *in vivo* experiments in experimental autoimmune neuritis (EAN) are discussed.

Experimental autoimmune neuritis (EAN) - a model to study immune regulation *in vivo*

Experimental autoimmune neuritis (EAN) is an animal model mediated by autoantigen-specific T-cells for human Guillain-Barré syndrome (GBS), an acute demyelinating inflammatory disease of the peripheral nervous system (PNS), (reviewed in (46). EAN can be actively induced in Lewis rats by immunization with bovine P2-protein or recombinant human P2-protein, with a peptide (amino acid 53-78) spanning the neuritogenic epitope, or by adoptive transfer of neuritogenic T-cells (AT-EAN) (47, 74). Further autoantigens that have

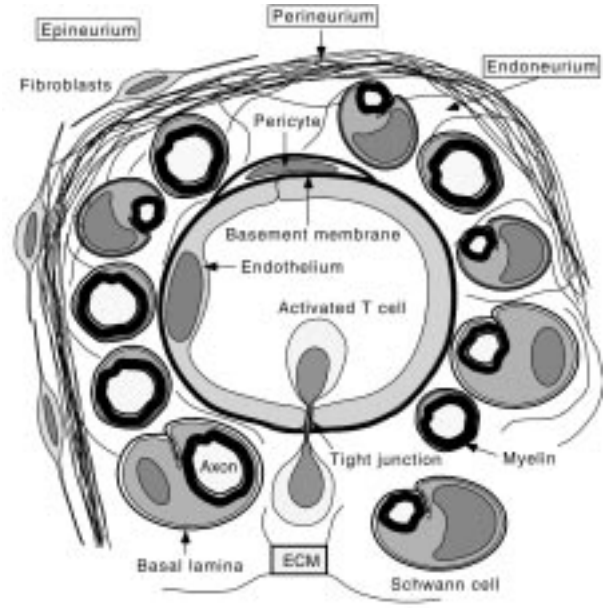

Figure 1. *The intact blood-nerve barrier (BNB) and the extracellular compartments in the PNS.* In GBS activated T cells and macrophages enter the PNS at the interface between the capillary compartment and the neural environment. Three anatomical compartments with a different cellular and ECM composition can be distinguished in the PNS: epineurium, perineurium and endoneurium. The epineurium surrounds the perineural ensheathment of the fascicles and contains blood vessels, lymphatics, fibroblasts, collagen type I bundels and fat. The perineurium consists of a lamellar arrangement of flattened perineurial cells, covered by BM and interspersed collagen fibrills and tenascin-C. Contiguous perineurial cells are linked together by tight junctions. They constitute the morphological basis of the perineurial diffusion barrier. The endoneurium is composed of collagen type I and III fibrills, and embeds blood vessels, axon-Schwann cell units, fibroblasts, and occasionally macrophages. The basal lamina surrounding Schwann cells is composed of classical ECM proteins such as collagen type IV, fibronectin, laminins, vitronectin, entactin, and heparan sulfate. These are secreted by the Schwann cell. The BNB consists of an endothelial cell lining with tight junctions, a basement membrane (BM), the pericytes and is less tight than the blood-brain barrier. Depending on the etiology, T cells and macrophages have a different distribution in these peripheral nerve compartments in human immune-mediated neuropathies.

been identified in EAN models in rats and mice are P0 protein (75), myelin basic protein (MBP) (1), possibly PMP22 (32) and galactocerebroside in rabbits. EAN replicates many clinical, electrophysiological and immunological aspects of the Guillain-Barré syndrome and hence has been widely used as a model to investigate disease mechanisms (50).

The immune response in EAN can be dissected into an induction and an effector phase. Adhesion molecules (AMs) are critically involved into these different phases (3, 21). In the induction phase the injected autoantigen is presented to "naive" T cells by professional antigen-presenting cells (APC) such as macrophages or dendritic cells resulting in T cell activation. Two external signals are crucially required for an effective T cell activation by antigen presentation: the antigen-specific signal provided by the immunogenic peptide and presented in the context of major histocompatibility complex molecules on APC, and the antigen-independent signal called costimulation and mediated by AMs of the integrin family such as $\alpha L\beta 2$ (LFA-1, CD11a/CD18), $\alpha 4\beta 1$ (VLA-4, CD49d/CD29), or $\alpha v\beta 3$ (CD51/CD61) and AMs of the immunoglobulin-superfamily such as ICAM-1 (CD54), VCAM-1 (CD106), CD2, CD58, and, of special functional relevance, CTLA-4 (CD152), B7-1 (CD80) and B7-2 (CD86) expressed both on T cells and APC.

Activated T cells then circulate in the blood, attach to the venular endothelium in the PNS and penetrate the blood-nerve barrier (BNB). This transendothelial migration gives rise to the effector phase of the immune response in EAN. In the PNS the autoantigen is presented by macrophages to T cells. The reactivated $CD4^+$ T cells amplify the immune response by recruiting further T cells and macrophages via chemokines and cytokines (51). The resulting breakdown of the BNB allows the passage of circulating autoantibodies which are thought to synergize with T cells to produce demyelination (52, 89).

Adhesion molecules in the induction phase of EAN

Antigen presentation is pivotal in T cell activation. The first important step in the pathogenesis of EAN is the presentation of autoantigen in the induction phase. This results in the physiological activation of disease-inducing T cells (51). AMs are essential for effective antigen presentation *in vitro* in rodents and humans. *In vivo*, inhibition of some of these coaccessory molecules such as CD2 (60), ICAM-1 (6) or LFA-1 (5) by monoclonal antibodies (mAbs) prevents adequate T cell activation and attenuates EAN in the rat.
After activation, T cells circulate and finally enter the target organ at the blood-nerve barrier (BNB)(Figure 1). In the acute phase of EAN, upregulation of AMs such as ICAM-1 and VCAM-1 at the endothelial tight junctions on lesion-associated blood vessels parallels clinical disease and parenchymal infiltration (30, 53, 103).

Central role of transendothelial migration in lesion formation. The transendothelial migration of immune

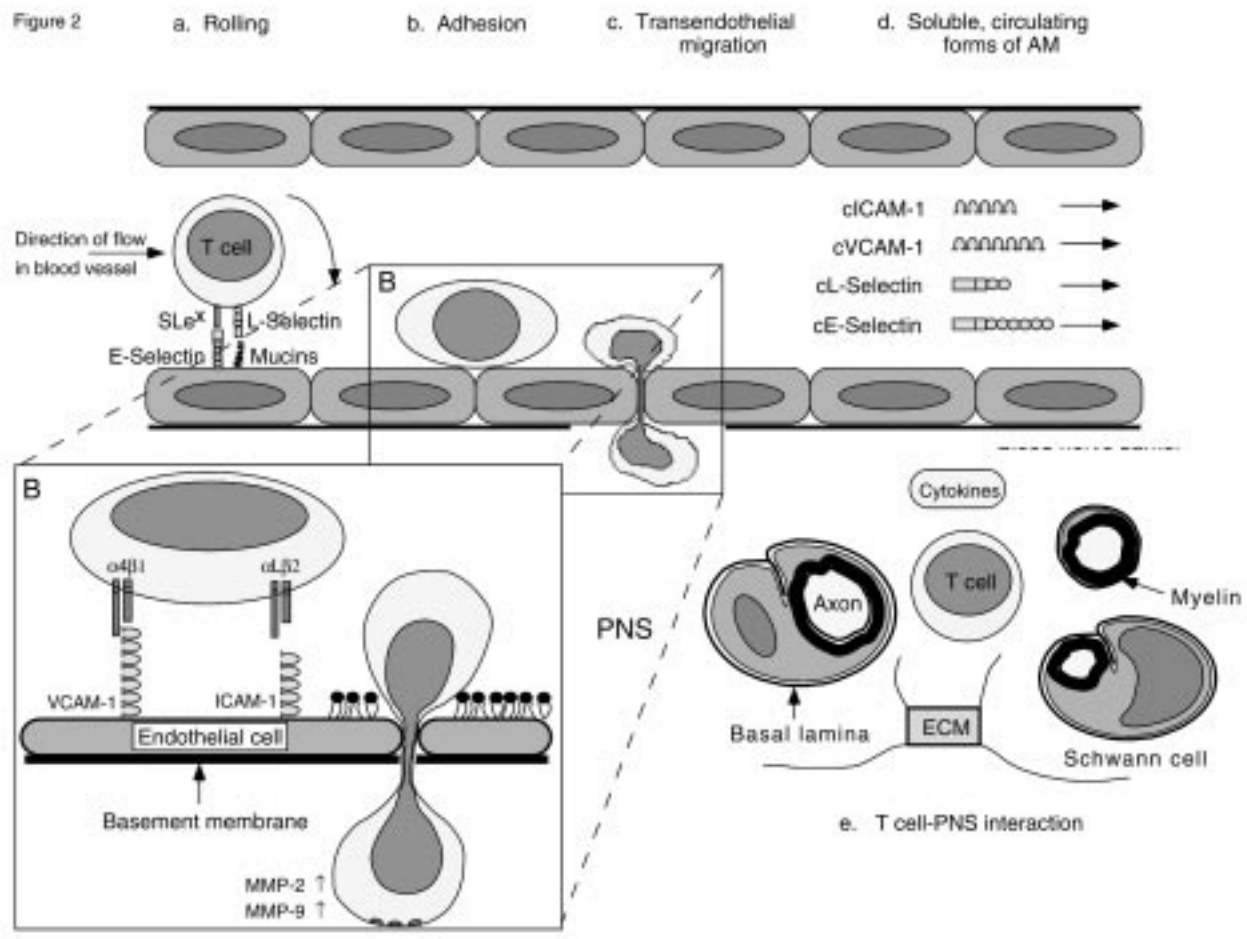

Figure 2. *Transendothelial migration of activated T cells across the BNB in EAN is mediated by integrins.* Through the action of selectins - a family of cell adhesion molecules - T cells establish a loose reversible contact with endothelial cells. This initial rolling is followed by a firm irreversible adhesion mediated by integrins $\alpha 4\beta 1$ (VLA-4) and $\alpha L\beta 2$ (LFA-1) on T cells and their immunoglobulin-like receptors on endothelium (VCAM-1, ICAM-1). Locally released chemokines (black circles) bound to endothelial glycoproteins (glycocalyx) increase integrin adhesiveness, and induce directed movement of T cells and monocytes. The subsequent transendothelial migration of T cells through the BNB is thought to be mediated mainly by a4b1/VCAM-1. Engagement of $\alpha 4\beta 1$ with VCAM-1 activates matrix metalloproteinases (MMP) which degrade certain ECM proteins.

The extracellular domain of VCAM-1, ICAM-1 and selectins is shed from the cell surface after T-cell-endothelial-cell interaction, and soluble forms circulate in the blood and cerebrospinal fluid (cVCAM-1, cICAM-1, cL-selectin, cE-selectin). Physiological levels of circulating forms of integrin ligands increase with pathology. T cells that have migrated into the PNS interact with local cellular and ECM components. The nature of this complex interaction determines the outcome of the immune response in the target tissue.

cells through the BNB is a crucial step in the pathogenesis of immune-mediated disease of the PNS. The presence of a variety of AMs on mononuclear cells and the upregulation of their ligands on endothelial cells during active disease point to a common pathogenetic role of AM in the initiation of tissue inflammation in EAN. This notion was reinforced by therapeutic manipulation with mAbs *in vivo*. Archelos and colleagues were the first to delineate the involvement of ICAM-1, LFA-1 and recently, L-selectin (4) in transendothelial migration in EAN. Enders *et al*. extended their findings on VLA-4 and its ligand VCAM-1 (30) (Figure 2). Blockade of VLA-4 ($\alpha 4\beta 1$) by mab was effective in EAN (Figure 3) and this integrin seems to be the most important AM in transendothelial migration of T cells in rodent EAN and hence the most promising target candidate for therapeutic intervention in GBS. Surprisingly VLA-4 and VCAM-1 blockade are equally effective in enhancing T cell apoptosis in the inflammatory lesion (Gold, Lobb in preparation).

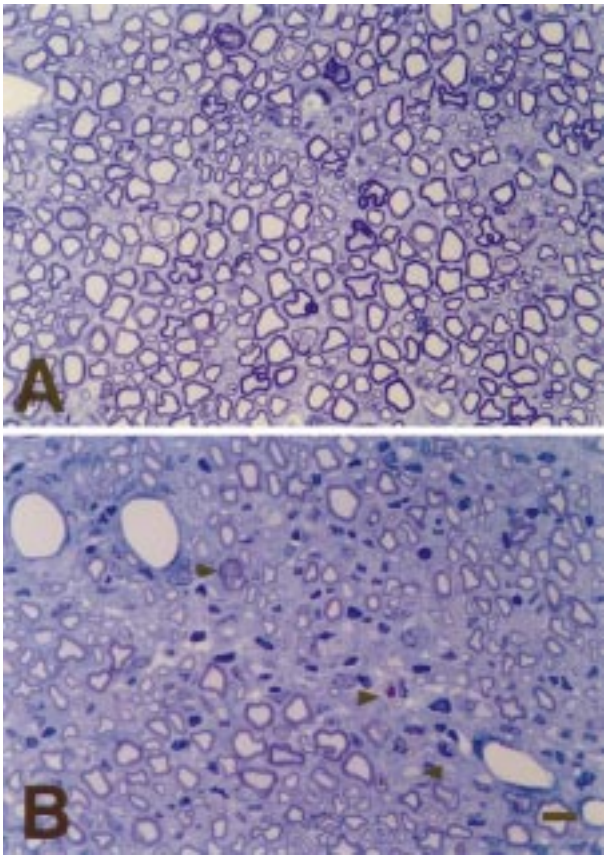

Figure 3. *Morphological analysis of adoptive-transfer (AT)-EAN treated with the VLA-4 specific mAb TA-2.* Detection of axonal damage and demyelination in semithin sections from sciatic nerves of the anti-VLA-4 group (**A**) and from control animals (**B**) receiving isotype control only. Arrowheads indicate Wallerian degeneration in B. Bar = 10 μm for A-B

Triggering of autoreactive T and B cell responses

The mechanisms underlying generation of autoreactive T cell and B cell responses are clear in the EAN model where the autoimmune cascade is deliberately and artificially set off. In both acute and chronic immune neuropathies in man, the conditions allowing emergence of peripheral nerve directed autoreactivity remain obscure.

It is conceivable that myelin-reactive T lymphocytes are contained in the immune repertoire of healthy individuals. Under normal conditions, they are inactive or silenced by various modes of peripheral tolerance. Tolerance may break down when the individual is confronted by an infective organism that happens to share epitopes with endogenous peripheral myelin proteins such as P0 or P2. Such mimicry has been hypothesized to underlie GBS associated with cytomegalovirus infection (2, 50b, 50c). Given the degeneracy of epitope recognition by T lymphocytes this notion may be disputed. Another possibility is antigen nonspecific T cell activation mediated by cytokines (13).

This hypothesis may be viewed as a revival of the time-honored concept of bystander demyelination. Evidence to support a variation on this theme is available.

The possibility of nerve injury mediated by activated, non-neural specific T cells was studied by systemic transfer of ovalbumin-specific T cells, followed by intraneural injection of ovalbumin (52, 89). Rapid endoneurial infiltration of T cells and macrophages occured on the side injected with ovalbumin and was associated with a marked increase in blood-nerve barrier permeability. In the casein-injected control nerve only degeneration and macrophage infiltration was observed that declined after 3 days. Histological and clinical features in this model were similar to those observed in P2-induced EAN. When given in combination with anti-myelin antibodies primary demyelination or axonal degeneration was demonstrable by electrophysiological studies, thus replicating typical features of GBS.

Molecular mimicry based on structural similarities between microorganisms and peripheral myelin may be more important for the generation of autoreactive B cell responses. There is a large body of evidence indicating that infection with certain strains of the gramnegative enteropathogen *Campylobacter jejuni* elicits antibody responses directed to gangliosides and related glycolipids on the myelin membrane and axolemma since carbohydrate sequences of these glycoconjugates are also contained in the lipopolysaccharide fraction of the microorganism (Figure 4) (47, 50c,117).

Adhesion molecules in the effector phase of EAN

Once T cells and monocytes have passed the blood-nerve-barrier they adopt to a completely different microenvironment in the PNS. There neural cells such as Schwann cells and resident macrophages and the extracellular matrix harbor relevant ligands for receptors on infiltrating immune cells. In this review we will focus on the role of AM with special emphasis on integrins expressed in the PNS and with the immune-regulating and inflammation terminating capacities of Schwann cells and macrophages.

In EAN, infiltrating macrophages express ICAM-1 (103) whereas T cells display on their surface VLA-4 (30) and LFA-1 (5) but the *in vivo* adhesion molecule phenotype has not yet been studied in detail. Matrix metalloproteinases (MMPs) which degrade extracellular matrix (ECM) molecules are upregulated on T cells

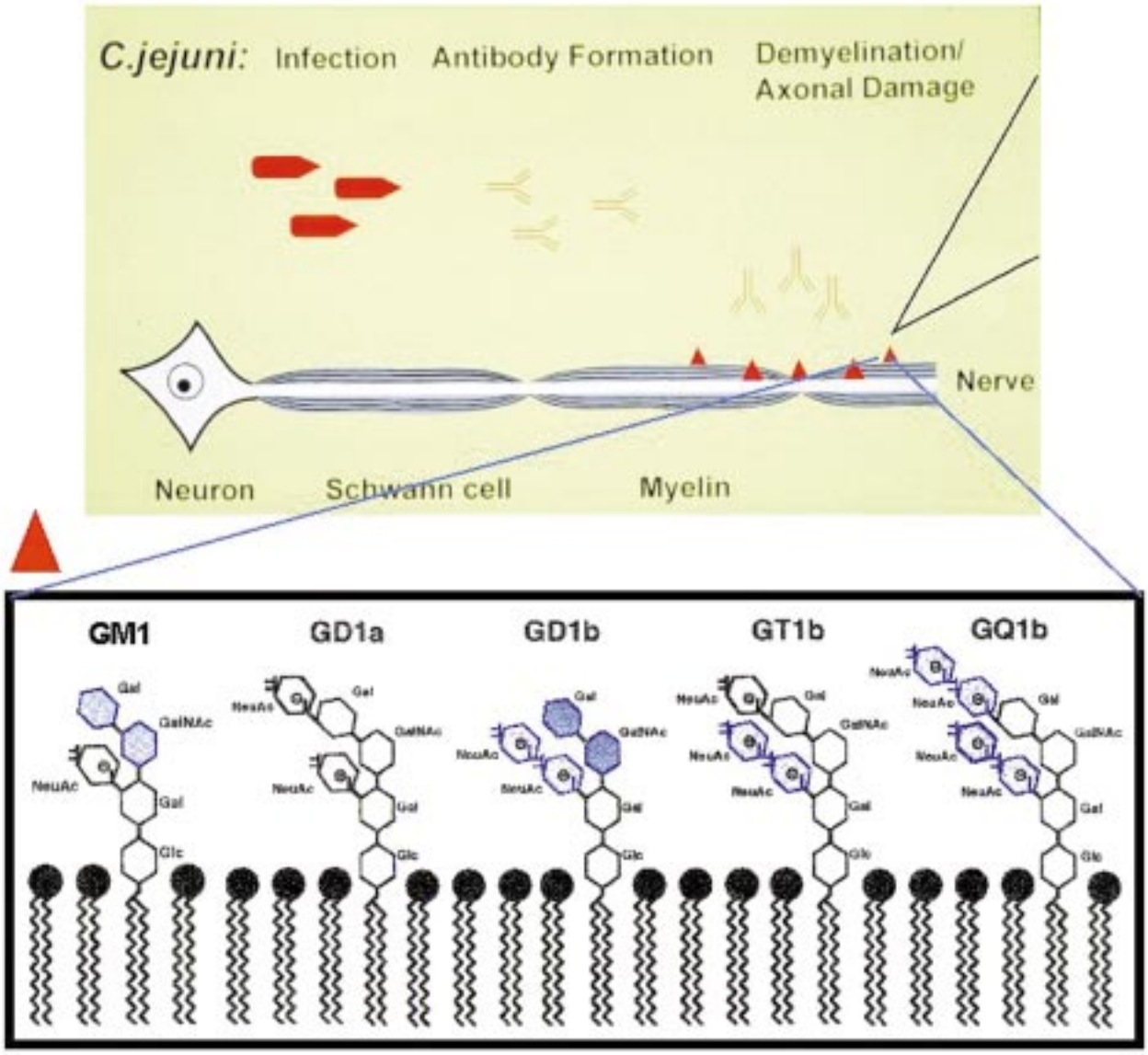

Figure 4. *Molecular mimicry: Cross-reactive antibody responses to C.jejuni and peripheral nerve glycoconjugates.* Approximately 30% of all Western patients with Guillain-Barré Syndrome develop the neuropathy some 1 - 3 weeks after infection with the gram-negative enteropathogen C.jejuni. The lipopolysaccharide fraction of serovars of C.jejuni associated with GBS contains epitopes shared with glycolipids present in the myelin sheath and axolemma of peripheral nerve. It is conceivable that cross-reactive antibody responses ("molecular mimicry") underlie nerve damage in C.jejuni-related Guillain- Barré Syndrome. Modified from (50c, 91b).

(65). In addition, there is growing evidence that AMs of the integrin family and their ECM receptors in the PNS are important in the effector phase and may contribute to tissue repair (7, 9).

Demyelination and axonal loss modulate integrins in EAN. In myelin-induced EAN, an EAN variant characterized by severe inflammation, prominent demyelination and axonal loss, the expression of integrins follows a distinct spatiotemporal pattern (90). Interestingly, integrin expression on Schwann cells is associated with distinct histopathological alterations in EAN and GBS (Figures 5, 6). Additional *in vitro* studies and observations from human non-inflammatory neuropathies (see below) suggest that integrin expression is modulated by several factors in the PNS. Demyelination, disruption of the basal lamina, and axonal loss appear to override some of the cytokine

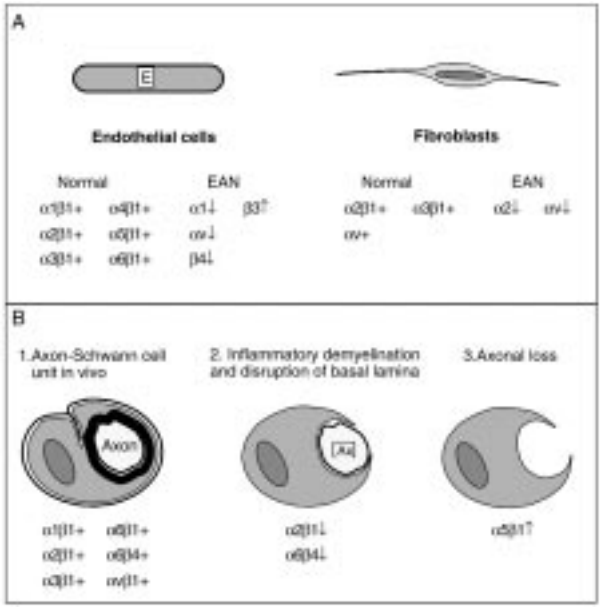

Figure 5. *Expression of integrins (**A**) on endothelium and fibroblats and (**C**) on Schwann cell/axon units in experimental autoimmune neuritis (EAN) and Guillain-Barré syndrome (GBS).* (**A**) Endothelial cells at the BNB express a variety of integrins in normal rats. In acute EAN, there is a up- and down-regulation of distinct integrin subunits on endothelial cells, Schwann cell/axon units and fibroblasts. Inflammatory demyelination and concomitant disruption of the basal lamina is associated with a transient downregulation of $\alpha 2\beta 1$ and $\alpha 6\beta 4$ in Schwann cells. If additional axonal loss is present due to severe inflammation a neo-expression of $\alpha 5\beta 1$ is observed. Integrin expression normalizes with progressive tissue repair in EAN. A similar pattern is observed in sural nerve biopsies obtained from patients with GBS.

effects on the expression of integrins by Schwann cells found *in vitro* (90).

AMs in immune-mediated neuropathies

The pathogenesis of GBS and other inflammatory neuropathies is only partially known. The nature and characteristics of the induction phase remain elusive and it is still obscure if the immune cells relevant for the disease exhibit a defined phenotype and specificity. Therefore, descriptive studies on AM in these diseases had to focus on T cells in general, the blood-nerve barrier and the repair phase (50,90,97).

Expression of AMs in GBS and human neuropathies. In GBS, we confirmed the association of the downregulation of $\alpha 6\beta 4$ integrin with inflammatory demyelination and the neo-expression of integrin subunit $\alpha 5$ with additional axonal loss, as was previously shown in EAN (90). Similarly, a neo-expression of $\alpha 5$ on Schwann cells and a transient downregulation of $\beta 4$ have been observed in non-inflammatory chronic axonal neuropathies in humans (91, 93). These studies in human neuropathies indicate that in the PNS structural features such as the presence or absence of a basal lamina, demyelination or axonal loss are important co-determinants of integrin regulation and that an altered integrin expression is involved in the repair process as suggested by studies in animal models of regeneration and inflammation (71, 90).

It is well known that some immune molecules are cleaved from the cell surface after ligand engagement at the BNB. Such shed molecules circulate in blood and are associated with disease severity in some cases. Circulating fragments of integrin $\alpha 6\beta 4$-like immunoreactivity have been detected in GBS and suggested to be a putative novel marker of myelin damage (102). Indeed, circulating integrin subunits would be helpful sensors of disease activity, but molecular identification of the $\alpha 6\beta 4$-like immunoreactivity in serum is still needed to exclude a casual crossreactivity with other proteins.

In neuropathies associated with vasculitis - an inflammatory reaction at the arterioles supplying the nerves - increased levels of integrin receptors ICAM-1 and VCAM-1 were noted on endothelial cells in the PNS and infiltrating cells were positive for $\alpha L\beta 2$ and $\alpha M\beta 2$ (17, 85). Further, raised levels of E-selectin, an AM expressed on activated endothelium, were measured in the blood of these patients (48).

Integrins and the effector phase in GBS

The complexity of the interactions between neural and immune cells, ECM and humoral inflammatory mediators such as antibodies, cytokines, chemokines, matrix metalloproteinases and trophic factors in the development of the demyelinating lesion in GBS make it difficult to study the role of AM such as integrins in the repair phase *in vivo*. However, abundant *in vitro* data combined with the described expression pattern of integrins *in vivo* suggest a possible central role of integrins in the genesis of the inflammatory lesion (7).

Integrins, as ECM receptors, may be involved in this repair process. Preliminary experimental evidence indicates that the complex processes of repair can be targeted therapeutically. Treatment of rats with a monoclonal antibody to the $\alpha 1$ subunit attenuates EAN and reduces immune cell infiltration and demyelination (own unpublished observation).

The obvious involvement of adhesion molecules in

the pathogenesis of EAN and GBS has relevant clinical implications (81). Antibody targeting of transendothelial migration of T cells governed by α4β1 provides a realistic novel therapeutic option in GBS (72).

Local immune activation - the role of Schwann cells

Cellular immune functions. The contribution of Schwann cells to the initiation and termination of an immune response in the PNS is still a matter of debate. In principle it has been shown that they possess immune molecules as a basic prerequisite to interact with invading T cells. Schwann cells *in vitro* constitutively express at low levels MHC class I but no significant numbers of MHC class II molecules. Upregulation of MHC class I or expression of MHC class II can be induced by stimulation with interferon-γ (IFN-γ) or upon coculture with activated T cells (8, 66). Interestingly, tumor necrosis factor-alpha (TNF-α) synergizes with IFN-γ and further increases MHC expression which also has functional importance (40). Moreover adhesion molecules such as ICAM-1 (CD54), which are constitutively expressed on cultured Schwann cells are upregulated by these cytokines and may exert costimulatory functions. Thus pretreated, Schwann cells have been shown to process exogenous P2 protein or its neuritogenic peptide for antigen presentation (40). Under certain conditions they can even present endogenous myelin proteins like myelin basic protein (MBP) to autoaggressive lymphocytes in a MHC class II restricted manner (114). In that case Schwann cells were prepared from older animals where myelination was already ongoing. These findings underscore that Schwann cells are able to phagocytose exogenous antigens and degrade them to antigenic peptides in endosomal compartments (8). Although the relevance of MHC class II expression on Schwann cells has been questioned in EAN (96) (see below), there is principle evidence that Schwann cells are capable of this immune function *in vivo* (14, 88). A possible explanation for this discrepancy between the in-vitro and *in vivo* situation may be provided by the findings of Tontsch *et al*. (108) and Neumann *et al*. (83) who have demonstrated that neurons indeed exert a regulatory function on glial cells.

It is of note that Schwann cells are also the source of a number of proinflammatory cytokines such as IL-1, IFNa and TNFa. Given the autocrine and paracrine functions of these molecules both feedback actions on Schwann cells and modulation of the pericellular immune circuitry are conceivable (76).

Schwann cells also express molecules that can terminate T cell inflammation and downregulate immune functions. Fas and its ligand are central molecules of a family of death factors that regulate T cell survival in the immune system (see review in (82). Recent findings from our group show that Schwann cells do not express Fas (CD95) or Fas ligand (FasL) constitutively. However, proinflammatory Th1 cytokines can upregulate FasL or Fas on the surface of Schwann cells within 48 hrs (Wohlleben, *et al*., manuscript in preparation - Figure 7). Thus several potential scenarios are conceivable. First, crosslinking of Fas molecules on invading T cells by membrane-bound or secreted FasL could eliminate the autoaggressive immune effectors. This could explain T cell apoptosis observed during the natural disease course of EAN (118); see below). Second, expression of Fas on Schwann cell membranes could render them susceptible to T cell attack. Apoptotic elimination of Schwann cells is observed during EAN (26b, Brück, Gold; unpublished) and may further augment demyelination in the PNS. The ultimate functional aspect of the expression of Fas/FasL on Schwann cells is not yet understood and currently under investigation.

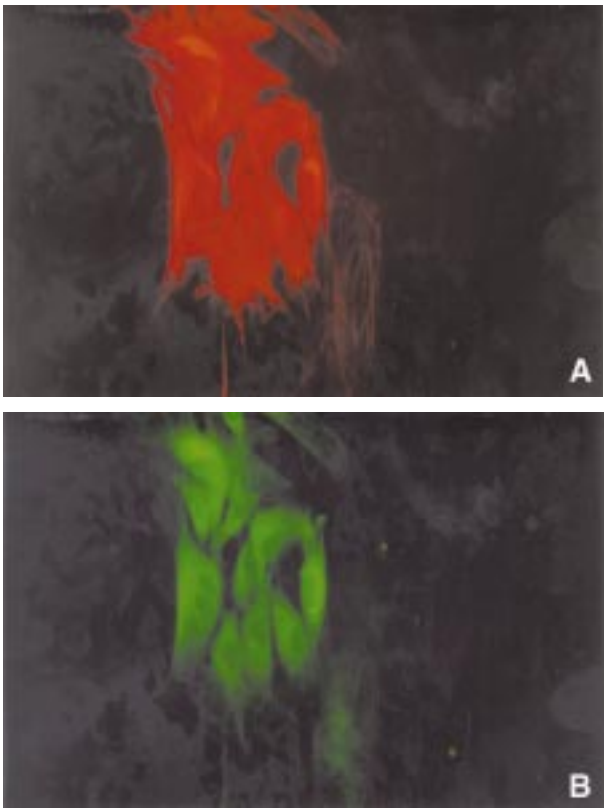

Figure 6. *Immunofluorescence detection of S100 (**A**) and α2 (**B**) on Schwann cell cultures.* Schwann cells were purified from newborn rat sciatic nerves. Magnification: A, B: x 340.

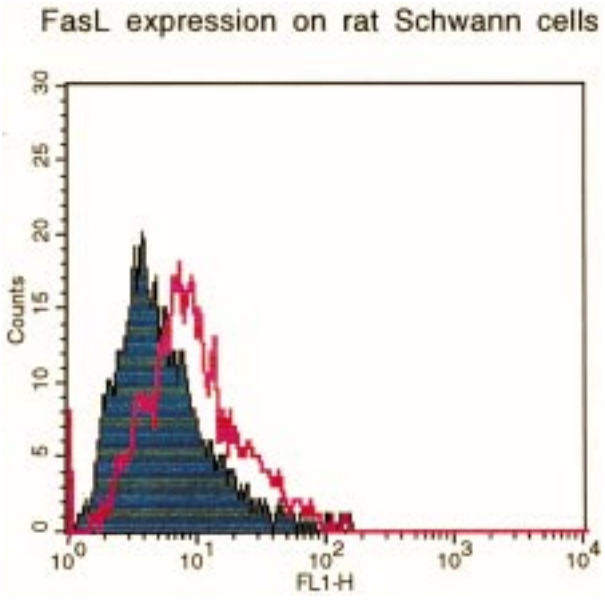

Figure 7. *FACS analysis of Fas ligand on rat Schwann cells.* Neonatal Schwann cells were treated for 2 days with 300 IU/ml IFNγ and TNFα. FACS analysis was performed using a biotinylated anti-rat FasL IgM antibody (red line) and an isotype control (green area). A weak but significant upregulation of FasL can be observed.

Humoral immune functions. Apart from these functions which are mediated by and dependent on cell-cell contact, Schwann cells may modulate local immune reactions in the PNS through the elaboration and release of humoral factors. Schwann cells produce IL-1, a potent cytokine which promotes T cell activation and proliferation (15), and also IL-6 which may bias the local cytokine milieu to a T helper 2 (Th2) type of reaction (16). Furthermore, Constable *et al*. (26) found that Schwann cells can be induced *in vitro* to secrete prostaglandin E2 and thromboxane A2. These immunomodulators can inhibit or stimulate T cells, depending on their level of production. Schwann cells are also endowed with a cytokine-inducible nitric oxide synthase (iNOS) (41). Simultaneous treatment with IFN-γ and TNF-α upregulates iNOS-specific mRNA in Schwann cells within 12 hrs (Figure 8). Nitrite secretion as a measure of NO production was detectable after 24 hrs and reached its plateau on day 3. Nitrite release was inhibited by N-monomethyl L-arginine, a competitive inhibitor of iNOS and not by N-nitro L-arginine, which preferentially blocks the noninducible NO-synthase. This finding ruled out unspecific release of NO by Schwann cell cultures and underscored the presence of a macrophage-like, inducible form of NO synthase. Secretion of NO by Schwann cells exerts a strong suppressive effect on T cell activation in a coculture model (41). This mechanism has been proven *in vitro* where reactive nitric oxide induced apoptosis of autoreactive T cell lines (119). Thus, Schwann cell-derived reactive oxygen intermediates have the potency to limit inflammatory demyelination. Importantly exogenous IL-2 could rescue T cells from apoptotic cell death *in vitro* (see below). Survival of T cells exposed to IL-2 could occur via upregulation of antiapoptotic molecules of the bcl-2 family (23) and provide an important counterplayer to local proapoptotic elements.

It is interesting to consider that in the local tissue milieu Schwann cells are in close contact with endoneurial fibroblasts, which have been shown to release interferon-beta (IFN-β). This could in turn downregulate NO production of Schwann cells, very similar to recent experiments with glial cells from the central nervous system (CNS) (54).

At present it is not clear whether Schwann cells may also act by production of endogenous steroid hormones. These neurosteroids are synthesized in CNS (116), but also in sciatic nerve (80) and may act by their mediators such as lipocortin-1 (annexin-1) to downregulate inflammatory reactions. Lipocortin-1, but not lipocortin-2 or -5 is a potent inhibitor of the antigen-specific activation of pathogenic T cell lines (37). Surprisingly truncation studies indicated that the activity of lipocortin-1 resides within the core region and not in the N-terminal fragment. In a side by side comparison with prednisolone lipocortin-1 suppressed T cell activation at a 30-fold lower dosage by weight. Importantly, lipocortin-1 expression in sciatic nerve is also increased during recovery from EAN and may contribute to termination of the inflammatory reaction (36).

Schwann cells are endowed on their membrane with a number of regulatory complement proteins such as CR1 (CD35), decay accelerating factor CD55, membrane cofactor protein CD46 and CD59 (70, 110, 111). This ensemble of proteins serves to attenuate the proinflammatory and demyelinating properties of activated complement factors that are involved in the pathogenesis of autoimmune demyelination in peripheral nerve (69, 95).

On the other hand, while macrophages constitute the major source of complement at inflammatory foci, Schwann cells can also be induced, e.g. by IFNγ, TNFα and IL-1β to generate the central complement component C3 (28). In Guillain-Barré Syndrome, nerve antibody titres are paralleled by levels of complement activation products in blood and CSF (C3a, C5a, soluble

membrane attack complex). These activated complement factors are also detected in situ (44, 45, 68).

Antigen-specific interaction of Schwann cells and T cells. At present we do not know which of these different cellular and humoral properties prevails *in vivo*. Finally it is clear that Schwann cells are only incomplete facultative antigen-presenting cells (APC). Antigen presentation by Schwann cells has two effects on T cell activation (41): (i) there is incomplete T cell activation after day 2 as reflected by low thymidine incorporation despite elevated IL-2 receptor, transferrin-receptor and costimulatory molecules such as CD28 on T cells. (ii) it increases the susceptibility of T cells to undergo apoptotic cell death when steroid hormones were added to the culture. The first effect implies that maximal proliferation of antigen-specific T cells occurs after 24 hrs, one day earlier than observed with thymic APC. Thereafter T cell activation is markedly diminished, irrespective of the pretreatment of the Schwann cells (41). Similar findings were obtained for astrocytes (39). Possible mediators of this effect have not yet been identified. Secondly, Schwann cells but not professional APC exert a priming proapoptotic effect on T cells. T cells are then rendered susceptible to steroid-mediated apoptosis. This may have consequences *in vivo* when invading T cells are also exposed to elevated systemic steroid levels that are measurable in experimental autoimmune models (78).

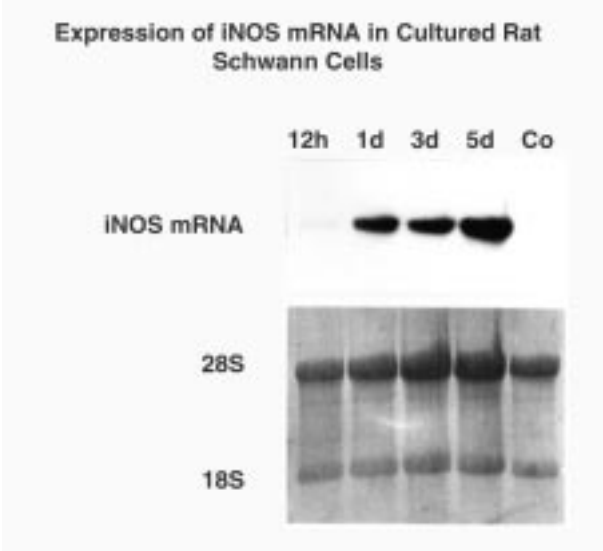

Figure 8. *Upregulation of inducible nitric oxide synthase in rat Schwann cells.* iNOS mRNA is specifically expressed in rat Schwann cells following stimulation with 300 U/ml g-IFN and TNF-a. Total RNA from 500.000 Schwann cells per sample was extracted and subjected to Northern blot analysis as described in material and methods. A specific mRNA species was detected as early as 12 hours after cytokine treatment, further increased by day 1 and remained elevated until day 5, whereas no specific mRNA was found in controls (Co, top panel). Total RNA was visualized by methylene-blue staining on the same blot (bottom panel). (reproduced from (41) with kind permission from Academic Press)

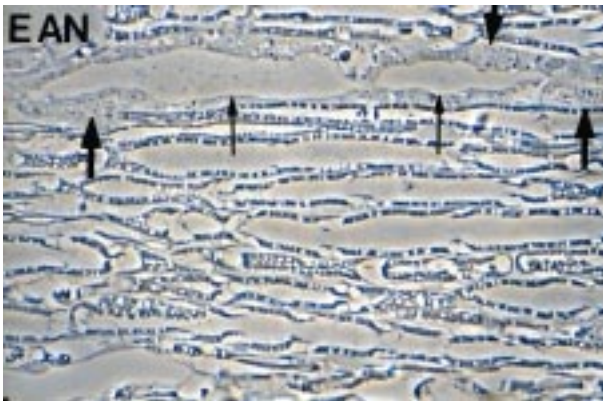

Figure 9. *Macrophages in experimental autoimmune neuritis.* Seventeen days after active immunisation of Lewis rats with peripheral nerve myelin and complete Freund's adjuvant, i.e. at the height of clinical disease, macrophages (bold arrows) are the chief cellular elements in the peripheral nerve lesion. Here they are shown as they have phagocytosed the myelin sheath in direct contact with the denuded axon (arrows). Semithin cryosection.

The role of macrophages

Macrophages are the predominant cell population in the affected nerves of animals with experimental neuritis (see below) and their selective elimination suppresses the disease (Figure 9) (49, 58). They also feature prominently in the lesions of GBS (53, 64). Unlike in the CNS, macrophages in the PNS lying near vessels or scattered in the parenchyma are phenotypically similar. They do not exhibit the sharp distinction that characterizes the perivascular macrophages and microglia in the CNS (reviewed in (42)). Experimental studies with chimeric rats suggest a rapid and significant turnover of macrophages within the PNS. Within three months after established chimerism up to 60% of macrophages were replaced by bone-marrow derived cells (109).

The PNS macrophages resemble their perivascular counterpart in the CNS in their constitutive expression of MHC class II molecules (43, 109). Also, in experimental neuritis the primary MHC class II positive cells are macrophages (96). The crucial role of macrophages in immune-mediated nerve damage centres around anti-

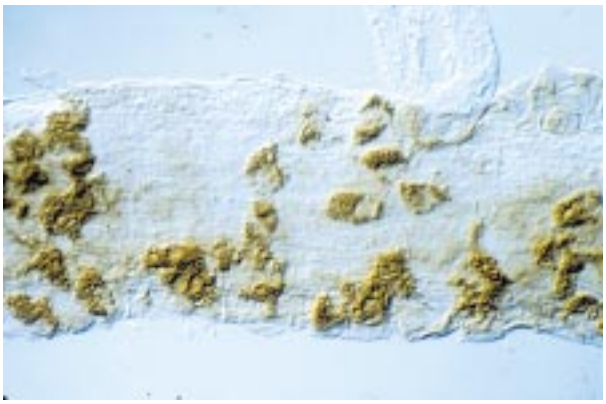

Figure 10. *TNFα in experimental autoimmune neuritis.* Seventeen days after active immunisation of Lewis rats with peripheral nerve myelin and complete Freund's adjuvant teased nerve fibre preparations were stained for TNFα. Massive upregulation is associated with macrophages. Large post-phagocytic macrophages at later stages of demyelination were TNFα-negative. Intraperitoneal application of an anti-TNFα antibody significantly reduced the degree of inflammatory demyelination and clinical expression of the disease (cf (104)).

gen presentation, direct phagocytic attack on myelin, and the release of proinflammatory cytokines including TNF-α, IL-1 and IL-6 and other noxious molecules (Figure 10). In coculture models release of proinflammatory mediators by macrophages can induce T cell apoptosis very efficiently (79b). This raises the possibility that macrophages may contribute to termination of inflammation by apoptosis (see below). Furthermore, strong expression of lipocortin-1 is seen in macrophages in experimental neuritis (36). In view of the immunosuppressive effect of lipocortin-1 on autoimmune T cells (see above) this may further help to downregulate autoimmune inflammation.

Investigation of proinflammatory enzymes involved in the pathogenesis of EAN may help to develop improved therapeutic approaches. Recent studies have focussed on the expression of matrix metalloproteinases (MMP). Upregulation and expression of MMP-9 (92-kd gelatinase) and MMP-7 (matrilysin), but not of other MMPs were found by immunocytochemistry, RT-PCR, and zymography (Figures 11, 12) (55, 65). Also, utilization of a comparable MMP pattern underlined pathophysiological similarities between EAN and GBS (Figure 13) (65). The selective expression of certain MMPs in inflammatory autoimmune disorders of the PNS and their varied cellular localization suggest that these enzymes may play a crucial , but multifactorial role in the process leading to disruption of the blood-nerve barrier. MMPs may therefore serve as potential targets for newer therapies that make use of specific

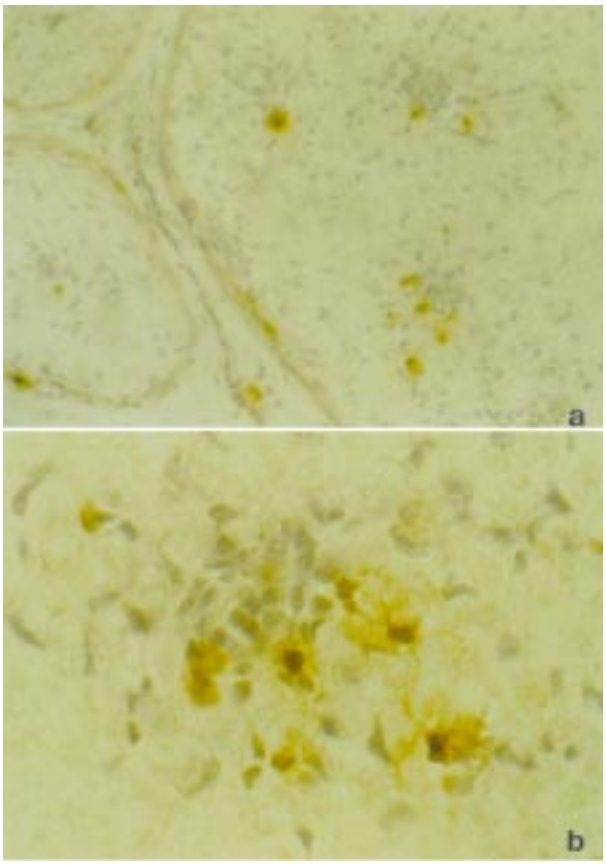

Figure 11. *Immunohistochemistry for Gelatinase B in sciatic nerves from Lewis rats with AT-EAN at peak of clinical severity.* Positive immunoreactivity was primarily found in the endo- and perineurium (**A**), and could be localized to infiltrating mononuclear cells and around blood vessels (**B**). A x 200; B x 600.

inhibitors capable of preventing activation of MMPs. Such an approach has already been shown to have a beneficial effect on the disease course of EAN (92).

Since EAN is a monophasic disease, endogenous counterregulatory mechanisms must operate to limit the extent of peripheral nerve inflammation. Potential mediators are immunoregulatory Th2 cytokines like transforming growth factor beta (TGF-β) or IL-10. Regulation of the isoform TGF-β1 and its cellular localization have been characterized during disease evolution in the AT-EAN model (62, 63). By quantitative Northern blot analysis and in situ hybridization immunohistochemistry TGF-β1 mRNA was upregulated in sciatic nerves and spinal roots, with peak levels just preceding the first clinical signs of recovery (Figure 14). In double labelling studies, macrophages and a subpopulation of T cells were identified as the major cellular source (Figure 15). Thus the physiological function of TGF-β1 in the

inflamed nerve not only pertains to cellular growth and matrix formation, but also implies a number of immunological mechanisms. This is underscored by *in vivo* data that confirm a beneficial effect of another isoform, TGF-β2 on the disease course of EAN induced by active immunization (59). Yet in rapidly developing AT-EAN intraperitoneal injection of TGF-β2 failed to abrogate the disease of diminish its consequences, suggesting that its therapeutic efficacy is limited. Since peak levels of TGF-β1 were reached before the height of the disease, one might speculate that other immunoregulatory cytokines such as IL-10 take over that role at later stages. This was indeed shown in studies comparing the expression of IL-10 in EAN as a model for immune mediated demyelination and Wallerian degeneration as a lesion model (34). Possible cellular sources of IL-10 are Schwann cells, macrophages, and T cells (57). Here again the pathophysiological relevance of these findings was highlighted by therapeutic studies with IL-10 (10). IL-10 effectively suppressed clinical EAN induced by active immunization with peripheral nerve myelin. Even in ongoing EAN administration of IL-10 ameliorated the disease. ELISPOT and isotype assays confirmed that this suppression was associated with downregulation of Th1 responses and upregulation of a Th2 type response.

T cell apoptosis in EAN - a physiological defense mechanism to terminate inflammation

In 1972 Kerr and colleagues used morphological criteria to define apoptosis as a specialized form of cell death (61). Traditionally apoptotic cells were identified by electron microscopy (115), but in the meantime newer techniques such as *in situ* tailing assays have become available (33, 38) which detect associated biochemical events of oligonucleosomal DNA fragmentation occuring in most apoptotic cells. Although apoptosis is a purely morphological term, it has an array of pathophysiological and functional implications for the immune system (25). Since the plasma membrane itself is not a primary target of apoptosis but rather provides specific signals to phagocytic cells (94), release of proinflammatory cytoplasmic constituents is avoided. In vulnerable organs such as the nervous system apoptosis would provide an ideal, noninflammatory mechanism to terminate the autoimmune T cell attack at vulnerable sites. It is assumed that the process of apoptosis is completed with in 4 to 5 hours (20).

Pender and colleagues drew attention to apoptosis as a possible mechanism of cell destruction in inflammatory brain lesions of Lewis rat autoimmune encephalomyelitis (EAE) (86). In the PNS it was not

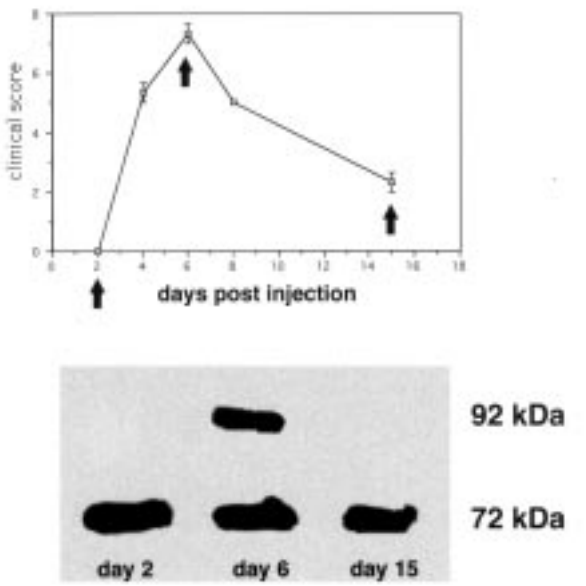

Figure 12. *Proteolytic activity in Lewis rat.* Detection of gelatinase activity in sciatic nerve from Lewis rat with AT-EAN by sodium dodecyl sulfate-substrate gel analysis during the clinical course of the disease. The graph demonstrates the disease course of AT-EAN after injection of neuritogenic T cells. Each arrow marks a time point proteolytic activity has been determined. Gel analysis is shown below: Each zone of clearing (black) represents proteinase activity. Only in animals with maximal disease activity - at day 6 - proteolytic activity is detectable at the 92 kDa level. In contrast, clearing remains unchanged at the 72kDa level throughout all time points investigated.

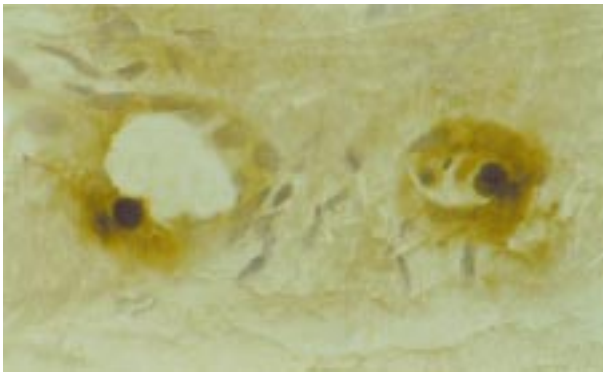

Figure 13. *Immunohistochemistry for Gelatinase B in sural nerve biopsy from a patient with GBS.* Positive immunoreactivity was primarily localized around blood vessels and to infiltrating mononuclear cells. X 600.

clear whether T cells become activated on encountering their target antigen in the peripheral nervous system or whether this occurs in lymphoid organs. Also, little was known about the fate of activated T cells once they have invaded the PNS. Conceivably, they could either leave

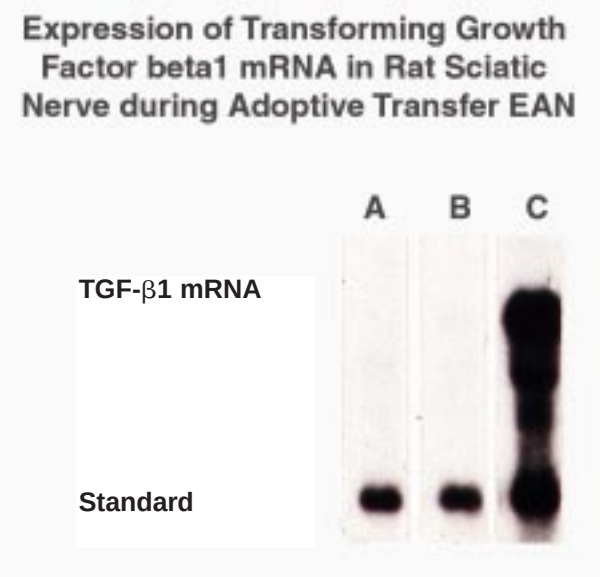

Figure 14. *Transforming growth factor beta mRNA expression in EAN.* Northern blot demonstrating upregulation of TGF-b1 mRNA in Lewis rats with P2-T cell transfer EAN at day 7 (**C**) compared to day 0 (**B**) and controls (**A**). Courtesy of Dr. R. Kiefer.

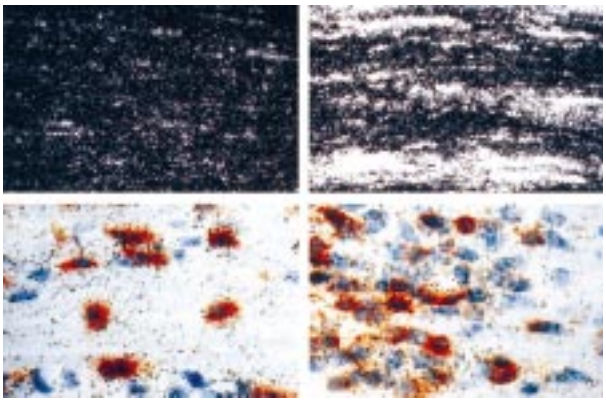

Figure 15. *TGF-β in EAN. In situ* hybridisation (upper panels) and *in situ* hybridisation combined with immunocytochemistry for macrophages (lower left panel) and T-lymphocytes (lower right panel) 18 days after immunisation of Lewis rats with peripheral nerve myelin. Upper left panel: control. Massive upregulation of signal for TGF β-1 mRNA, mostly associated with T-cells but also with macrophages. Courtesy of Dr. R. Kiefer.

their target tissue and end up in peripheral immune organs or may be eliminated *in situ*. Sites and mode of elimination and activation were studied in EAN induced by autoreactive T cell transfer or by active immunisation with purified bovine PNS myelin (118). In both models, T cell activation as reflected by incorporation of bromodeoxyuridine (BrdU) occured in lymphoid organs before disease onset and was only rarely observed *in situ* in the inflamed sciatic nerve. There, however, T cells exhibiting morphological signs of apoptosis were detected immediately after disease onset. T cell apoptosis reached its maximum at day 7 after cell transfer in AT-EAN and around day 17 in active EAN and led to elimination of about 10% of the inflammatory T cells. Bearing in mind that programmed cell death is a dynamic process, this could render a substantial number of T cells apoptotic, amounting up to 50% of the original T cells within 24 hours. Apoptosis of macrophages or Schwann cells was only very rarely seen.

These findings allow some important conclusions to be drawn regarding immune protection in the central and peripheral nervous system. Both tissues obviously take advantage of specialized, physiological defense mechanisms to limit inflammatory processes. The magnitude is much higher in the CNS (99) than in the PNS and almost zero in various human inflammatory myopathies (101). Even in the immunological setting of HIV infection, which is characterized by an increased frequency of apoptosis in blood and lymphoid organs, T cell apoptosis is not augmented in nerve or muscle (100) speaking for similar pathomechanisms of HIV-associated neuromuscular disorders and their idiopathic counterparts. T cell activation in the PNS appears to follow mechanisms quite similar to those operating in the CNS (84) and may therefore not account for this disparity. These data underline that T cell apoptosis in autoimmune disorders occurs apparently only in immune-protected sites (reviewed in (35).

At present the molecular mediators are still in large part unknown. To better understand the mechanisms of apoptosis *in vivo* it is important to know whether antigen-specific cells alone or also recruited T cells are destroyed within the nervous system. So far this has only been studied in EAE. Pender and colleagues induced EAE by a T cell clone using Vβ8.2 T cell receptor, the predominant T cell receptor element in MBP-induced EAE of the Lewis rat (24), and characterized apoptosis and T cell receptor usage in lymphocytes isolated and enriched from spinal cord (106, 107). They found that the frequency of Vβ8.2+ T cells was sevenfold higher in the apoptotic population than in non-apoptotic T cells. Furthermore they could not detect recirculation of Vβ8.2+ T cells in peripheral lymphoid organs. This work suggests, although by indirect evidence, that antigen-specific T cells are the prime target for destruction by apoptosis. Similar experiments in EAN are very difficult to perform since quantitative

recovery of T cells from inflamed nerve is not that easily achieved as from spinal cord (R. Gold, unpublished). Experiments comparing T cell apoptosis in EAN induced by different autoantigens may also help to delineate the relative contribution of the specific antigen and subsequent T cell receptor activation. Again, only in the CNS counterpart EAE data have been generated showing that the extent of T cell apoptosis in the parenchyma is comparable in MBP, S-100 or MOG induced EAE (11). In the CNS a specific histopathological pattern of lesion distribution associated with a distinct autoantigen (67) may account for differences in T cell apoptosis. In the future studies in mouse mutants may yield important insights with regard to the involved partners. Recently detailed investigations have provided evidence that the contribution of the Fas/FasL system in EAE is obviously not as important as in other autoimmune disorders (77).

Therapeutic induction of T cell apoptosis in autoimmune diseases: studies in experimental autoimmune neuritis

Treatment regimens in patients with immune-mediated disorders of the nervous system aim to reduce inflammation, demyelination, to prevent primary and secondary axonal damage, and to accelerate recovery. Therapeutic efficacy of steroids in inflammatory disorders of the nervous system has been well established for immune neuropathies (29, 87), multiple sclerosis and optic neuritis (12). Corticosteroids exert their beneficial antiinflammatory effect at multiple levels. Besides their antiedematous properties, they modulate cell activation, cytokine expression, secretion of inflammatory mediators, and leucocyte migration (reviewed in (18, 19). These effects are all mediated by the cytosolic glucocorticosteroid receptor. At high doses, glucocorticosteroids can also directly promote cell death, and this is thought to reflect a nongenomic, physicochemical action of glucocorticosteroids (reviewed in (22). Possible candidates for this molecular effect are membrane-bound, ligand-gated purinergic P2X receptors (56), which upon activation cause a perturbation in intracellular ion homeostasis.

Based on the therapeutic use of high-dose glucocorticosteroids we first investigated whether steroid 'pulse-therapy' could induce T cell apoptosis *in situ* (111). Since a predictable and synchronized disease course was highly desirable for these investigations, we focussed on AT-EAN of the Lewis rat which was produced by transfer of activated, P2-specific T lymphocytes. To delineate whether the effect of steroid hormones is stage-specific, two pulses of glucocorticosteroids (10 mg/kg body weight) were administered after disease onset or at its presumed maximum. Due to the rapid elimination of apoptotic cells (13) rats had to be sacrificed 6 hours after the second steroid pulse, before a clinical effect became apparent. At both time points there was a massive reduction of T cell inflammation and a four- to fivefold increase of T cell apoptosis in the inflamed sciatic nerve. In addition, reduced cellular pro-

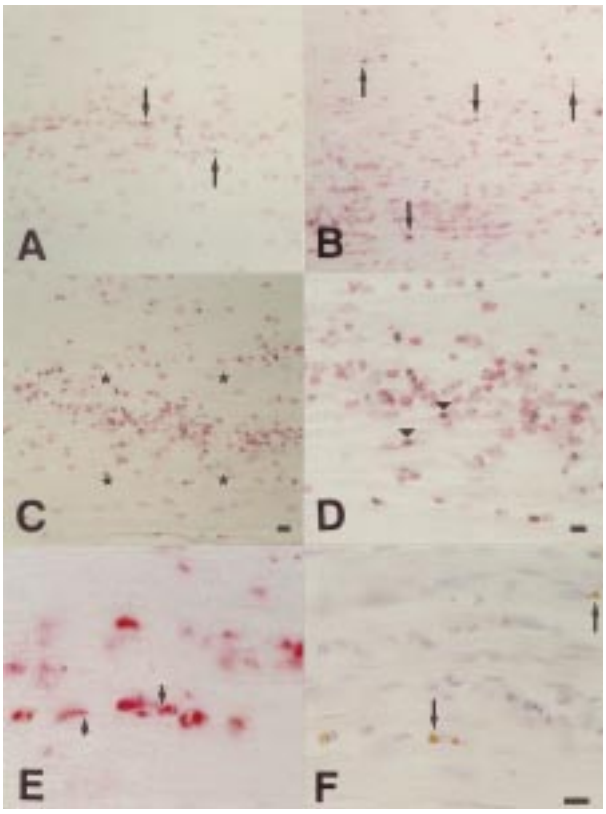

Figure 16. *Immunocytochemical characterization of apoptotic cells and BrdU incorporation in sciatic nerve from Lewis rat EAN.* **A-D**) T-cell infiltration in AT-EAN on day 7 in sciatic nerve rats receiving control serum only (**A**), rhP2 recipients (**B**), and rats treated with rhP2 followed by 10 mg/kg methylprednisolone (**C,D**); double-labelling of apoptotic T-cells. Nuclei with fragmented DNA are labeled black by *in-situ* tailing followed by anti-T-cell immunochemistry visualized with alkaline phosphatase and fast red salt. Microphotographs show only some apoptotic T cells in control rats (arrows in **A**), whilst their number increases after P2 injection (**B**) and is further augmented by glucocorticosteroids (**C**). (**D**) shows a higher magnification from the area marked by squares in (**C**). In some apoptotic T cells the membrane signal is still preserved (arrowheads) bar = 20 μm for A-C, 10 μm for D. **E**) BrdU positive fragments (brown, indicated by arrows) are incorporated in ED-1 positive, red-labelled macrophages. **F**) BrdU positive cells in sciatic nerve after antigen therapy (arrows) bar = 10 μm for E-F

liferation in lymphoid organs and loss of thymus weight were observed in steroid recipients reflecting typical genomic actions of steroids. These results support the use of pulsed high dose corticosteroid therapy in autoimmune neurological disorders. In EAE, dose-response relations have been delineated more clearly (98). Here doses of at least 10 mg/kg body weight methylprednisolone were needed to enhance T cell apoptosis.

Another approach that has been developed for the treatment of experimental autoimmune disorders is antigen-specific therapy. This is based on the observation that T cell receptor reengagement at an appropriate stage of the cell cycle eventually leads to T cell apoptosis (reviewed in (73). Its efficacy has been demonstrated by Critchfield *et al*. in EAE in mice by intravenous administration of MBP (27). Taking advantage of an approach similar to that described above for glucocorticosteroid hormones, we demonstrated that intravenous treatment with recombinant P2 protein can prevent AT-EAN and active EAN of the Lewis rat, and causes a profound increase in T cell apoptosis in the sciatic nerve (Figure 16) (112). With regard to induction of apoptosis in AT-EAN antigen therapy was at least as effective as steroid hormones. When both treatment strategies were applied, they acted synergistically and enhanced T cell apoptosis *in situ* (Figure 16). Under these circumstances even T cell proliferation in the nervous system could be observed which does not normally occur in untreated EAN (118). Interestingly, the native P2 protein showed a much higher efficacy than the neuritogenic peptide spanning aa 53-78 which is recognized by these neuritogenic T cell lines *in vitro*, supporting the concept that antigen presentation must occur *in situ* (113) and may be executed by activated Schwann cells in the PNS (114). Antigen therapy may prove useful as highly specific treatment of autoimmune disorders of the nervous system. In ongoing investigations TNF-α has been identified as the principal mediator of the effects of antigen therapy *in situ* (Weishaupt, Gold, submitted for publication). The intracellular signalling mechanisms that are associated with high-dose antigen therapy have not yet been elucidated.

Conclusions and perspectives

The peripheral nervous system, due to the lack of a strong anatomic barrier at strategic sites (most proximally at the root exit zone and most distally at the motor terminals), is jeopardized by immunoinflammatory responses from the systemic immune compartment. Hence, tight control mechanisms are required to ensure its viability. These are manifold and include the elaboration of various down-regulatory molecules by resident partially immunocompetent cells such as Schwann cells and endoneurial macrophages. Recently, elimination of invading autoaggressive T cells by apoptosis has been identified as a major process to contain neural inflammation and protect the integrity of peripheral nerve. Elucidation of the immunoregulatory circuitry in the PNS and its disturbance in human disease and experimental models will aid in the development of improved treatments for immune-mediated neuropathies.

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